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Vancomycin Resistance in *Staphylococcus haemolyticus* Causing Colonization and Bloodstream Infection

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The increase in the incidence of infections due to beta-lactam-resistant coagulase-negative staphylococci has resulted in expanded use of vancomycin for such infections. Despite this, coagulase-negative staphylococci have remained susceptible to vancomycin in recent years. This report describes a strain of *Staphylococcus haemolyticus* with increased resistance to vancomycin (MIC, 8.0 to 16 μg/ml). *S. haemolyticus* was initially isolated from a patient with acute leukemia and neutropenia in surveillance throat and stool cultures. The microdilution vancomycin MICs for these isolates were 1.0 to 2.0 μg/ml. Subsequent *S. haemolyticus* isolates from the bloodstream and tracheal aspirate occurred in the setting of prolonged empirical vancomycin therapy. MICs for these isolates were 8.0 to 16 μg/ml. Further vancomycin resistance (MIC, 32 μg/ml) could be selected for in vitro in all four isolates. Restriction endonuclease analysis of plasmid DNA indicated that the isolates were very closely related and likely to be of the same strain. We conclude that colonization with a vancomycin-susceptible strain of *S. haemolyticus* was subsequently linked to a nosocomial bloodstream infection with an apparently identical strain with intermediate levels of vancomycin resistance. Prolonged empirical vancomycin therapy was temporally associated with this episode.

The incidence of infection caused by coagulase-negative staphylococci has increased dramatically in recent years. Between 1980 and 1987 at the University of Iowa Hospitals, the rates of nosocomial bacteremia due to coagulase-negative staphylococci increased from 5.2 to 42 episodes per 10,000 admissions, and the proportion of all nosocomial bloodstream infections due to coagulase-negative staphylococci increased from 8 to 26% (6). Although treatment may include the removal of infected intravascular devices or prosthetic materials, nearly all patients with coagulase-negative staphylococcal bloodstream infection receive antibiotic therapy. Beta-lactam antibiotics are generally considered to be the first-line agents for treatment of staphylococcal infections. However, 35 to 80% of coagulase-negative staphylococcal isolates are resistant to these antibiotics (14), and vancomycin is currently the only readily available therapeutic alternative for such infections.

Until recently, there had been no reports of clinically important staphylococcal isolates resistant to vancomycin, and attempts to induce resistance to vancomycin in vitro were unsuccessful (1). Moreover, it was thought that coagulase-negative staphylococci lacked the genetic capacity to develop resistance to vancomycin (2). However, peritonitis caused by *Staphylococcus haemolyticus* with relative resistance to vancomycin in a patient undergoing chronic ambulatory peritoneal dialysis was described in 1987 (14). We report a case of coagulase-negative staphylococcal bloodstream infection caused by *S. haemolyticus* with increased resistance to vancomycin. In this patient, routine microbiological surveillance indicated colonization with a susceptible strain before bloodstream infection. The similarity of restriction endonuclease digest patterns of plasmid DNA from susceptible colonizing strains and subsequent resistant strains causing infection suggests in vivo selection for resistance. Furthermore, the demonstration of rapid in vitro development of resistance by initially susceptible organisms exposed to vancomycin lends support to this concept.

CASE REPORT

An 82-year-old woman (patient 1) was admitted with complaints of sudden onset of left-sided headache followed by vertigo, nausea, vomiting, and diplopia. The physical examination and magnetic resonance imaging findings were consistent with a pontine infarction. Tests performed upon admission demonstrated pancytopenia, and a bone marrow aspirate and biopsy revealed acute promyelocytic leukemia. On day 4 of hospitalization, the patient developed a fever of 38.4°C orally, and because of persistent neutropenia antibiotic therapy with tobramycin, ceftazidime, and vancomycin was begun. A double-lumen Hickman catheter was placed, and the patient underwent chemotherapy with cytosine arabinoside and daunorubicin. Although blood cultures were negative, the fever persisted, resulting in the addition of clindamycin (day 10) and amphotericin B (day 11). When erythema multiforme developed (day 17), aztreonam was substituted for ceftazidime. A repeat bone marrow examination revealed that the induction regimen had failed, and a second course of chemotherapy was begun. The neutropenia and fever persisted, and therapy with tobramycin, vancomycin, aztreonam, and amphotericin B continued. The peak and trough levels of vancomycin in serum throughout this time were 27 to 42 and 9 to 23 μg/ml, respectively. On hospital days 28 to 32, the patient was afebrile, but on day 32, erythema and tenderness around the Hickman catheter site were noted. On the following day, a yellow-green discoloration of the margins was present and the patient developed a fever of 39.3°C. Blood cultures drawn on day 33 were positive (two of four bottles) for *S. haemolyticus* with intermediate susceptibility to vancomycin (MIC, 8.0 to 16

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μg/ml). Persistent leukemia was documented on bone marrow examination, and therapy was withdrawn. The fever continued, increasing lethargy and respiratory distress developed, and the patient died on hospital day 38. No postmortem examination was performed.

In addition to the bloodstream isolate, *S. haemolyticus* was recovered from this patient on three other occasions. Routine weekly surveillance cultures of the throat, urine, and stool are obtained from patients with acute leukemia at the University of Iowa Hospitals, and all staphylococcal and fungal isolates are identified and saved. Importantly, all surveillance cultures from this patient were initially negative for any staphylococcal species. However, *S. haemolyticus* was subsequently isolated from throat (day 17) and stool (day 24) surveillance cultures. These isolates were susceptible to vancomycin (MIC, 1.0 to 2.0 μg/ml). A tracheal aspirate culture (day 34) was also positive for *S. haemolyticus*, but this isolate had an antibiotic susceptibility pattern similar to that of the vancomycin-intermediate bloodstream isolate. These four isolates were the subject of microbiologic analysis.

MATERIALS AND METHODS

Bacterial isolates. Isolates of *S. haemolyticus* obtained from patient 1 (throat, stool, blood, and tracheal aspirate) and nine epidemiologically unrelated patients (patients 2 to 10; all nosocomial bloodstream isolates) were stored frozen at −20°C in skim milk until use in the study. All isolates were identified as coagulase-negative staphylococci by their appearance on blood agar, Gram stain morphology, and positive catalase and negative coagulase tests. Species identification was performed with the API Staph Trac system (Analytab Products, Plainview, N.Y.).

Epidemiologic typing. The isolates from patient 1 were further characterized by biotype, antimicrobial susceptibility profile, and restriction endonuclease analysis of plasmid DNA (REAP) to determine whether they constituted a single strain of *S. haemolyticus*. The similarities and differences in the epidemiologic markers among the four isolates from patient 1 were compared with those observed among the nine epidemiologically unrelated *S. haemolyticus* bloodstream isolates.

Biotype. The biochemical profile (biotype) of each isolate was determined with the Staph Trac identification system (Analytab). All isolates were tested in parallel on the same day to ensure comparability of results. Isolates were considered to belong to different strains if there were at least two differences in the results of the biochemical reactions.

Antimicrobial susceptibility profile. The antimicrobial susceptibility profile (antibiotype) was determined by a standard microdilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS) (10). The antibiotics were obtained from their manufacturers and included chloramphenicol, clindamycin, erythromycin, kanamycin, methicillin, gentamicin, tetracycline, and vancomycin. All isolates were tested on the same day. Isolates were considered to belong to different strains if there were differences of a factor of 4 or more in the MICs of at least three antibiotics in the panel.

REAP. Stored isolates of *S. haemolyticus* were plated on blood agar and incubated at 35°C for 48 h. Multiple (three to five) colonies were removed from the plate with a sterile swab, inoculated into 10 ml of tryptic soy broth, and incubated overnight at 35°C. The organisms were lysed, and DNA was extracted by the method of Nahaie et al. (8). Restriction endonuclease digestion of plasmid DNA was performed with *Eco*RI (New England BioLabs, Inc., Beverly, Mass.) according to the instructions of the manufacturer. DNA was digested at 37°C for 2 h, and the restriction fragments were separated by electrophoresis on 0.7% agarose gels containing ethidium bromide. Electrophoresis was performed with Tris-borate-EDTA buffer at 100 V for 4 h. Bacteriophage lambda DNA digested with *Hin*dIII to yield fragments of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.5 kilobases was included to provide molecular weight markers in each gel. The gels were photographed under UV light, and the restriction digest patterns were compared. As described by Mickelsen et al. (7), isolates which differed by no more than three bands were considered to belong to the same strain.

In vitro susceptibility to vancomycin. Vancomycin was supplied by Eli Lilly & Co. Research Laboratories (Indianapolis, Ind.). The initial in vitro susceptibility determinations on the four clinical isolates from P1 were performed by using a Vitek instrument (Vitek Systems, Inc., Hazelwood, Mo.) and a microdilution method (10). All subsequent determinations of the MIC of vancomycin were performed by the broth macrodilution method as recommended by the NC CLS (10). The tests were performed in cation-supplemented Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, Md.) with vancomycin concentrations ranging from 0.06 to 64 μg/ml. The inoculum was adjusted to 5×10^5 CFU/ml, and MICs were recorded after 24 h of incubation at 35°C. MBCs were determined after the MIC readings according to NCCLS guidelines (9) by sampling each clear tube and plating a 0.01-ml sample onto the surface of a blood agar plate. Plates were incubated at 35°C for 24 h, and the number of visible colonies was recorded. The MBC was recorded as the lowest concentration of antibiotic that produced killing of 99.9% or more of the initial inoculum.

Selection for vancomycin resistance. In vitro selection for vancomycin resistance among the four isolates from patient 1 was performed by broth and agar dilution techniques. Broth selection of vancomycin resistance was performed by incubating the four test isolates (5×10^5 CFU/ml) at 35°C in cation-supplemented Mueller-Hinton broth with and without vancomycin at a concentration equal to the macrodilution MIC for the organism. Following a 48-h incubation, the cells were harvested by centrifugation and washed three times with saline, and vancomycin MICs and MBCs were determined by the macrodilution method.

Agar selection of vancomycin resistance was performed by the pour plate technique described by Schwalbe et al. (14). The four isolates from patient 1 were grown in tryptic soy broth (BBL), harvested, and resuspended at an inoculum concentration of 10^9 CFU/ml. One hundred microliters of inoculum (10^8 CFU) was added to 20 ml of melted Mueller-Hinton agar (BBL) containing vancomycin at concentrations ranging from 4.0 to 128 μg/ml. The vancomycin-containing agar was poured into sterile plastic petri plates (100-mm diameter; Fisher Scientific Co., Pittsburgh, Pa.), solidified at room temperature, and incubated at 35°C for 48 h. Colonies visible on plates containing the highest concentration of vancomycin were selected and grown in vancomycin-free tryptic soy broth, and the pour plate procedure was repeated. After a total of seven pour plate cycles, the numbers of CFU on the vancomycin-containing and control plates were counted, and the frequency of resistance to vancomycin was calculated as the ratio of CFU on vancomycin-containing agar to CFU on control agar. Colonies were selected from the plates containing the highest concentration of vancomycin, and MICs and MBCs were deter-

TABLE 1. Comparison of biotypes, MICs, and REAP subtypes of isolates of *S. haemolyticus* from patient 1 and epidemiologically unrelated patients

Patient no.	Isolate source	Biotype[a]	MIC (μg/ml)[b]								REAP subtype
			CH	CL	ER	KA	ME	GE	TE	VA	
1	Throat	6616151	4.0	16	16	64	32	16	4.0	2.0	A
1	Stool	6616151	8.0	16	16	64	32	4.0	4.0	2.0	A
1	Blood	6616151	4.0	16	16	64	32	4.0	4.0	8.0	A
1	Tracheal aspirate	6616151	4.0	16	16	64	32	4.0	4.0	8.0	A
2	Blood	6636151	8.0	16	16	64	32	4.0	2.0	4.0	B
3	Blood	6636151	8.0	16	16	64	32	32	2.0	4.0	C
4	Blood	6636151	8.0	16	16	64	32	32	2.0	4.0	D
5	Blood	6636151	8.0	16	16	64	32	32	2.0	4.0	E
6	Blood	6636151	4.0	0.13	8.0	64	32	32	2.0	4.0	F
7	Blood	6636151	8.0	16	16	64	32	32	2.0	4.0	G
8	Blood	6636151	4.0	0.13	16	64	32	32	2.0	4.0	H
9	Blood	6636151	8.0	16	16	64	32	16	4.0	4.0	I
10	Blood	6636151	8.0	16	16	64	32	16	2.0	4.0	J

[a] API Staph Trac biotype number.
[b] Abbreviations: CH, chloramphenicol; CL, clindamycin; ER, erythromycin; KA, kanamycin; ME, methicillin; GE, gentamicin; TE, tetracycline; VA, vancomycin.

mined by repeated passage of each strain in vancomycin-free tryptic soy broth for a total of 4 weeks (six passages) followed by determination of the macrodilution MIC.

RESULTS

Epidemiologic typing. All four isolates from patient 1 and the nine epidemiologically unrelated isolates of *S. haemolyticus* had virtually identical biochemical profiles (biotypes) as determined by the API Staph Trac assay (Table 1). Likewise, the antimicrobial susceptibility profiles (antibiotypes) of all 13 isolates were highly resistant and not significantly different. The plasmid profiles of the four isolates from patient 1 before digestion with *Eco*RI differed by no more than two minor (faintly staining) bands (data not shown). Likewise, the REAP DNA patterns of the isolates from patient 1 were all similar, if not identical (Fig. 1), and were significantly different from the patterns of the nine epidemiologically unrelated isolates (Fig. 2). Thus, the four isolates from patient 1 were closely related and probably represented the same strain (subtype) of *S. haemolyticus*, and each of the epidemiologically unrelated isolates represented distinctly different subtypes (Table 1).

In vitro susceptibility to vancomycin and selection for vancomycin resistance. The initial vancomycin MICs for the four isolates from patient 1, obtained by the Vitek instrument and microdilution methods, were 1.0 to 2.0 μg/ml for the two surveillance isolates (throat and stool) and 8.0 to 16 μg/ml for the blood and tracheal aspirate isolates (Table 2). Testing of these isolates by the broth macrodilution method after storage at −20°C yielded MICs of 1.0 μg/ml for the surveillance isolates and 4.0 μg/ml for the blood and tracheal aspirate isolates. After broth selection with vancomycin, the MICs increased to 4.0 μg/ml for the surveillance isolates, 16 μg/ml for the bloodstream isolate, and 32 μg/ml for the tracheal aspirate isolate (Table 2). The MBCs were 32 μg/ml for both the blood and tracheal aspirate isolates and 16 and 64 μg/ml for the surveillance throat and stool isolates, respectively.

The agar pour plate technique employed a large inoculum (10^8 CFU in 20 ml of agar) and allowed for the selection of resistant clones from the initial population of each of the four test isolates (Table 2). Sequential passage of each isolate in progressively higher concentrations of vancomycin produced clones with MICs of 16 to 32 μg/ml and MBCs of 32 to >64 μg/ml. The frequency of resistance (the ratio of CFU in vancomycin medium to CFU in control medium) at a vancomycin concentration of 16 μg/ml ranged from $1:10^3$ (blood isolate) to 1:10 (all others). Resistance to vancomycin was stable at 16 μg/ml in all four isolates following repeated passage in vancomycin-free tryptic soy broth over a period of 4 weeks (Table 2).

DISCUSSION

Since the introduction of penicillin therapy in the 1940s, staphylococcal organisms have repeatedly demonstrated their ability to develop resistance to antibiotics. Although the propensity of *Staphylococcus aureus* to develop antibiotic resistance has been recognized since 1945 and is now well known, the historical perspective on coagulase-negative staphylococci is limited because these organisms have been

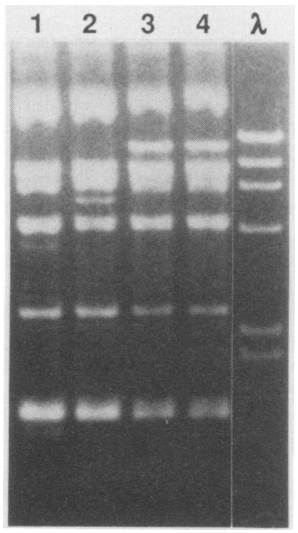

FIG. 1. *Eco*RI digest patterns of plasmid DNA of *S. haemolyticus* isolates from blood (lane 1), tracheal aspirate (lane 2), throat (lane 3), and stool (lane 4) from patient 1. Lane λ, Molecular weight standard of lambda phage DNA digested with *Hin*dIII.

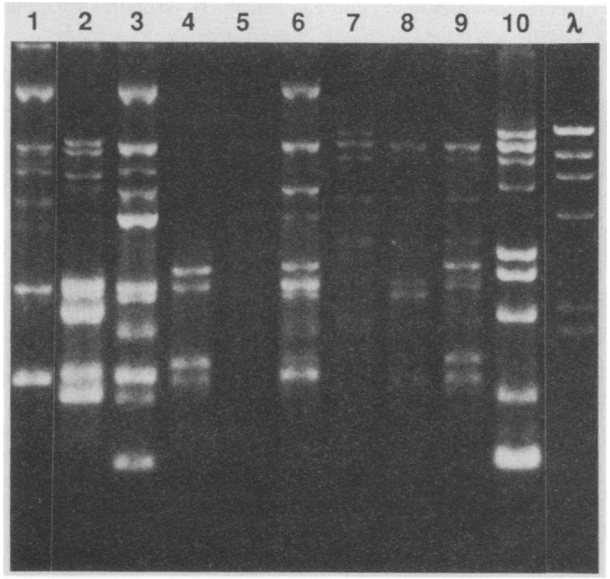

FIG. 2. Comparison of *Eco*RI digest patterns of plasmid DNA of *S. haemolyticus* bloodstream isolates from patient 1 (lane 1) and nine epidemiologically unrelated patients (lanes 2 to 10). Lane λ, *Hin*dIII digestion of lambda phage DNA (molecular weight standard).

recognized as prevalent pathogens only in the past decade. The occurrence of beta-lactam resistance in strains of coagulase-negative staphylococci has indeed increased over this period of time (6, 15), but susceptibility to vancomycin until recently has remained uniform. This report, however, describes colonization with vancomycin-susceptible *S. haemolyticus* and subsequent infection with a closely related but vancomycin-intermediate strain. Similarly high vancomycin MICs were recently reported in a laboratory-based study of nosocomial *S. haemolyticus* isolates in which 62% of 47 multiply resistant strains had vancomycin MICs equal to or greater than 6.25 μg/ml (3). These strains were isolated from various anatomical sites, and none of the vancomycin-intermediate *S. haemolyticus* isolates were associated with active infection.

In our patient with acute leukemia, neutropenia persisted throughout the 38-day hospital course and broad-spectrum antibiotic therapy was administered continuously from hospital day 4. Vancomycin was included in her regimen because of the increasing frequency of gram-positive infections in neutropenic patients, although the role of empirical use of vancomycin in this setting has been debated (4, 11). The recurrence of fever and the pain and erythema about the Hickman line site were very compatible with a true infection due to *S. haemolyticus*; however, the contribution of the infection to her death is unclear.

There are many similarities between this patient and the patient with peritonitis discussed by Schwalbe et al. (14). In both, the species of coagulase-negative staphylococcus involved was *S. haemolyticus*, a prosthetic device was in place (Tenckhoff catheter or Hickman catheter), and the infections occurred in a setting of prolonged antibiotic therapy. Schwalbe et al. (14) demonstrated a stepwise increase in vancomycin resistance in eight clinical isolates obtained sequentially over a period of 88 days of vancomycin therapy; vancomycin MICs increased from 2.0 to 8.0 μg/ml. In our patient the similarity of REAP patterns in the earlier, susceptible isolates and the later, more-resistant isolates and the fact that they were significantly different from REAP patterns of epidemiologically unrelated strains of *S. haemolyticus* suggest an in vivo selection of resistance. This is supported, furthermore, by the fact that in vitro the development of increased resistance to vancomycin following exposure to that agent was readily demonstrated. All isolates had a four- to eightfold increase in vancomycin MICs following a 48-h incubation in vancomycin-containing broth.

Subpopulations with vancomycin MICs of 16 to 32 μg/ml were recovered from all four isolates after sequential passage in vancomycin-containing agar. The expression of resistance was apparently stable, since the vancomycin MICs after serial passage in vancomycin-free tryptic soy broth over a 4-week period were 16 μg/ml. Schwalbe et al. (14), through the same process of expansion of resistant clones and repetition of the pour plate procedure, isolated a strain with a vancomycin MIC of 128 μg/ml. Thus, *S. haemolyticus* clearly possesses the ability to express vancomycin resistance. This may be an increasingly common phenomenon, as evidenced by a recent report documenting intermediate susceptibility to vancomycin (MIC, ≥6.25 μg/ml) in a high percentage of nosocomial *S. haemolyticus* isolates (3). In addition, vancomycin resistance in clinical isolates of species of bacteria other than *S. haemolyticus* has been described (5, 12, 16). The genetic and biochemical mechanisms responsible for this resistance are not clear, although it is of interest that plasmids carrying vancomycin resistance genes have recently been described for *Enterococcus faecium* (5). Although the *Eco*RI digest patterns of the susceptible isolates (throat and stool) of *S. haemolyticus* in the present study contained an 18-kilobase fragment not seen in the more-resistant (blood and tracheal aspirate) isolates (Fig. 1), the relationship between this fragment and expression of vancomycin resistance was unclear and was not investigated further.

If the strain of *S. haemolyticus* with relative resistance to vancomycin causing bloodstream infection in patient 1 was derived from the susceptible, colonizing isolate, some ap-

TABLE 2. Effects of in vitro vancomycin exposure on susceptibility of *S. haemolyticus* isolates to vancomycin

Specimen	Hospital day	Microdilution vancomycin MIC (μg/ml)	Macrodilution MIC of vancomycin (μg/ml) after:			
			No selection	Broth selection	Agar selection	Passage in vancomycin-free media[a]
Throat surveillance	17	1.0–2.0	1.0	4.0	32	16
Stool surveillance	24	1.0–2.0	1.0	4.0	32	16
Blood	33	8.0–16	4.0	16	32	16
Tracheal aspirate	34	8.0–16	4.0	32	16	16

[a] After the agar selection process, the isolates were passaged six times in vancomycin-free media before MIC determinations.

proaches to minimize the in vivo selection of subpopulations with increasing vancomycin resistance need to be developed. There are many events associated with hospitalization that may contribute to selection of resistant strains. However, in a group of 40 patients in our hospital with *S. haemolyticus* bacteremia, antibiotic exposure was found to be significantly associated with the isolation of strains that were multiply antibiotic resistant (unpublished observations). Although the role of antimicrobial agent use as primary cause or confounder for the occurrence of a resistant strain may be debated, the selection of resistance after exposure to vancomycin has now been amply documented; our findings and those of Schwalbe et al. (13, 14) reveal both in vitro and in vivo selections of vancomycin resistance in clinically important isolates of *S. haemolyticus*. Thus, the development of increasing resistance to vancomycin should be considered a potential adverse effect of extended vancomycin therapy.

Although the abilities of the various antimicrobial susceptibility-testing systems to predict strains of *S. haemolyticus* which contain vancomycin-resistant subpopulations are currently unknown, the recent findings of Schwalbe et al. (13) suggest that a double zone of growth around an imipenem disk may provide a useful phenotypic marker for the potential for vancomycin resistance in these isolates. This propensity of *S. haemolyticus*, apparently unique among the coagulase-negative staphylococci, to develop resistance upon exposure to vancomycin suggests that it may be important for clinical laboratories to identify isolates of coagulase-negative staphylococci routinely to the species level. Certainly, many questions remain concerning the frequency and clinical significance of these strains of *S. haemolyticus* and will require additional clinical and laboratory investigation.

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