

## Nosocomial Infection by *Staphylococcus haemolyticus* and Typing Methods for Epidemiological Study

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A patient with chronic myelogenous leukemia became colonized with a *Staphylococcus haemolyticus* strain and experienced a septic episode caused by this strain during a cytostatic course. The strain was multiply resistant to antibiotics; the MIC and MBC of vancomycin were 2 and 4 mg/liter, and the MIC and MBC of teicoplanin were 4 and 16 mg/liter, respectively. We performed a surveillance study on the carriage of *S. haemolyticus* in medical and nursing staff of the hospital ward where the patient was treated. *S. haemolyticus* was isolated from 18 sites on 12 of the 39 people tested. A number of typing methods were performed in order to investigate the possible relationships among the isolates. Methods used were immunoblotting of staphylococcal peptides, plasmid analysis, restriction fragment length polymorphism of chromosomal DNA, and pulsed-field gel electrophoresis of total DNA. Compared with the immunoblotting technique, the molecular methods were more discriminative. The strain colonizing the patient showed a consistent pattern by all typing methods during isolation. When the immunoblot technique was used, similar patterns were found with isolates from hospital staff and isolates from unrelated sources. With the molecular techniques, no evidence of a local spread of the patient's strain was found. However, plasmid profiles and restriction fragment length polymorphism and pulsed-field gel electrophoresis patterns showed that *S. haemolyticus* isolates collected from hospital ward personnel were related, which was not the case with isolates collected from unrelated sources. Restriction fragment length polymorphism analysis was more discriminative when IS431 was used as a DNA probe instead of a probe based on the 16S rRNA gene. *S. haemolyticus*, as in this case, may develop resistance to vancomycin and teicoplanin. These antibiotics are considered the last-resort drugs for the therapy of nosocomial gram-positive infections. Thus, local spread of staphylococci resistant to these drugs is an important problem, which should be prevented by strict hygienic measures and antibiotic policy.

Coagulase-negative staphylococci are common causes of infection in febrile neutropenic patients. These gram-positive bacteria may cause bacteremia, especially in the presence of indwelling venous catheters (10, 11). The antimicrobial susceptibility of coagulase-negative staphylococci is unpredictable. Nosocomial strains frequently show resistance, which has been shown to be correlated with antimicrobial drug use (14).

Vancomycin and teicoplanin are antistaphylococcal antibiotics to which resistance is rarely seen. These drugs have been recommended in the empirical treatment of febrile neutropenic patients (10). Nevertheless, resistance of coagulase-negative staphylococci to vancomycin and teicoplanin has been recently reported (1, 7, 19, 21). The spread of such strains in hospitals may constitute an additional threat for the immunocompromised patient.

We report a case of infection with serious illness and bacteremia in a leukemic patient with a *Staphylococcus haemolyticus* strain that showed reduced susceptibility to vancomycin and teicoplanin. Some of the hospital staff were found to be carriers of *S. haemolyticus*. By means of five different typing methods, we sought evidence of the local spread of this strain.

### MATERIALS AND METHODS

**Case.** Patient A is a 60-year-old man with a history of chronic myelogenous leukemia diagnosed in 1980. In November 1990, he was treated for a blast crisis. During cytostatic treatment, the patient developed leukocytopenia, for which he was placed in an isolation unit and received oral prophylaxis with ciprofloxacin, polymyxin B, and amphotericin B. He developed signs of an ileus, accompanied by bacteremia with *Klebsiella* sp., which was treated successfully with ceftazidime. He also had a *Candida* esophagitis. In January 1991, he again became septic, and blood cultures were positive with coagulase-negative staphylococci. Surveillance cultures from the skin, nose, throat, and sputum showed growth of gentamicin-resistant, coagulase-negative staphylococci 31 days before blood cultures became positive. Gentamicin-resistant, coagulase-negative staphylococci, 10<sup>3</sup> to 10<sup>4</sup> CFU/g, were isolated from the feces. Eleven days later, the blood cultures became positive. Antimicrobial therapy was changed to ceftazidime, fluconazole, and vancomycin. The patient's condition nevertheless worsened, and he died 2 days after the blood cultures became positive with the coagulase-negative staphylococcus. The bacteriological culture of a Hickman catheter removed postmortem remained negative. The strain from the blood culture was identified as *S. haemolyticus* with reduced susceptibility to vancomycin and teicoplanin.

**Isolates.** Resistant coagulase-negative staphylococci were isolated by streaking the swabs over a selective blood agar plate containing 5% sheep erythrocytes and gentamicin (16 mg/

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liter). Plates were incubated for 24 h at 37°C. Morphologically different colonies from pure cultures were identified with the API-Staph method (API-Staph; API bioMérieux). Sixty-eight isolates of coagulase-negative staphylococci were thus collected from the patient on 20 different days at intervals of 1 to 3 days during the 37-day period. These isolates were found in cultures taken from the nose (12 isolates), axilla and groin (12 isolates), feces (9 isolates), throat or palate (13 isolates), blood (6 isolates), urine (12 isolates), sputum (3 isolates), and pus (1 isolate). One week after identification of the patient's strain from the blood culture, samples from all 39 medical and nursing staff of the ward on which the patient was treated were taken from both hands, axilla, groin, and nose. Only isolates identified as *S. haemolyticus* were used for susceptibility testing and further typing. The *S. haemolyticus* isolates obtained from the hospital personnel were then compared with the *S. haemolyticus* isolate from the patient. For demonstrating a possible epidemiological relatedness of the isolates isolated in the patient's ward, a set of *S. haemolyticus* isolates from other hospitals was obtained in this study. These *S. haemolyticus* isolates had been isolated from clinical material from epidemiologically unrelated sources and were kindly provided by B. I. Davies (De Weever Hospital, Heerlen, The Netherlands) and R. P. Mouton (University Hospital, Leiden, The Netherlands). Another set of isolates was collected in our laboratory from leukemic patients in other hospitals.

**Susceptibility tests.** Susceptibility tests were performed on all *S. haemolyticus* isolates with a standard agar dilution method (16), using Iso-Sensitest agar (Oxoid). MICs of vancomycin, teicoplanin, penicillin, amoxicillin, cloxacillin, cefuroxime, imipenem, gentamicin, amikacin, erythromycin, clindamycin, doxycycline, chloramphenicol, rifampin, and ciprofloxacin were determined.

Stepwise resistance was provoked in the strain from the patient's blood and in a control *S. aureus* strain, ATCC 29213. By the method of Schwalbe et al. (19), 0.1 ml of an inoculum of approximately  $10^9$  CFU/ml was poured from an overnight culture in Iso-Sensitest broth (Oxoid) into an agar plate (Iso-Sensitest agar) containing vancomycin in a one-log two-step-higher concentration than the MIC for the strain. With colonies grown on this agar plate, the procedure was repeated on agar plates containing vancomycin concentrations in a range of 2 to 64 mg/liter.

**Immunoblotting.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) were performed as described previously (12, 20, 22) with some modifications. Briefly, different strains of *S. haemolyticus* were grown overnight in 5 ml of tryptone soy broth (Oxoid Ltd., Basingstoke, United Kingdom) and harvested by centrifugation. The pellet was resuspended in 100  $\mu$ l of distilled water and lysed with lysostaphin, 100  $\mu$ g/ml (Sigma). Twenty microliters of lysate was heated at 100°C for 5 min with 60  $\mu$ l of sample buffer (62.5 mM Tris-HCl [pH 6.8] containing 2.6% SDS, 10% glycerol, 5.6%  $\beta$ -mercaptoethanol, and 0.001% bromophenol blue). For electrophoresis, the suspensions were applied to a 4% acrylamide stacking gel over a 10% acrylamide separating gel in a discontinuous buffer system. Separated proteins were immediately transferred to nitrocellulose paper, using a Miniprotean II Trans-Blot cell (Bio-Rad). After blocking of nonspecific binding sites with phosphate-buffered saline containing 0.5% Tween 20 (PBS-Tween 20), the nitrocellulose membrane was incubated overnight at 25°C with hyperimmune rabbit serum diluted 1:100 in PBS-Tween 20. The membranes were washed in PBS-Tween 20 and incubated for 1 h at 25°C with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G-immunoglobulin M (ITK) diluted 1:300. Devel-

opment of immunoblots was carried out with nitroblue tetrazolium and indolyl sulfate (Sigma) (20).

**DNA techniques.** Bacterial strains were grown as described above. Plasmid DNA was extracted and analyzed with ethidium bromide-stained agarose gels (8, 18).

For restriction fragment length polymorphism (RFLP) analysis, extraction of chromosomal DNA and Southern blotting were performed as described previously (9, 18). In brief, genomic DNA was extracted from a 1.5-ml overnight culture by using lysostaphin, SDS, and proteinase K. DNA was further purified by extraction with phenol-chloroform and ethanol precipitation. Finally, the DNA was resolved in 100  $\mu$ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). *Cla*I was used to digest DNA from all isolates according to the manufacturer's recommendations (Boehringer Mannheim) (3). The digested DNA was analyzed by electrophoresis in a 1% agarose gel at 25 V overnight in TBE buffer (89 mM Tris, 89 mM boric acid, 25 mM EDTA, pH 8.2) and stained in ethidium bromide, 0.5  $\mu$ g/ml. Southern blots were prepared on a Hybond N<sup>+</sup> membrane (Amersham), using a vacuum miniblot system (Millipore Corp., Bedford, Mass.), and stored at 4°C until use. For hybridization of Southern blots, two different probes were used: one probe was based on the IS431 sequence, an insertion-like element found frequently among *Staphylococcus* spp. (2), and the other probe was homologous to a broadly conserved 16S rRNA gene region in the prokaryotic chromosome (6). The probes were produced with the PCR, using primer 1, 5'-TACATCATGTTAATAAGGG, and primer 2, 5'-TTGC GTGAGTGTGGCGAAGC, to amplify an 800-bp sequence from IS431. Primer 3, 5'-GAGTCTTCTGTATGAACTA, and primer 4, 5'-CGCTTAACCTGCTAGCATG, were used for amplification of a 1,700-bp fragment from the 16S rRNA gene. The oligonucleotides were provided by the Molecular Microbiology Unit of the National Institute of Public Health and Environmental Protection. The target for amplification was 5  $\mu$ l of any *S. haemolyticus* DNA suspension, which was also used for the digestions, as described above. Amplification was performed as described before (17) for 35 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C, using a thermal cycler (Perkin-Elmer). After amplification, the PCR products were divided into aliquots of 10  $\mu$ l and purified by agarose gel electrophoresis, using low-melting agarose (preparative grade; Bio-Rad). After separation of the DNA fragments, agarose blocks containing DNA with the expected molecular weight were cut from the gel and used directly for labelling or stored at -20°C. Nonradioactive labeling of probe DNA with horseradish peroxidase was performed directly in the agarose blocks by using the enhanced chemiluminescence gene detection system (Amersham International plc, Amersham, United Kingdom). One agarose block containing labeled DNA was used for hybridization of one blot. For analysis of DNA by pulsed-field gel electrophoresis (PFGE), intact chromosomal DNA was prepared by in situ lysis of bacteria in agarose (13). Briefly, cells were embedded in 2% low-melting-point agarose, and the agarose blocks were incubated for 18 h at 37°C with lysostaphin, 100  $\mu$ g/ml. The blocks were stored in 5 $\times$  TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0). For restriction endonuclease digestion, agarose blocks were preincubated at 4°C in 75  $\mu$ l of appropriate buffer for the restriction enzyme. After 30 min, the buffer was removed and 75  $\mu$ l of fresh buffer containing 5 U of *Sma*I (Boehringer Mannheim) was added. Restriction digestion was performed for 2 to 3 h at 37°C. After digestion, blocks were inserted in the slots of a 1% agarose gel and electrophoresed by PFGE (4) for 17 h at 200 V, with pulse times ranging from 1 to 30 s, using a pulse wave power supply (Bio-Rad 760, model 200/2.0). The forward-to-reverse ratio

TABLE 1. Results of susceptibility tests with *S. haemolyticus*

Antibiotic	MIC (mg/liter) for <i>S. haemolyticus</i> isolates from:						
	Patient (n = 14)		Hospital staff (n = 17)		Different hospitals (n = 13)		
	Day 1 <sup>a</sup>	Days 20-37 Range	MIC <sub>50</sub> <sup>b</sup>	Range	MIC <sub>50</sub>	Range	MIC <sub>50</sub>
Penicillin	4	2-64	64	0.06-64	32	0.01-64	0.5
Amoxicillin	4	1->64	64	0.25-64	64	0.01-<64	2
Cloxacillin	0.12	0.25->16	>16	0.25-16	<16	0.125-<16	0.5
Imipenem	2	0.25->16	1	0.03-<16	1	0.03-<16	0.06
Cefuroxime	0.25	2->16	16	0.5-<16	<16	1-<16	2
Vancomycin	2	0.25-4	2	0.25-2	1	0.5-2	1
Teicoplanin	2	1->16	>16	0.5-16	2	0.5-16	4
Gentamicin	32	8-32	32	0.06-<32	32	0.03-<32	32
Amikacin	8	4-8	4	0.5-16	4	0.125-<16	4
Clindamycin	0.03	0.03-8	2	0.03-2	0.03	0.01-<16	0.3
Erythromycin	0.06	2-8	4	0.125-<16	8	0.03-<16	0.125
Doxycycline	2	0.5-2	1	0.5-16	0.5	0.03-<16	0.5
Chloramphenicol	1	4-8	4	2-<16	4	2-4	4
Rifampin	0.03	0.06	0.06	0.03-0.06	0.06	0.06	0.06
Ciprofloxacin	>16	>16	>16	0.125-<16	0.25	0.06-<16	0.25

<sup>a</sup> Day of admission to the hospital; see text.

<sup>b</sup> MIC<sub>50</sub>, MIC for 50% of isolates tested.

was 1:1. After electrophoresis, gels were stained with ethidium bromide, and DNA fragments were visualized with UV light.

## RESULTS

**Isolates.** In order to identify colonies as belonging to *S. haemolyticus*, pure cultures from morphologically different colonies were investigated with the API-Staph identification system. Coagulase-negative staphylococci isolated from the specimens from the patient during the 37-day period from the first surveillance day until death were identified as *S. haemolyticus*, *S. epidermidis*, and *S. hominis*.

*S. haemolyticus* was isolated in 26 of the 68 cultures tested. *S. haemolyticus* was isolated from the skin on the first day of admission. During the following period of 20 days, cultures were collected at various sites, and no *S. haemolyticus* strains were found. In the second period of 16 days, until the patient's death, *S. haemolyticus* was isolated from various sites during the whole period in which cultures were collected. From the cultures of specimens obtained from different sites from the 39 hospital personnel, *S. haemolyticus* was recovered from 18 sites on 12 persons. The hands were involved 14 times.

**Susceptibility tests.** The results of the agar dilution tests with 15 antibiotics are shown in Table 1. The isolate that was found on the day of admission was notably more susceptible than the majority of *S. haemolyticus* isolates found from day 21 on. This can be concluded from the MIC for at least 50% of the latter isolates. The wide range of MICs for the isolates on days 21 to 37 is due to one of the 13 isolates being more susceptible to multiple antibiotics. All isolates were highly resistant to ciprofloxacin. The isolates from the hospital personnel showed more variable patterns of susceptibility. MICs of teicoplanin tended to be lower. This was also the case with clindamycin. Most isolates were susceptible to ciprofloxacin. We found one ciprofloxacin-resistant isolate with a susceptibility pattern similar to those found in most of the isolates from the patient. Furthermore, this strain appeared to possess molecular properties similar to those of the patient's blood culture strain (see Table 2). The MIC of gentamicin for one isolate found on the gentamicin-containing plate appeared to be low (0.06 mg/liter) when the isolate was tested further. There is no other expla-

nation than that this isolate had lost its resistance during storage in the course of testing.

The strains derived from geographically different sources showed variable patterns of susceptibility. In contrast to isolates from the patient and from hospital personnel, more penicillin-susceptible strains were found. Only a few strains were resistant to clindamycin or ciprofloxacin.

In the strain derived from the patient's blood culture, stepwise resistance was provoked with vancomycin, reaching a MIC as high as 20 mg/liter at a resistance frequency of 10<sup>-7</sup> (18). It was not possible to provoke resistance in a control strain, *S. aureus* ATCC 29213.

**Immunoblotting.** Most isolates showed identical immunoblot profiles. The first isolate from clinical material from the patient showed a different profile when compared with the other 13 isolates from the patient (Table 2, profiles a and b). However, most of the isolates from ward personnel showed similar patterns, as did a number of strains from unrelated sources.

**Plasmid analysis.** Plasmids could be detected in all 14 strains from the patient (Table 2). Six different patterns were recognized. Patterns a to e showed high homology; pattern f (first patient isolate) was different. The most frequently found pattern was pattern a, which was found nine times among the patient's isolates. In the isolates from the hospital staff, eight different patterns were found. Five isolates harbored no plasmids. The most common pattern among the patient's strains, pattern a, was found only once among the isolates found on the hand of a member of the hospital staff. Pattern c, found once among the patient's isolates, was also recognized in the group isolates from the nursing staff (Fig. 1). Pattern a and c strains were found on the hands of two members of ward personnel. Eight strains from other hospitals harbored no plasmids, and the other strains had different patterns, none of which were found among the strains isolated from the patient or from hospital staff.

**RFLP.** Chromosomal DNA digested with *Cla*I and hybridized with a 16S rRNA probe generated only eight different patterns. Pattern a was most frequently found among the nonrelated strains. When IS431 was used as a probe, 24

TABLE 2. Genotypic and immunoblot characteristics of *S. haemolyticus* strains

Typing method	No. of isolates showing characteristic		
	From patient (n = 14)	From hospital staff (n = 18) <sup>a</sup>	From different hospitals (n = 13)
<b>Immunoblot profiles</b>			
a	13	16	9
b	1		
0 <sup>b</sup>		2 (2) <sup>c</sup>	4 (4)
<b>Plasmid profiles</b>			
a	9	1	
b	1		
c	1	1	
d	1		
e	1		
f	1		
0		10 (8)	5 (5)
No plasmids		5	8
<b>RFLP profiles</b>			
<b>16S rRNA probe</b>			
a	12	11	11
b	1	1	
c	1	4	
0		1	2 (2)
<b>IS431 probe</b>			
a	10	1	
b	2		2
c	1		
d	1		
0		16 (12)	11 (11)
<b>PFGE profiles</b>			
a	13	1	
b	1		
0		16 (9)	13 (13)

<sup>a</sup> One isolate died before the genotypic characteristics could be assessed.

<sup>b</sup> 0 = other patterns or profiles.

<sup>c</sup> Numbers in parentheses indicate the number of patterns.

different patterns were observed. The patient's strain could be divided into four patterns, of which pattern a was the most common, pattern b was found on two occasions, and patterns c and d were found only once (Table 2). Among the isolates from the hospital staff, pattern a was found once. There were no other similarities between isolates found on the patient and those from hospital staff. Four isolates showed identical patterns. A selection of RFLP profiles with the IS431 probe is shown in Fig. 2. In the collection of epidemiologically nonrelated strains from different hospitals, no similarity among strains was found.

**PFGE.** PFGE divided the patient strains into only two distinguishable patterns. The isolates from hospital staff showed 10 patterns, and the nonrelated strains showed 13 patterns.

All of the patient isolates, with the exception of the first isolate, yielded the same PFGE pattern, indicated as pattern a. This pattern was also found in a strain found on the hand of a member of the hospital staff (Fig. 3). No other similarities were detected between isolates found on hospital staff and nonrelated strains. One pattern was found seven times in the isolates from hospital staff. The nonrelated strains showed no relation to patient isolates or the isolates from hospital staff.

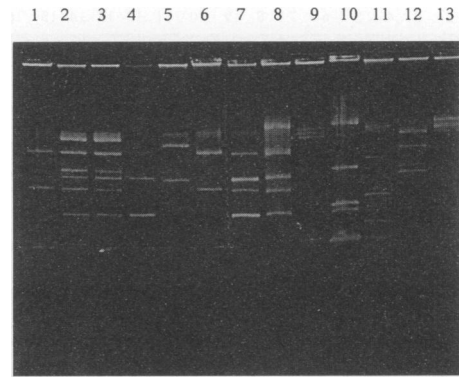


FIG. 1. Agarose gel electrophoresis of plasmid DNA purified from isolates of *S. haemolyticus* from the patient and hospital staff. Lanes 2 and 12 show patient isolates with pattern a, which was found most frequently. Lanes 1, 3 to 11, and 13 show the different patterns among isolates from nursing staff. Lane 4 shows a pattern c with high resemblance to one of the patient isolates (not shown). Lane 3 shows a pattern similar to the most common pattern of the patient isolates.

**DISCUSSION**

Multiple antibiotic resistance of coagulase-negative staphylococci is a recognized problem in situations where these species may cause clinical problems (5, 11, 14, 15). In recent years, vancomycin has been used to overcome multiple resistance in the treatment of leukemic patients and in the treatment of catheter-related infections, e.g., continuous ambulatory peritoneal dialysis-related peritonitis (5, 10). Recently, a number of reports have focused on the emergence of vancomycin-resistant coagulase-negative staphylococci, which were identified as *S. haemolyticus* (1, 7, 19, 21).

Schwalbe et al. were able to show a stepwise increase in vancomycin resistance in a strain during vancomycin therapy (19). When we were confronted with a patient who developed

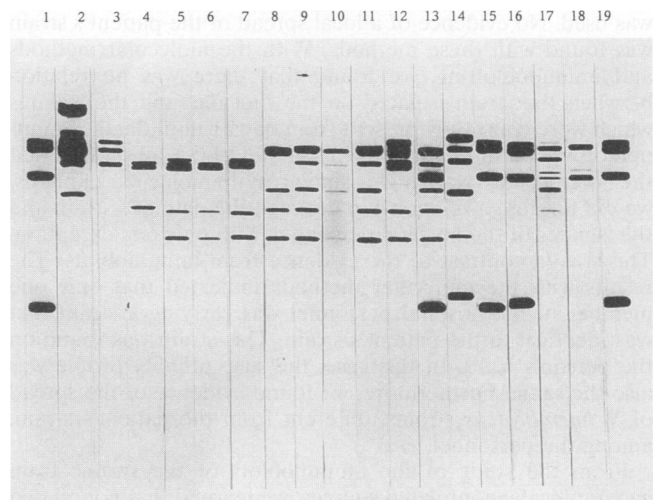


FIG. 2. RFLP analysis of chromosomal DNA of *S. haemolyticus* isolates. Southern blot analysis of *Cla*I-digested DNA hybridized with IS431 from isolates isolated from the patient and hospital staff. Lanes 1, 16, and 19 show the most common pattern among the patient's isolates. Lanes 2 to 15, 17, and 18 are isolates from members of the hospital staff, rearranged according to their similarity. Lane 15 shows a pattern similar to the most common pattern of the patient isolates.

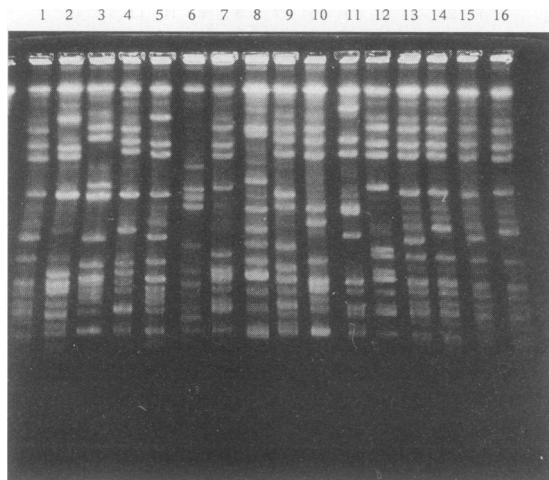


FIG. 3. PFGE separation of restriction fragments of total DNA digested with *Sma*I of *S. haemolyticus* isolates from the patient, hospital staff, and other hospitals. Lanes 1 and 16 show the most common pattern among the patient's isolates. Lane 15 shows a pattern from an isolate from the hospital staff with high homology to the patient isolates. Lanes 2 to 14 show patterns from isolates from other hospitals.

a serious infection with a strain with reduced susceptibility to vancomycin, we were able to trace susceptible isolates, which had been colonizing the patient prior to infection. A similar case has been described by Veach et al. (21). As previously described, it was possible to select for in vitro resistance in initially susceptible strains by exposure to vancomycin.

Isolates were characterized by means of immunoblotting, RFLP, PFGE, and plasmid profile analysis. The molecular methods were more discriminative than the immunoblot technique. Of the molecular methods, PFGE and RFLP have proved to be useful tools. When the 16S rRNA gene probe was used, RFLP was less discriminative than when the IS431 probe was used. No evidence of a local spread of the patient's strain was found with these methods. With the molecular methods and immunoblotting, we found that there was no relation between the strain isolated on the first day and the isolates which were constantly present from day 21 until death. Immunoblotting, plasmid analysis, RFLP, and PFGE all showed that the latter isolates were highly similar. With molecular methods, we did not find a relationship between the patient's strain and the isolates of the hospital personnel, with only one exception. This was in contrast to the evidence from immunoblots. The results with the molecular methods indicated that only one member of the hospital personnel was carrying a strain that was identical to the patient's strain. This strain was found on the person's hand. In this case, the susceptibility profile was also the same. Furthermore, we found evidence of the spread of *S. haemolyticus* strains, different from the patient's strain, among the personnel.

From the study of the immunoblots of the strains from geographically nonrelated sources, we learned that nonrelated *S. haemolyticus* strains may show highly similar polypeptide patterns as well as dissimilar patterns. Immunoblots of polypeptides may provide only a relative method to show the relatedness of strains in a situation of nosocomial infection.

In our hands, fingerprinting coagulase-negative staphylococci by means of plasmid profile analysis, RFLP, and PFGE has been a valuable tool in differentiating nosocomial *S.*

*haemolyticus* strains from incidentally colonizing strains. The emergence of vancomycin resistance in *S. haemolyticus* strains is a disturbing finding. Molecular techniques may be used to show evidence of local spread of such strains. In such situations, preventive measures such as the restriction of vancomycin use and strict hygienic protocols are possible means of infection control.

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