

Vancomycin-Resistant *Staphylococcus aureus* Isolate from a Patient in Pennsylvania

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A vancomycin-resistant *Staphylococcus aureus* (VRSA) isolate was obtained from a patient in Pennsylvania in September 2002. Species identification was confirmed by standard biochemical tests and analysis of 16S ribosomal DNA, *gyrA*, and *gyrB* sequences; all of the results were consistent with the *S. aureus* identification. The MICs of a variety of antimicrobial agents were determined by broth microdilution and macrodilution methods following National Committee for Clinical Laboratory Standards (NCCLS) guidelines. The isolate was resistant to vancomycin (MIC = 32 µg/ml), aminoglycosides, β-lactams, fluoroquinolones, macrolides, and tetracycline, but it was susceptible to linezolid, minocycline, quinupristin-dalfopristin, rifampin, teicoplanin, and trimethoprim-sulfamethoxazole. The isolate, which was originally detected by using disk diffusion and a vancomycin agar screen plate, was vancomycin susceptible by automated susceptibility testing methods. Pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested genomic DNA indicated that the isolate belonged to the USA100 lineage (also known as the New York/Japan clone), the most common staphylococcal PFGE type found in hospitals in the United States. The VRSA isolate contained two plasmids of 120 and 4 kb and was positive for *mecA* and *vanA* by PCR amplification. The *vanA* sequence was identical to the *vanA* sequence present in Tn1546. A DNA probe for *vanA* hybridized to the 120-kb plasmid. This is the second VRSA isolate reported in the United States.

In 1988, when the first *Enterococcus faecium* isolate with transmissible vancomycin resistance was reported in France (24), public health officials and infection control specialists were concerned that the *vanA* determinant, which mediated high-level vancomycin resistance in the enterococcal isolate, would be transferred to *Staphylococcus aureus* (6, 18, 39), an organism considerably more virulent than *E. faecium* (25, 37). In 1992, Noble and coworkers successfully transferred the *vanA* determinant from an *E. faecalis* donor to an *S. aureus* recipient (29), further heightening the concern about the potential spread of *vanA* to *S. aureus*.

Thus, it surprised many scientists that the first *S. aureus* isolate reported to manifest reduced susceptibility to vancomycin did not contain *vanA* or any of the other known vancomycin resistance determinants. Instead, the reduced susceptibility has been attributed to unusually thickened cell walls containing D-alanyl-D-alanine targets capable of binding vancomycin (11, 15, 17). The appearance of similar vancomycin-intermediate *S. aureus* strains in the United States (33, 34, 36), France (31), Brazil (30), Korea (22), and elsewhere during the next several years (14) fueled speculation that the transfer of *vanA* from enterococci to staphylococci may not occur in nature. Such speculations were discarded in June 2002 when a *vanA*-containing vancomycin-resistant *S. aureus* (VRSA) isolate was ob-

tained from a dialysis patient in Michigan (8). The isolate was also resistant to oxacillin, levofloxacin, and rifampin. Only 2 months later, a second VRSA isolate was obtained from a patient in Pennsylvania (7). This report describes the characterization of the second VRSA strain.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* HIP11983 was obtained from a patient at the Hershey Medical Center, Hershey, Pa. *S. aureus* HIP11714, for which the vancomycin MIC was 1,024 µg/ml (8), was obtained from the Michigan Department of Community Health. *Enterococcus faecalis* A256 was described by Shlaes et al. (32). All cultures were grown in brain heart infusion (BHI) broth or BHI agar with appropriate antimicrobial agents.

Confirmation of species identification. VRSA isolates were identified as *S. aureus* using traditional biochemical tests, including catalase, coagulase, slide agglutination, and acid production from glucose, D-maltose, D-trehalose, D-mannitol, sucrose, and β-D-fructose (2). Species identification was confirmed genotypically by DNA sequence analysis of 16S rDNA, *gyrA*, and *gyrB*. PCR assays were used to screen for enterococcal ligase genes (see below).

Susceptibility testing. MICs were determined by broth microdilution and broth macrodilution methods using cation-adjusted Mueller-Hinton broth (Difco) and National Committee for Clinical Laboratory Standards (NCCLS) procedures (28). The antimicrobial agents were obtained from the following manufacturers. Amikacin, gentamicin, kanamycin, minocycline, oxacillin, penicillin G, rifampin, tetracycline, and trimethoprim-sulfamethoxazole were from Sigma Chemical Co. (St. Louis, Mo.). Meropenem was from Astra-Zeneca Pharmaceuticals (Wilmington, Del.). Quinupristin-dalfopristin, teicoplanin, and telithromycin were from Aventis Pharmaceuticals, Inc. (Sommeret, N.J.). Clarithromycin was from Abbott Laboratories (Abbott Park, Ill.). Erythromycin, oritavancin, tobramycin, and vancomycin were from Lilly Research Laboratories (Indianapolis, Ind.). Ciprofloxacin, faropenem, and moxifloxacin were from Bayer (West Haven, Conn.). Garenoxacin and gatifloxacin were from Bristol-Myers Squibb (Princeton, N.J.). Daptomycin was from Cubist Pharmaceuticals, Inc. (Lexington, Mass.), and TD-6424 was from Theravance (South San Fran-

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FIG. 1. Disk diffusion and Etest analysis of the PA-VRSA isolate on Mueller-Hinton agar. A zone of complete growth inhibition can be observed within a wider zone of reduced growth around both the Etest strip and the 30- μ g vancomycin disk. The arrows indicate the presence of small colonies within the inner zone of inhibition that impact the reading of the MIC result.

cisco, Calif.). Imipenem and ertapenem were from Merck and Co. (Rahway, N.J.), levofloxacin and RWJ54428 were from Johnson & Johnson (Raritan, N.J.), and azithromycin and clinafloxacin were from Pfizer, Inc. (Groton, Conn.). Linezolid was from Pharmacia-Upjohn (Kalamazoo, Mich.), ranbezolid was from Ranbaxy Laboratories (New Delhi, India); sitafloxacin was from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), iclaprim was from Arpida AG (Münchenstein, Switzerland), clindamycin was from United States Pharmaceuticals (Rockville, Md.), and tigecycline was from Wyest-Ayerst Laboratories (Pearl River, N.Y.). Etest strips were used in conjunction with Mueller-Hinton agar as described by the manufacturer (AB Biodisk, Piscataway, N.J.). Automated susceptibility testing with the Vitek (bioMérieux, Inc., Durham, N.C.), Vitek2 (bioMérieux), and MicroScan (MicroScan Walkaway SI, Dade Behring, Inc., West Sacramento, Calif.) was performed according to the manufacturers' instructions, except that two separate inocula were prepared for each analysis: one was grown on blood agar plates and the other was grown on BHI vancomycin agar screen plates containing 6 μ g of vancomycin per ml. Quality control strains included *S. aureus* ATCC 29213, *S. aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *E. faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 (28).

Time-kill studies. Time-kill studies with the VRSA isolate were conducted using cation-adjusted Mueller-Hinton broth supplemented with antimicrobial agents at the MIC of the agent, two times the MIC, and four times the MIC. Growth controls were included in each experiment. The initial inoculum was 5×10^5 to 5×10^6 CFU/ml. Viability counts of antimicrobial agent-containing cultures were performed at 0, 3, 6, 12, and 24 h. Colony counts were determined from plates containing 30 to 300 bacterial colonies. The upper limit of sensitivity was 300 CFU/ml. Time-kill assays were analyzed to determine which antimicrobial agents produced a $\Delta \log_{10}$ CFU/ml of -1 (90% killing), -2 (99% killing), and -3 (99.9% killing). Assay readings were taken at 3, 6, 12, and 24 h and compared with bacterial counts at 0 h. Bactericidal activity was defined as the lowest antimicrobial agent concentration that reduced the original inoculum by $\geq 3 \log_{10}$ CFU/ml (99.9%), and bacteriostatic activity was defined as a reduction in the original inoculum of $< 3 \log_{10}$ CFU/ml.

PCR and DNA sequence analysis. The 16S ribosomal DNA sequences were amplified and sequenced by the method of Ibrahim et al. (19). Oligonucleotide primers for the *S. aureus gyrA* sequence were designed from gene sequence data published by Margerrison et al. (26). The *gyrA* forward primer, GYRA1 (5'-G AACTCTTGATGGCTGA), and reverse primer, GYRA2R (5'-TGACGGCTC TCTTCATTAC), encompassed the quinolone resistance-determining region and flanking nucleotide sequences, producing a 484-bp gene fragment. The primers were selected using Oligo 6 software (Molecular Biology Insights, Inc.,

TABLE 1. MICs of antimicrobial agents against the PA-VRSA isolate

Target	Class	Antibacterial	MIC (μ g/ml)		
Cell wall	Glycopeptide	Vancomycin	32		
		Teicoplanin	8		
		Oritavancin	0.25		
		TD-6424	0.5		
	β -Lactam	Penicillin G	32		
		Oxacillin	>64		
		Imipenem	4		
		Meropenem	16		
		Ertapenem	32		
		Faropenem	16		
		RWJ54428	0.5		
		Cell membrane	Lipopeptide	Daptomycin	0.5
				Ribosome	Macrolide
		Erythromycin	>64		
Clarithromycin	>64				
Azithromycin	>64				
Telithromycin	>64				
Clindamycin	>64				
Streptogramin	Quinupristin-dalfopristin	1			
Aminoglycoside	Kanamycin	Kanamycin	>64		
		Gentamicin	64		
	Tobramycin	>64			
	Amikacin	32			
	Tetracycline	Tetracycline	64		
		Minocycline	0.12		
	Glycylcycline	Tigecycline	0.12		
		Oxazolidinone	Linezolid	1	
		Ranbezolid	1		
	Topoisomerase	Quinolone	Ciprofloxacin	>64	
Levofloxacin			32		
Gatifloxacin			8		
Moxifloxacin			4		
Clinafloxacin			2		
Sitafloxacin			1		
Garenoxacin			2		
RNA polymerase			Rifampicin	Rifampin	≤ 0.06
	Folic acid metabolism	Trimethoprim-sulfamethoxazole		0.25	
Diaminopyrimidine	Iclaprim	Iclaprim	0.25		

Cascade, Colo.). Oligonucleotide primers for *gyrB* were those described by Kaatz and Seo (20). Amplification of *vanA* (9), *mecA* (38), and *aac(6')-aph(2'')* (21) were performed as previously described. An additional 19 PCR primers specific for the components of the *vanA* transposon Tn1546 (i.e., *vanR*, *vanS*, *vanH*, *vanX*, *vanY*, and *vanZ*) described by Arthur et al. (1) were used. Other primers designed using Oligo 6 software and the known sequence of Tn1546 (GenBank accession no. M97297) included *vanS4* (forward) (5'-CTT CCA GCG ATG CCA GAC TTG) (corresponding to bases 5762 to 5782) and *vanS2* (reverse) (5'-CAA GTC TGG CAT CGC TGG AAG) (bases 5782 to 5762). Primer pairs p9-p10, p11-vanS2, p13-p14, p15-p16, p17-p18, and p19-p1 yielded products of the pre-

TABLE 2. Results of automated susceptibility testing of VRSA isolates

<i>S. aureus</i> strain ^a	MICs ^b (μ g/ml) by method:		
	MicroScan	Vitek	Vitek2
PA-VRSA (uninduced)	4, ≤ 2 , ≤ 2	2, 1, 4	2, 2, 2
PA-VRSA (induced)	≤ 2 , ≤ 2 , 4	1, 16, 16	8, 8, 8
ATCC 29213	≤ 2 , ≤ 2 , ≤ 2	≤ 0.5 , 1, 1	≤ 1 , ≤ 1 , ≤ 1

^a Organisms were grown overnight on BHI agar containing 6 μ g of vancomycin per ml prior to testing (induced). Uninduced strains were grown on sheep blood agar overnight.

^b Tests were run in triplicate.

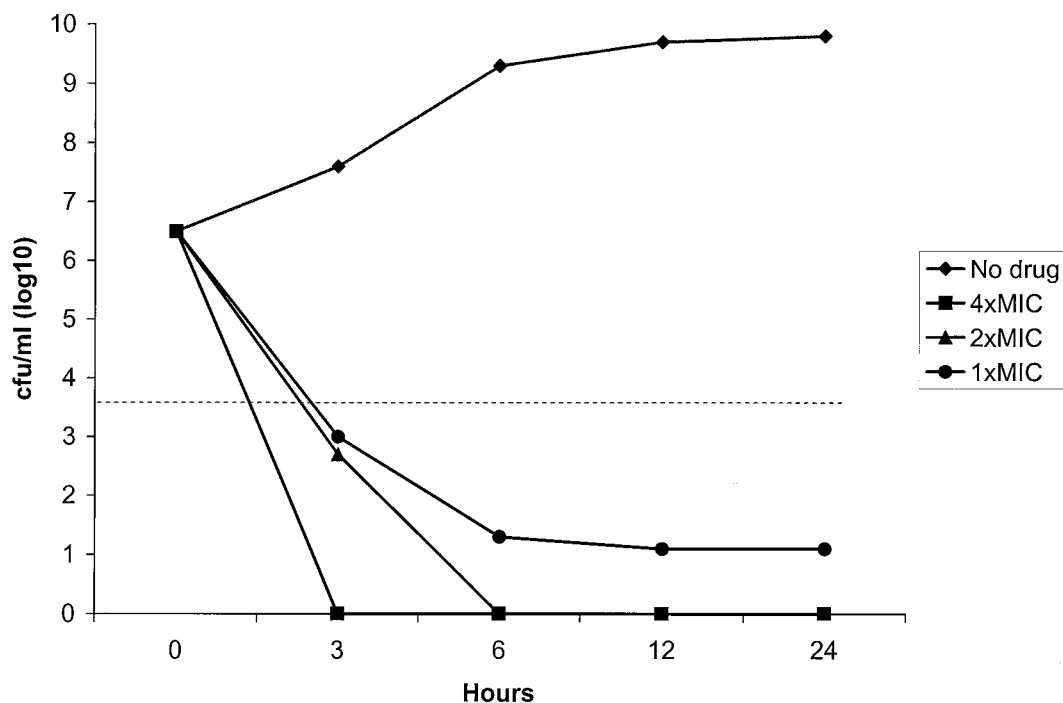


FIG. 2. Time-kill studies with the PA-VRSA isolate and daptomycin. The organism was grown in the absence of drug (growth control), at the MIC (1x) for the organism, and at twice (2x) and four times (4x) the concentration of daptomycin required to inhibit the growth of the organism. The decrease in viable counts of >3 log units indicates that daptomycin is bactericidal for this organism.

dicted sizes of 1,225, 1,087, 1,793, 1,965, 1,584, and 428 bp, respectively. PCR for enterococcal ligase genes was performed by the method of Dutka-Malen et al. (12). The DNA sequence of *vanA* was determined with the dRhodamine terminator cycle sequencing kit (Applied Biosystems [ABI] using products of independent PCRs to determine sequences of the forward and reverse strands. Reactions were analyzed on an ABI 377 automated DNA sequencer. DNA and inferred amino acid sequences were compared using DNAsis for Windows (version 2.5; Hitachi).

Plasmid isolation. *S. aureus* plasmids were isolated using a modified protocol for the Midi- or Maxi-plasmid purification kit (Qiagen, Valencia, Calif.). *S. aureus* cells were harvested from mid-log-phase cultures grown in BHI broth with 10 μ g of vancomycin per ml. Cell pellets were suspended in lysis buffer containing 50 μ g of lysostaphin per ml and incubated at 37°C for 30 min before beginning the standard protocol.

PFGE. Genomic DNA was digested with the *Sma*I restriction enzyme, and DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) (3, 27) to compare the pulsed-field types of *S. aureus* HIP11983, the Pennsylvania VRSA isolate (PA-VRSA), with those of *S. aureus* HIP11714, the Michigan VRSA isolate (MI-VRSA). A *Sma*I digest of *S. aureus* NCTC 8325 was used to normalize the gel and for restriction fragment size reference (35) using the BioNumerics software package (Applied Math, Kortrijk, Belgium). Pulsed-field gel patterns were interpreted using standard criteria (27, 35).

Southern hybridization. Plasmid DNA from a 0.8% agarose gel was transferred to a Zeta-Probe GT genomic blotting membrane (Bio-Rad) and hybridized with a digoxigenin (DIG)-labeled *vanA* probe (complete coding region) amplified from *E. faecalis* A256 using oligonucleotide primers Van1 and Van2 (9). The probe was generated with the PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, Ind.) and hybridization was visualized with the DIG nucleic acid detection kit (Roche). A DIG-labeled *tetK* probe was generated by amplification of *tetK* sequences from *S. aureus* CDC82-7701 harboring plasmid pRC701 (10) using primers TETKF (5'-TAGGGGGAATAATAGCATT) and TETKR (5'-AATCCGCCATAACAAATA).

RESULTS

Case summary. The second VRSA isolate from the United States was obtained from a 70-year-old morbidly obese patient who presented to the Hershey Medical Center with a several

week history of increased somnolence, intermittent fever, chills, malaise, night sweats, shortness of breath, and dyspnea on exertion. He was admitted to the hospital in September 2002. The patient had a chronic heel ulcer producing purulent drainage. Multiple organisms were grown from swab specimens taken from the heel ulcer, including group B beta-hemolytic streptococci, *P. aeruginosa*, *Stenotrophomonas maltophilia*, and *S. aureus*.

Species identification. The *S. aureus* isolate, which grew as a small cream-colored colony, was identified using traditional biochemical tests. DNA sequence analysis of the 16S ribosomal DNA genes, *gyrA* and *gyrB*, confirmed the identification of the isolate as *S. aureus* (data not shown). The PCR assay for enterococcal ligase sequences was negative.

Antimicrobial susceptibility test results. On initial testing of the *S. aureus* isolate, a small area of clearing was noted within a zone of reduced growth around a 30- μ g vancomycin disk, suggesting possible resistance (Fig. 1). Growth was also observed on a BHI agar screen plate containing 6 μ g of vancomycin per ml (data not shown). The vancomycin MICs were 64 μ g/ml by the Etest method and 32 μ g/ml by the NCCLS broth microdilution reference method, although the teicoplanin MIC was only 8 μ g/ml by broth microdilution. The Etest MICs took into account small colonies growing within the apparent inner zone of inhibition (Fig. 1). Repeat testing of the isolate by broth microdilution demonstrated vancomycin MICs of 16 to 64 μ g/ml and teicoplanin MICs of 4 to 8 μ g/ml. The broth MICs of other antimicrobial agents are shown in Table 1. The isolate was resistant to aminoglycosides, macrolides, oxacillin, and tetracycline, but it was susceptible to chloramphenicol, linezolid, minocycline, quinupristin-dalfopristin, rifampin, and

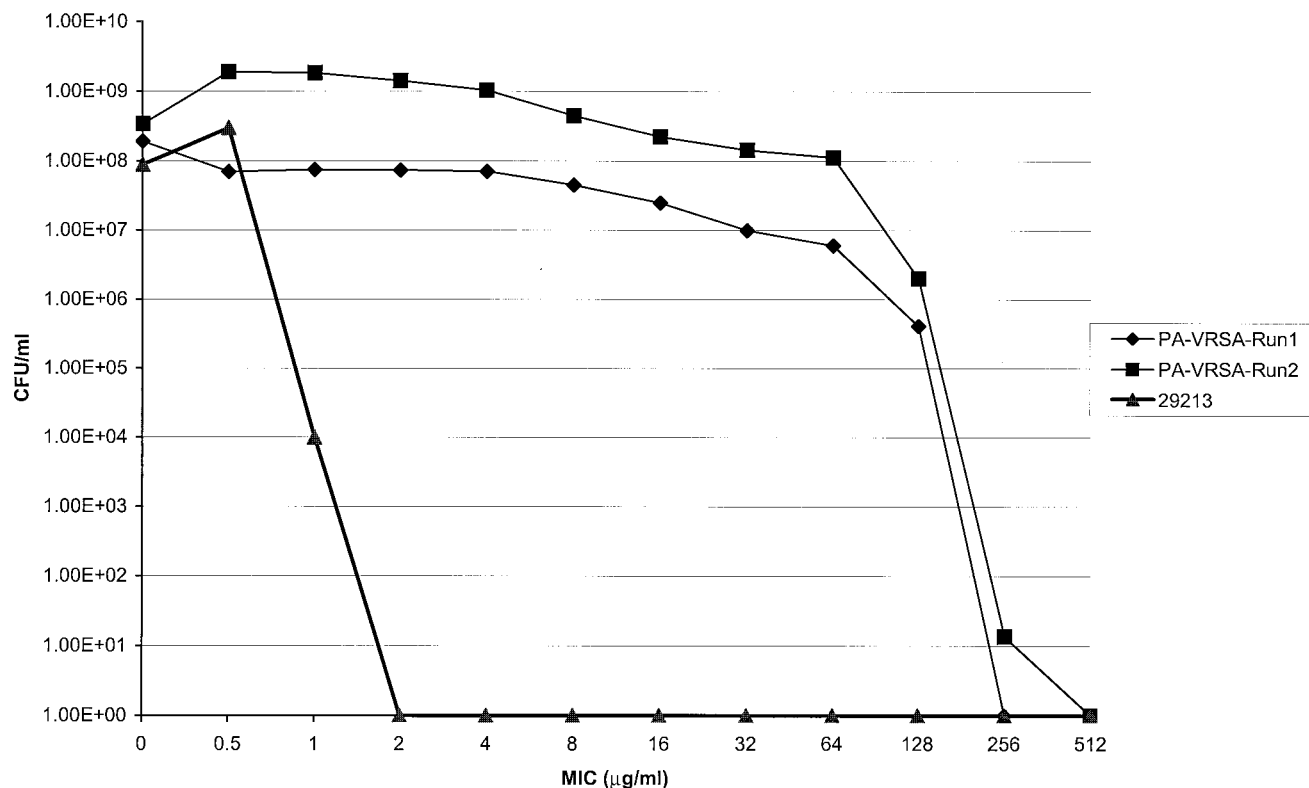


FIG. 3. Population analysis of the PA-VRSA isolate. Serial dilutions of the organism were plated on increasing concentrations of vancomycin. The PA-VRSA isolate was tested on two separate occasions (Run1 and Run2). *S. aureus* ATCC 29213 was used as a vancomycin-susceptible control. $1.00E+10$, 1×10^{10} .

trimethoprim-sulfamethoxazole. The MICs of two experimental glycopeptides, oritavancin and TD-6424, and several other experimental agents, including RWJ54428, tigecycline, ranbezolid, and iclaprim were consistent with vancomycin-susceptible *S. aureus* isolates. Vancomycin susceptibility was tested by three automated methods, i.e., MicroScan conventional MIC plates, Vitek cards, and Vitek2 cards, and each method was tested on three separate days. The combined results are shown in Table 2. None of the vancomycin MIC results were in the resistant range based on NCCLS interpretive criteria (i.e., MIC of ≥ 32 $\mu\text{g/ml}$), although after overnight growth on BHI agar containing 6 μg of vancomycin per ml, the vancomycin MICs tended to be higher by the Vitek and Vitek2 cards than when the bacteria were propagated on a nonselective medium (Table 2).

Time-kill studies and population analysis. Although daptomycin was rapidly bactericidal for the VRSA strain (Fig. 2), linezolid and quinupristin-dalfopristin were only bacteriostatic (data not shown). By population analysis, the organism appeared to be homogeneously resistant to vancomycin (Fig. 3) with no apparent shoulder in the curve that would indicate a heterogeneously resistant subpopulation.

Molecular tests. The organism was positive by PCR for the *mecA*, *vanA*, *erm(A)*, and *aac(6')-aph(2'')* determinants. Additional PCR primers specific for the components of the *vanA* transposon, Tn1546, confirmed the presence of *vanR*, *vanS*, *vanX*, *vanY*, and *vanH*, suggesting that a significant portion of

Tn1546 was present. However, two primer sets directed to regions of ORF1 and ORF2 at the 5' end of Tn1546 failed to amplify the expected products of 1,309 and 1,132 bp, suggesting that these sequences may be absent or truncated. Two other primer sets directed to the regions between the ORF1

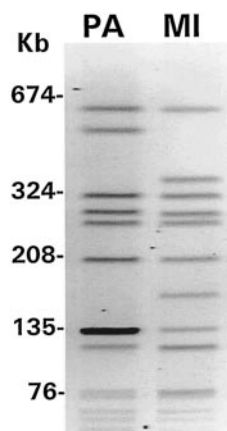


FIG. 4. PFGE of *Sma*I macrorestriction fragments of genomic DNA from the PA-VRSA (PA) and MI-VRSA (MI) strains. The *Sma*I macrorestriction fragments for the two VRSA isolates indicate that both are related to the USA100 pulsed-field type, also known as the New York/Japan clone.

and ORF2 genes and the *vanS* and *vanH* genes yielded products larger than the predicted sizes of 1,299 and 593 bp, suggesting the integration of additional DNA in these areas. DNA sequence analysis of the *vanA* gene was consistent with the prototype *vanA* sequence of *E. faecalis* A256 (32).

Plasmid analysis revealed two plasmids of 120 and 4 kb. The *vanA* gene was localized to the 120-kb plasmid by Southern hybridization (data not shown). The *tetK* gene, which mediates resistance to tetracycline, but not minocycline (4), was present on the 4-kb plasmid (data not shown).

The *Sma*I macrorestriction digestion patterns of genomic DNA from the PA-VRSA and MI-VRSA isolates are shown in Fig. 4. Although both patterns fall within the USA100 lineage (also known as the New York/Japan clone), the two patterns are readily distinguished from one another, indicating that the PA-VRSA isolate is not epidemiologically linked to the MI-VRSA isolate.

DISCUSSION

This organism represents the second confirmed VRSA isolate from patients in the United States. Like the Michigan isolate, this VRSA isolate, although oxacillin resistant, was susceptible to a number of antistaphylococcal agents, including linezolid, quinupristin-dalfopristin, and trimethoprim-sulfamethoxazole, and the novel agent, daptomycin (8). However, time-kill studies showed that linezolid and quinupristin-dalfopristin exhibited only bacteriostatic activity against this strain, while daptomycin was rapidly bactericidal.

Although both the MI-VRSA and PA-VRSA isolates contained *vanA*, the PA-VRSA isolate demonstrated only moderate resistance to vancomycin (MIC range, 16 to 64 $\mu\text{g/ml}$). In addition, the teicoplanin MIC (range, 4 to 8 $\mu\text{g/ml}$) for the PA-VRSA isolate was in the susceptible range defined by the NCCLS. This is inconsistent with the VanA phenotype typically observed in enterococci. The reason for the low vancomycin MIC is unknown, but it may be related to the level of expression of the *vanA* gene that is present on a large, 120-kb plasmid. Plasmids of this size are atypical of *S. aureus* (13). Thus, this plasmid may represent either an enterococcal plasmid or a cointegrate plasmid composed of enterococcal and staphylococcal plasmid sequences. In contrast to the Michigan case, no putative donor enterococcus was isolated from the Pennsylvania patient to help clarify this issue.

PCR analysis suggests that sequences corresponding to the *vanR*, *vanS*, *vanX*, *vanY*, and *vanH* of the Tn1546 genetic element are present on the 120-kb plasmid (5, 16, 23); however, the 5' end of the transposon may be truncated. It would also appear that there is additional DNA in two regions of the transposon, although the nature of these sequences will require further analysis. Thus, the plasmids in the PA-VRSA and MI-VRSA isolates are significantly different, indicating two independent events of interspecies transfer, most likely from enterococci. Both the MI-VRSA and PA-VRSA isolates have PFGE patterns that are consistent with the most common PFGE types of hospital-acquired staphylococcal infections in the United States (27).

Perhaps the most disconcerting finding of the study was that the automated susceptibility testing systems, i.e., MicroScan, Vitek, and Vitek2, did not identify the PA-VRSA isolate as resistant to vancomycin. Even after overnight growth on a

vancomycin-containing medium (which would be impractical to implement as a routine susceptibility procedure in most clinical laboratories), the organism was not identified as fully resistant to vancomycin. Thus, when performing automated susceptibility testing of *S. aureus* strains, and particularly oxacillin-resistant *S. aureus*, microbiologists should consider including a vancomycin agar screen plate (BHI containing 6 μg of vancomycin per ml) in the testing battery or performing nonautomated broth- or agar-based MIC tests to ensure that vancomycin-resistant strains do not go undetected.

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