

Brief Report

THE DEVELOPMENT OF VANCOMYCIN
RESISTANCE IN A PATIENT
WITH METHICILLIN-RESISTANT
STAPHYLOCOCCUS AUREUS INFECTION

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OVER the past two decades, vancomycin has been considered the antibiotic of choice for methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Indeed, multidrug-resistant clones of MRSA for which the only available effective antibacterial agent is vancomycin have recently been identified. Recent reports describing the therapeutic failure of vancomycin for MRSA infections have aroused considerable concern regarding the emergence of MRSA strains for which there will be no effective therapy.¹⁻³ The mechanism of reduced susceptibility in these staphylococcal strains has not been identified, although data indicate that it is not the same as the vancomycin-resistance mechanism in enterococcal strains.⁴

We describe here microbiologic properties of MRSA strains recently isolated from a patient receiving vancomycin therapy. The last of the three isolates, recovered shortly before the patient's death, showed resistance to vancomycin.

CASE REPORT

A 79-year-old man with end-stage renal disease due to hypertension began hemodialysis in August 1996. In November and December 1997, repeated thrombosis of his arteriovenous Gore-Tex graft occurred, and on December 15 an internal jugular catheter was inserted. On December 31, 1997, he was admitted to the hospital with fever and altered mental status, and both of two blood cultures were positive for MRSA. One gram of vancomycin had been given intravenously at the outpatient dialysis center three hours before these positive blood-culture results were obtained. He had also received 1 g of intravenous vancomycin 12 months and again 6 weeks before this admission, when thrombectomies of the arteriovenous graft were performed. He received 500 mg of vancomycin intravenously on days 2 and 4 of this admission and twice weekly thereafter. A culture of blood taken four days

after admission was negative. The internal jugular catheter broke and was removed on January 10, 1998; cultures of the catheter tip were positive for MRSA. The atrioventricular graft became thrombosed, and a new graft was inserted on January 20. The original graft, however, was left in place. The patient was discharged on January 30, 1998, and continued to receive 500 mg of vancomycin intravenously twice weekly until February 13, 1998. During therapy, the concentration of vancomycin in five random serum samples ranged from 6.3 to 17.3 μg per milliliter.

The patient was readmitted on March 20, 1998, with fever (temperature, 40°C), altered mental status, and shortness of breath. Blood cultures on admission were positive for MRSA. Intravenous vancomycin, tobramycin and ceftriaxone were administered, but he died the next day. An autopsy was not performed.

METHODS

Bacterial strains used in this study and their relevant properties are listed in Table 1. Species present in the isolates were identified with use of the API-Staph Test System (BioMerieux Vitek, Hazelwood, Mo.). Tryptic soy broth and tryptic soy agar (Difco, Detroit) were used for the cultivation and analysis of bacterial cultures, which were grown at 37°C with vigorous aeration. The antibiotics used were oxacillin, nafcillin, cefazolin, cefotaxime, gentamicin, clindamycin, erythromycin, ciprofloxacin, and vancomycin (obtained from various manufacturers).

Chromosomal DNA was prepared and digested with the *Sma*I endonuclease (New England Biolabs, Beverly, Mass.), and the DNA fragments were separated by pulsed-field gel electrophoresis with a CHEF-DRII apparatus (Bio-Rad, Hercules, Calif.), as described previously.⁷

Testing for Susceptibility to Antimicrobial Agents

Susceptibility to antibiotics was determined by broth dilution and, in the case of vancomycin and β -lactam antibiotics, by population analysis⁸ as well. In the population-analysis method, bacterial cultures grown overnight with aeration ($\geq 10^9$ colony-forming units per milliliter) were plated at several dilutions each on a set of tryptic-soy-agar plates containing serial dilutions of the test antibiotic. Plates were incubated at 37°C for 48 hours, and the bacterial colonies were then counted. Plotting colony counts against drug concentrations provided a graphic representation (a population-analysis profile) of the composition of the bacterial culture in relation to the homogeneity or heterogeneity of the antibiotic-susceptibility phenotype. The minimal inhibitory concentration was defined as the lowest concentration of the antibiotic that prevented the appearance of 99.9 percent of the bacterial colonies.

Characterization of Vancomycin-Resistant Isolates

Morphologic changes in the vancomycin-resistant isolates grown in the presence of vancomycin were assessed by phase-contrast microscopy and thin-section electron microscopy, as described previously.⁹ Induction of autolysis was measured with Triton X-100,¹⁰ and the concentration of vancomycin in the growth medium was determined by bioassay.¹⁰ The stability of vancomycin resistance was determined by serial culture (passage) of the bacteria for more than 70 generations in drug-free culture medium.

RESULTS

Reduced Susceptibility to Vancomycin and Resistance to Other Antibiotics in MRSA Strain PC-3

MRSA isolates labeled PC-1 and PC-2 were recovered from this patient on December 31, 1997, and January 10, 1998. The clinical sources of PC-1 and PC-2 were blood and the site of catheter exit, respectively. MRSA isolate PC-3 was recovered from blood on March 20, 1998, the day before the patient died.

The minimal inhibitory concentrations of vanco-

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TABLE 1. PROPERTIES OF MRSA STRAINS.

STRAIN	DATE OF ISOLATION	MINIMAL INHIBITORY CONCENTRATION OF VANCOMYCIN μg/ml	FREQUENCY OF SUBPOPULATIONS ACCORDING TO VANCOMYCIN CONCENTRATION*			SOURCE OF DATA
			2 μg/ml	4 μg/ml	8 μg/ml	
PC-1	12/31/97	2.0	5×10^{-4}	6×10^{-6}	$\leq 10^{-9}$	Current study
PC-2	1/10/98	2.0	10^{-3}	5×10^{-7}	$\leq 10^{-9}$	Current study
PC-3	3/21/98	8.0	1	1	2×10^{-3}	Current study
PC-3*	—†	16.0	1	1	1	Current study
II/13	1996	1.0	10^{-5}	8×10^{-8}	$\leq 10^{-9}$	Roberts et al. ⁵
IV/5	1996	1.0	2×10^{-5}	6×10^{-7}	5×10^{-8}	Roberts et al. ⁵
LHH-3	1994	1.0	6×10^{-7}	3×10^{-8}	$\leq 10^{-9}$	de Lencastre et al. ⁶
MMC-2	1994	1.0	5×10^{-6}	5×10^{-9}	$\leq 10^{-9}$	de Lencastre et al. ⁶
NYH-2	1994	1.0	10^{-4}	8×10^{-5}	9×10^{-7}	de Lencastre et al. ⁶
NYH-2*	—†	8.0	1	6×10^{-1}	6×10^{-4}	Current study
QNS-2	1994	2.0	5×10^{-6}	5×10^{-7}	7×10^{-9}	de Lencastre et al. ⁶
SH-3	1994	1.0	8×10^{-7}	7×10^{-8}	$\leq 10^{-9}$	de Lencastre et al. ⁶
WMC-1	1994	2.0	10^{-3}	8×10^{-6}	4×10^{-7}	de Lencastre et al. ⁶

*Frequencies were determined by population analysis.

†PC-3* and NYH-2* are in vitro derivatives of the corresponding clinical isolates.

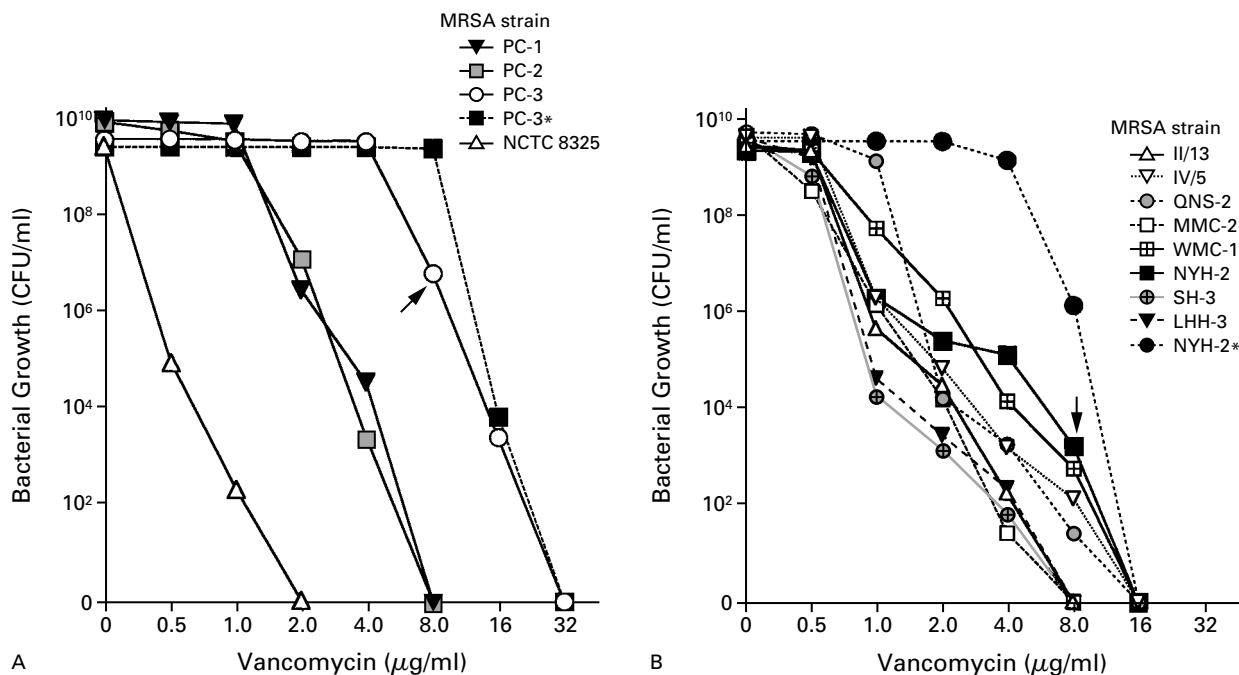


Figure 1. Susceptibility of MRSA Strains to Vancomycin.

MRSA strains PC-1, PC-2, PC-3, and control strain NCTC 8325 were grown in tryptic soy broth overnight and plated on vancomycin-containing agar at various cell concentrations for population analysis (see the Methods section). A colony of PC-3 capable of growing on agar containing 8 μg of vancomycin per milliliter was picked (arrow) and used to inoculate fresh, antibiotic-free tryptic soy broth. After overnight growth, this culture, named PC-3*, was also plated for population analysis (dashed line) (Panel A). Cultures of eight MRSA isolates, closely related genetically and representing an MRSA clone widespread in New York City, were recovered in eight hospitals and also tested by population analysis.³ A colony of strain NYH-2 growing on agar containing 8 μg of vancomycin per milliliter was picked (arrow) and used to inoculate antibiotic-free tryptic soy broth. A culture called NYH-2* was generated, and the population-analysis profile constructed (Panel B). CFU denotes colony-forming units.

mycin, as determined by the broth-dilution method and from the population-analysis profiles, were 2 μg per milliliter for PC-1 and PC-2 and 8 μg per milliliter for PC-3 (Fig. 1A). All three of these isolates were resistant to gentamicin, clindamycin, erythromycin, and ciprofloxacin, and all three gave a positive signal with a *mecA*-specific DNA probe.⁶ The minimal inhibitory concentrations of oxacillin, as determined by broth dilution, were 3 μg per milliliter for PC-1 and PC-2 and 0.8 μg per milliliter for PC-3. Population-analysis profiles showed that each isolate had heterogeneous resistance to oxacillin (data not shown).

Genetic Relatedness of MRSA Isolates PC-1, PC-2, and PC-3

Figure 2 shows that the *Sma*I digestion of chromosomal DNA from isolates PC-1, PC-2, and PC-3 produced identical DNA-fingerprint patterns, indi-

cating their genetic identity. This finding and the clinical history of the patient suggest that PC-3 was derived from the earlier isolates PC-1 and PC-2, presumably as a result of in vivo selection during therapy with vancomycin.

Selection of Highly Vancomycin-Resistant *S. aureus* in Vitro

Bacteria capable of growing on agar plates containing vancomycin at a concentration of 8 μg per milliliter were present at a frequency of approximately 10^{-3} in cultures of strain PC-3. A colony picked from agar containing 8 μg of vancomycin per milliliter (Fig. 1A, arrow) was dispersed in broth and used at a low cell concentration (about 100 cells per milliliter) as inoculum to initiate a broth culture that contained no vancomycin. The next day, the culture was turbid with growth and was plated for population

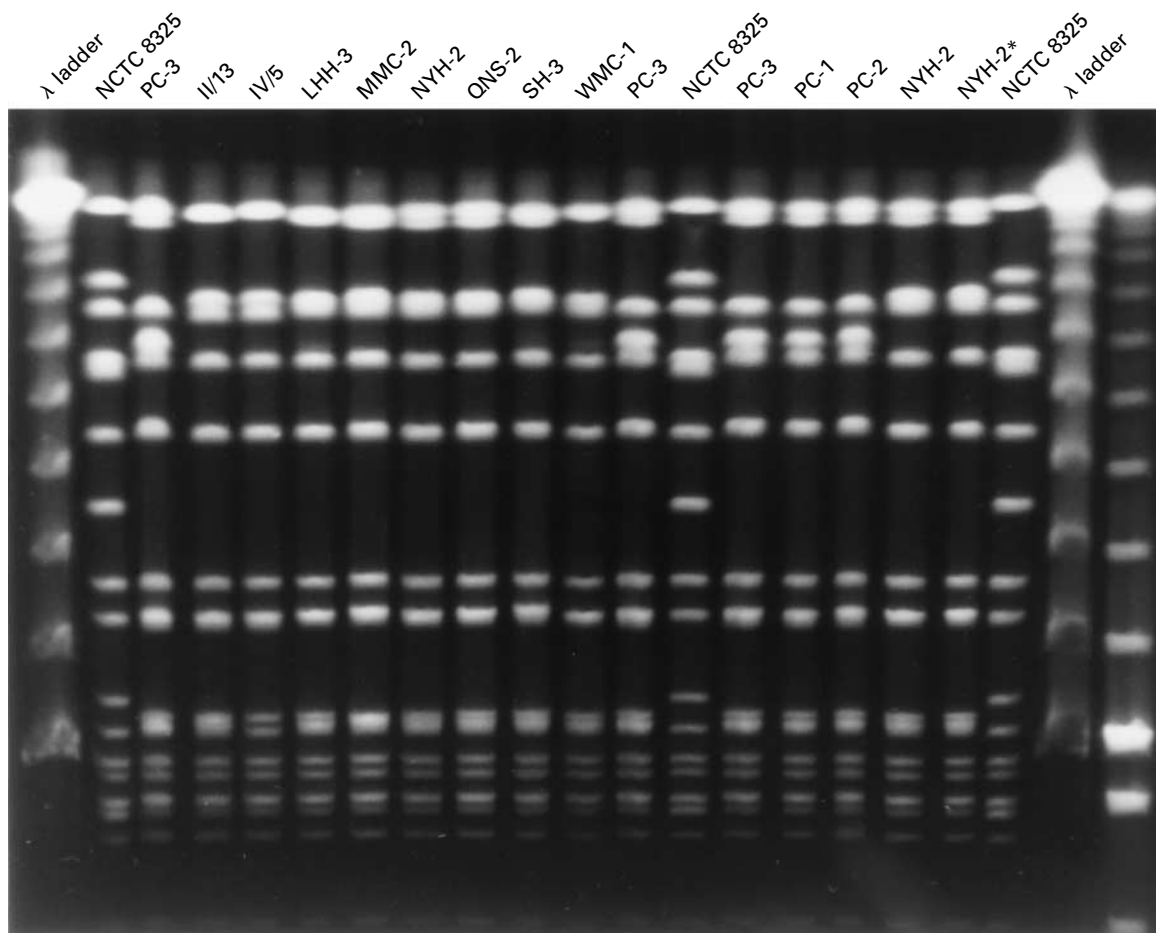


Figure 2. Pulsed-Field Gel Electrophoretic Patterns of the Vancomycin-Resistant Isolate PC-3 and MRSA Isolates Recovered in Eight Hospitals in New York City.

For identification of the strains, see Table 1 and Figure 1. The right-hand lane is a low-molecular-weight ladder, and the λ ladders are λ -phage DNA molecular-weight markers.

analysis (Fig. 1A). All the cells in this culture, denoted PC-3*, could grow on agar containing 8 μg per milliliter of vancomycin, and the minimal inhibitory concentration for the majority of the cells in the culture increased to 16 μg per milliliter. The culture of PC-3* remained heterogeneous and contained (at a frequency of approximately 10^{-5}) bacteria capable of growing even on 16 μg of vancomycin per milliliter.

Genetic Relatedness to Strain PC-3 of an MRSA Clone Widely Spread in Metropolitan New York City

The patterns of the three PC isolates on pulsed-field gel electrophoresis were compared with that of a multidrug-resistant MRSA clone that was recently shown to be widely distributed in hospitals in metropolitan New York (the "New York MRSA").^{5,6} Figure 2 shows the electrophoretic patterns of representatives of this MRSA that were recovered in eight hospitals in metropolitan New York. Comparison of the fingerprints clearly showed that the PC isolates were close relatives (subtype variants on pulsed-field gel electrophoresis) of this highly prevalent MRSA clone.

Susceptibility to Vancomycin

We used population analysis to study the susceptibility to vancomycin of MRSA isolates recovered in hospitals in the New York metropolitan area and sharing the pulsed-field electrophoretic type that was closely related to the pattern of our patient's isolates (Fig. 1B and Table 1). All the strains examined showed heterogeneous vancomycin-resistance phenotypes. For instance, both strains QNS-2 and WMC-1 contained large subpopulations (frequency, approximately 10^{-1} and 10^{-2} , respectively) of bacteria that could grow on agar containing 1 μg of vancomycin per milliliter, and bacteria with even higher minimal inhibitory concentrations were also present, at low but measurable frequencies (Fig. 1B). A colony of strain NYH-2 picked from the agar plate with 8 μg of vancomycin per milliliter (Fig. 1B) and used to inoculate drug-free broth was grown overnight and replated for population analysis. For this strain (NYH-2*), the majority of bacteria had a minimal inhibitory concentration of vancomycin of 8 μg per milliliter, with a population profile similar to that of strain PC-3.

Some Properties of the Vancomycin-Resistant Strain PC-3

Thin sections of PC-3 examined by electron microscopy showed the typical appearance of *S. aureus*, with no thickening of the cell wall. However, growth of the bacteria in the presence of vancomycin resulted in the formation of multicellular aggregates with large quantities of material on their surface and with staining properties similar to those of cell walls (Fig. 3). Measurement of free vancomycin in cultures of PC-3 grown in the presence of 8 μg of the antibiotic per milliliter showed that it gradually decreased in con-

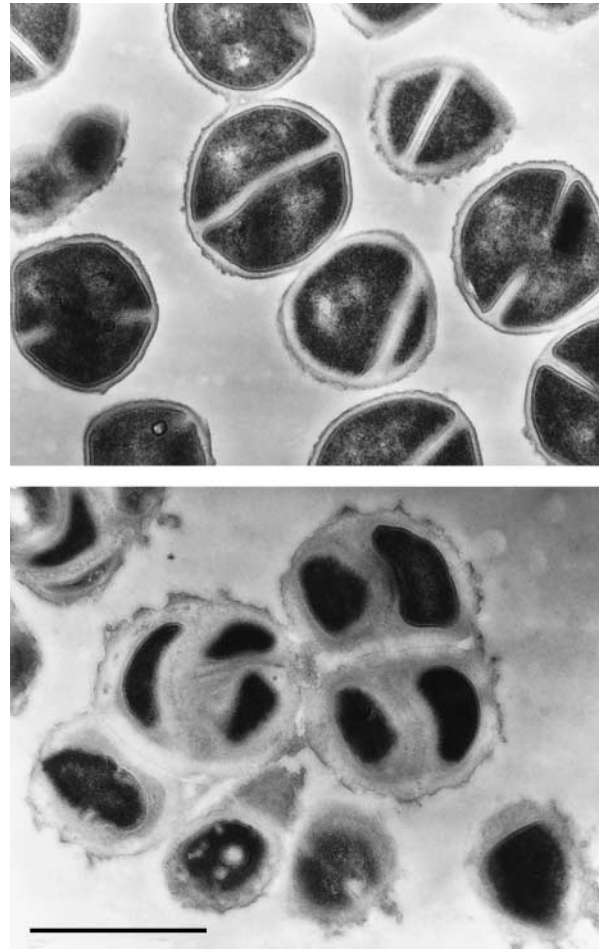


Figure 3. Morphologic Abnormality of the Vancomycin-Resistant Isolate PC-3 Grown in the Presence of Vancomycin.

The top panel shows a culture of PC-3 grown in tryptic soy broth without antibiotic. The bottom panel shows the same bacteria grown in the presence of 8 μg of vancomycin per milliliter. Cultures were harvested at the midexponential phase of growth and were prepared for transmission-electron microscopy. The bar represents 1 μm .

centration and eventually disappeared from the medium during growth of the bacteria. Vancomycin that disappeared from the medium could then be recovered in its biologically active form from the purified cell walls of PC-3 (Fig. 4). In addition, vancomycin inhibited autolysis in PC-3 cultures (data not shown).

These properties are similar to those recently observed in a vancomycin-resistant laboratory mutant of an MRSA strain.¹⁰ Yet another similarity between the vancomycin-resistant clinical isolate described here and the vancomycin-resistant laboratory mutant VM was the inverse relation between their minimal inhibitory concentrations of vancomycin and methicillin.¹⁰ Thus, the minimal concentrations of oxacillin that inhibited the growth of strains PC-1 and PC-2 (mini-

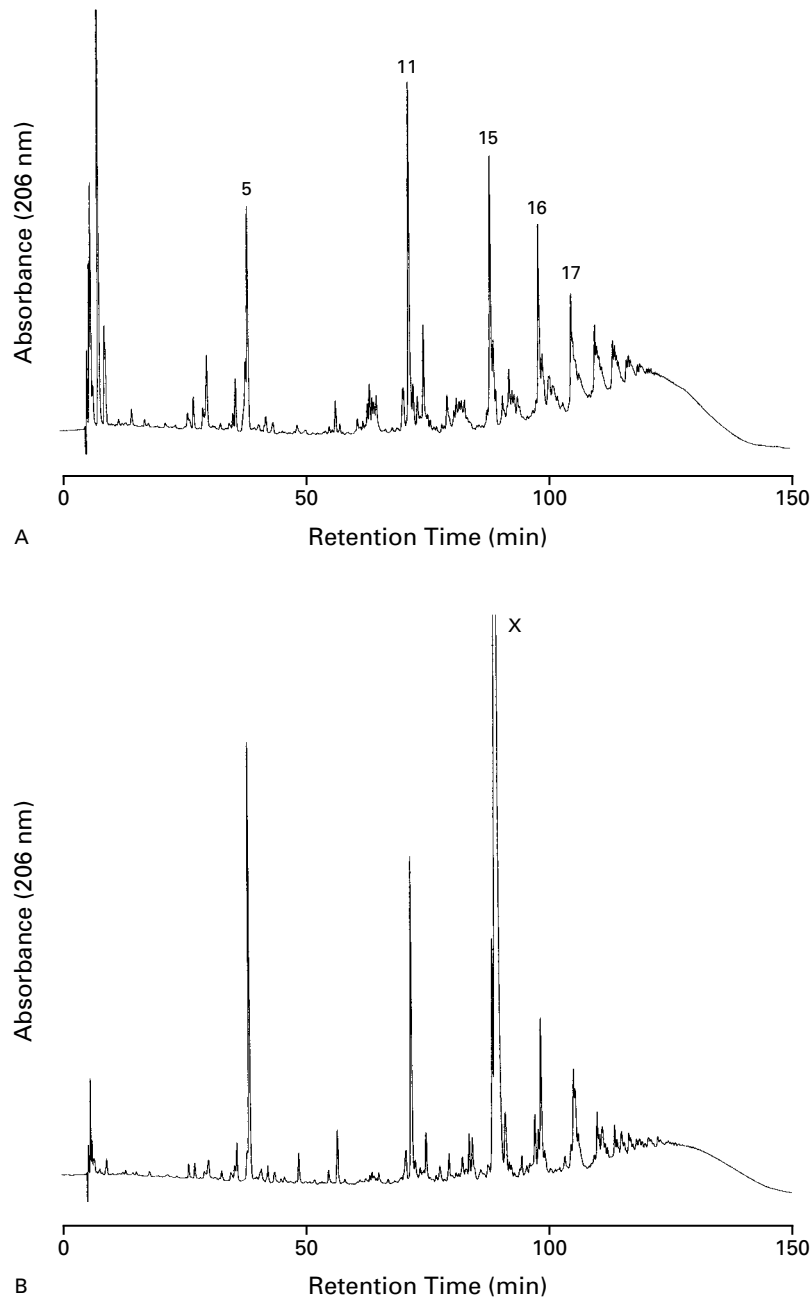


Figure 4. High-Performance Liquid Chromatography of Cell Walls of Strain PC-3 Grown in the Presence of Vancomycin.

Cell walls of PC-3 grown without vancomycin (Panel A) or with 8 μg of vancomycin per milliliter (Panel B) were analyzed by high-performance liquid chromatography. Numbers identify major mucopeptide components.⁵ X denotes a peak representing recovered vancomycin.

mal inhibitory concentration of vancomycin, 2 μg per milliliter) were 3 μg per milliliter, whereas strain PC-3 (for which a higher concentration of 8 μg of vancomycin per milliliter is required) was inhibited with only 0.8 μg of oxacillin per milliliter.

Effect of Synergistic Combinations of Vancomycin and β -Lactam Antibiotics

Previous observations indicated that inhibitors of early cell-wall synthesis, including vancomycin, can reduce methicillin resistance in MRSA.¹¹ In addition, the minimal inhibitory concentrations of several β -lactam antibiotics were reduced in a highly vancomycin-resistant MRSA strain¹⁰ and in strain PC-3 and its derivative PC-3*. On the basis of these findings, we decided to test the effectiveness of combinations of vancomycin and several β -lactam antibiotics.

Figures 5A and 5B show the effect of a combination of oxacillin and vancomycin on the survival of vancomycin-resistant strain PC-3*, an in vitro derivative of clinical isolate PC-3. The population-analysis profile (Fig. 5A) indicates that inclusion of oxacillin at a concentration of 0.4 μg per milliliter, a value below the minimal inhibitory concentration, in the agar plates containing vancomycin caused a significant reduction in the minimal inhibitory concentration of vancomycin, from 16 to 1 μg per milliliter. With van-

comycin at a concentration of 16 μg per milliliter or greater in combination with 0.4 μg of oxacillin per milliliter, no surviving colonies of PC-3* could be detected. Figure 5B shows a similar elimination of the vancomycin-resistant *S. aureus* by the β -lactam-vancomycin combination when vancomycin was incorporated at a constant concentration of 8 μg per milliliter into agar plates containing various concentrations of oxacillin. The minimal inhibitory concentration of oxacillin decreased (from 0.8 to <0.1 μg per milliliter) with the addition of vancomycin to the growth medium. At an oxacillin concentration of 3 μg per milliliter or greater in combination with 8 μg of vancomycin per milliliter, no surviving colonies of PC-3* could be detected. Similar results were obtained when oxacillin was replaced with nafcillin, cefazolin, or cefotaxime.

DISCUSSION

There has been concern about the development of vancomycin resistance in multidrug-resistant strains of *S. aureus*, especially since the demonstration of successful transfer of the *vanA* gene from enterococci to *S. aureus* under laboratory conditions.¹² Acquisition of the enterococcal vancomycin-resistance mechanism by staphylococci has not yet been observed in clinical isolates. On the other hand, reduced suscep-

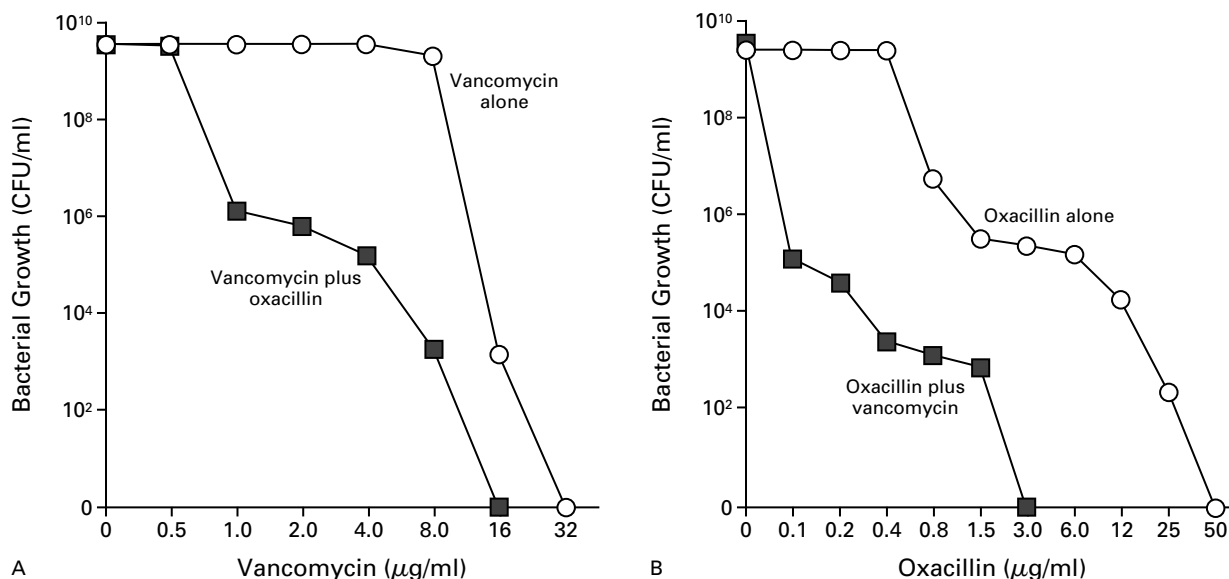


Figure 5. Inhibition of the Vancomycin-Resistant Strain PC-3* by a Combination of Vancomycin and Oxacillin.

A culture of PC-3* grown overnight was plated at different cell concentrations on two sets of tryptic soy agar plates: the first set contained various concentrations of vancomycin, and the second contained the same concentrations of vancomycin as well as a constant concentration of oxacillin (half the minimal inhibitory concentration, or 0.4 μg per milliliter) (Panel A). The same culture was also plated on two additional series of tryptic soy agar plates: the first set contained various concentrations of oxacillin; the second contained various concentrations of oxacillin as well as a constant concentration of vancomycin (half the minimal inhibitory concentration, or 8 μg per milliliter) (Panel B). CFU denotes colony-forming units.

tibility to vancomycin has now been described in both Japan and the United States in association with the failure of vancomycin treatment of MRSA infections.¹⁻³ Although the mechanism of staphylococcal resistance to vancomycin is not clear, a mechanism involving alterations in the bacterial cell wall and capture of antibiotic molecules at a distance from the sites of cell-wall synthesis has been proposed on the basis of properties of a vancomycin-resistant laboratory mutant.¹⁰ Clearly, elucidation of the mechanism of staphylococcal resistance to vancomycin is urgently needed, since current efforts in drug development are directed against the enterococcal mechanism of vancomycin resistance, which is distinct from that of staphylococci.¹⁰

The susceptibility of the MRSA strain PC-3 to vancomycin is similar to those of recently described *S. aureus* isolates with intermediate levels of vancomycin resistance from hospitals in Japan and the United States.¹⁻³ However, bacteria with minimal inhibitory concentrations of vancomycin as high as 16 µg per milliliter could easily be selected under laboratory conditions by inoculating drug-free growth medium with the more resistant subpopulations. The current experiments demonstrate the relative ease with which bacteria with elevated minimal inhibitory concentrations can take over a culture. The genetic identity among strains PC-1, PC-2, and PC-3 on DNA fingerprinting indicates that such a selection or takeover by the more highly resistant bacteria occurred in vivo, presumably as a consequence of suboptimal therapy with vancomycin in patients with an intravascular foreign body. Our data demonstrate that selection for increased resistance to vancomycin can occur during therapy.

The close genetic relatedness of vancomycin-resistant strain PC-3 to an MRSA clone that is widespread in hospitals in the New York metropolitan area is of obvious concern. On examination, several isolates belonging to this MRSA clone and recovered in eight hospitals did not include isolates associated with a reduced minimal inhibitory concentration of vancomycin similar to that for strain PC-3. However, several of the MRSA isolates from the New York hospitals contained subpopulations of bacteria with minimal inhibitory concentrations of vancomycin greater than those of the majority of cells and surpassing those of strains PC-1 and PC-2. The continued extensive use of vancomycin, together with the possibility of in vivo selection, as demonstrated in the current study, makes surveillance of vancomycin-susceptibility levels in MRSA isolates of primary importance.

Our experiments with the combination of β-lac-

tam antibiotics and vancomycin showed that at easily achievable concentrations such combinations were highly effective against the vancomycin-resistant MRSA strain. No surviving bacteria could be detected after exposure of the vancomycin-resistant strain to a combination of 8 µg of vancomycin and 3 µg of oxacillin per milliliter. It remains to be seen whether this combination of antibiotics is equally effective against other *S. aureus* isolates with intermediate levels of resistance to vancomycin. If the results of this experiment can be applied to the clinical setting, our data suggest that a combination of vancomycin and commonly available β-lactam agents may be an effective therapeutic regimen against vancomycin-resistant staphylococci.

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