Development of Reduced Vancomycin Susceptibility in Methicillin-Susceptible Staphylococcus aureus

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Background. Most cases of reduced vancomycin susceptibility in Staphylococcus aureus reported in the literature have been in methicillin-resistant strains. We report the development of reduced vancomycin susceptibility in a series of clonally related, methicillin-susceptible S. aureus (MSSA) clinical isolates. This isogenic series permitted us to determine whether the evolution of reduced vancomycin susceptibility in MSSA is similar to that seen in MRSA.

Methods. Differences in vancomycin population analysis profiles; chemical autolysis; vancomycin, oxacillin, and daptomycin minimum inhibitory concentrations; and bactericidal activities were examined.

Results. Progressive vancomycin resistance correlated with increasing daptomycin nonsusceptibility. Chemical autolysis and the bactericidal activity of vancomycin, oxacillin, and daptomycin were reduced in the final, vancomycin-intermediate S. aureus isolate, compared with the vancomycin-susceptible MSSA progenitor.

Conclusions. Clinicians should recognize that reduced vancomycin susceptibility can occur in S. aureus irrespective of background methicillin susceptibility and that development of intermediate vancomycin susceptibility in MSSA may result in increased tolerance to several classes of anti-staphylococcal antibiotics.

Methicillin-resistant Staphylococcus aureus (MRSA) is a major cause of community and nosocomial infections. Treatment with vancomycin has been the standard of care for serious MRSA infection for >40 years. However, over the past decade there have been reports of vancomycin treatment failures for MRSA infection [1–3]. True vancomycin-resistant S. aureus (VRSA; defined as a minimum inhibitory concentration [MIC] of ≥16 μg/mL [4]), which is associated with the incorporation of the enterococcal vanA cassette into S. aureus, has been reported only sporadically [5]. In addition to VRSA, reduced vancomycin susceptibility (RVS) is seen among vancomycin-intermediate S. aureus (VISA; MIC of 4–8 μg/mL) and hetero-VISA (susceptible to vancomycin by routine susceptibility testing, but containing resistant subpopulations with MICs ≥4 μg/mL) [4, 6]. Development of hetero-VISA and/or VISA does not involve incorporation of the vanA cassette, is associated with altered expression of multiple regulatory genetic elements, and is phenotypically characterized by increased cell wall thickness, altered cell wall penicillin-binding protein profiles, and reduced rates of cell wall autolysis [7–13]. Decreasing vancomycin susceptibility is a predictor of failing vancomycin therapy [2, 3, 14].

Most reported cases of hetero-VISA and VISA have arisen from MRSA progenitors. There are few detailed reports of RVS in methicillin-susceptible isolates [15–17]. In fact, in some of these cases the methicillin-susceptible S. aureus (MSSA) with RVS may have arisen from MRSA backgrounds. For instance, in 1 report of MSSA-RVS, molecular investigation revealed the presence of the genetic element conveying methicillin-resistance, mecA, and the authors demonstrated an inverse correlation between oxacillin and vancomycin MICs in the series of S. aureus isolates recovered from the index patient [15]. A similar inverse correlation between oxacillin and vancomycin MICs has been reported in other mecA-positive S. aureus isolates [18]. In another case, the source patient harbored genetically related methicillin-resistant and methicillin-susceptible...
VISA, and the authors postulated that the methicillin-susceptible VISA arose from deletion of the mecA element [16]. Bobin-Debrueux et al [17] described the clinical recovery of a mecA-negative, methicillin-susceptible hetero-VISA isolate; however, there was no vancomycin-susceptible progenitor to serve as a comparator.

In this study, we investigated a series of clinical MSSA isolates recovered from a patient who experienced vancomycin therapy failure (case 12 in Table 1 from reference [6]). Briefly, a patient with an infected foot ulcer initially completed a 2-week course of antibiotic therapy with oxacillin for MSSA bacteremia without evidence of remote sites of infection. The patient, complaining of back pain, returned 3 weeks after the original bacteremia, and was found to have vertebral osteomyelitis, for which oxacillin was reinitiated. The development of abnormal liver function, presumed to represent oxacillin hepatotoxicity, prompted a treatment change to intravenous vancomycin. Nine weeks after the initial bacteremia, because of ongoing back pain, debridement of the infected vertebral bodies was undertaken with implantation of spinal hardware. Operative bone cultures were positive for MSSA, which was susceptible to vancomycin in the clinical microbiology laboratory. The patient continued receiving vancomycin with trough concentrations ranging from 15 to 20 μg/mL. Three weeks after the operative debridement and 2 months into vancomycin therapy, the patient developed increasing back pain and intermittent fevers. Blood cultures again yielded MSSA, for which the vancomycin MIC was re-tested to be 0.12 mg/mL by the clinical microbiology laboratory.

Because of the development of VISA in this series of MSSA isolates, the following study was undertaken to characterize the natural history of RVS development in a methicillin-susceptible S. aureus progenitor, S. aureus A9635, and the final VISA isolate, S. aureus A9639, was assessed as follows. Overnight cultures of the 2 isolates were diluted in brain-heart infusion broth and incubated at 35°C with shaking at 250 rpm. At mid-exponential

### METHODS

The isolates described in this study are listed in Table 1. Vancomycin MIC, pulsed field gel electrophoresis, and delta-lysin activity were assessed for 2 isolates in this series, S. aureus A9635 and S. aureus A9639, which have been described elsewhere as part of an unrelated study [19]. Vancomycin (Sigma-Aldrich) and oxacillin (Sigma-Aldrich) susceptibilities were assessed in our laboratory by agar dilution as described by the Clinical Laboratory Standards Institute [4], with a slight modification in that incremental concentrations of vancomycin were used to assess for subtle changes in MIC. Daptomycin (Cubist Pharmaceuticals) susceptibility testing was performed using standard broth macrodilution technique, with a starting inoculum of ~5 × 10^6 colony forming units (CFU)/mL prepared by direct colony suspension, in Mueller-Hinton broth (Becton Dickinson) supplemented to a final calcium concentration of 50–55 mg/L. Vancomycin population analysis was performed for S. aureus A9635-A9637, S. aureus A9639, a vancomycin-susceptible control (ATCC 29213), and a known VISA (PC-3) [20]. Serial 1:10 dilutions of an approximately ~1 × 10^6 CFU/mL inoculum were plated on brain-heart infusion agar (Becton Dickinson) containing varying concentrations of vancomycin and incubated at 35°C for 48 h, as described elsewhere [7]. The lower limit of detection for this assay was 1.6 log CFU/mL.

Pulsed-field gel electrophoresis with SmaI-digested genomic DNA of S. aureus A9635-A9639 was performed using published methods [21, 22]. In addition, polymerase chain reaction was performed using previously published mecA primers [23] and genomic DNA isolated from S. aureus A9635, MRSA 32 (mecA-positive control [24]), S. aureus ATCC 29213 (mecA-negative control) (GenElute Bacterial Genomic DNA kit; Sigma-Aldrich). A method published elsewhere [7] for determining delta-hemolysin activity was used to assess agr function.

Chemical-induced autolysis of the vancomycin-susceptible progenitor, S. aureus A9635, and the final VISA isolate, S. aureus A9639, was assessed as follows. Overnight cultures of the 2 isolates were diluted in brain-heart infusion broth and incubated at 35°C with shaking at 250 rpm. At mid-exponential

### Table 1. Antibiotic Susceptibility Results (Agar and Macro-Broth Dilution) for Study Isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Time from original bacteremia, weeks</th>
<th>Minimum inhibitory concentration, μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9635</td>
<td>Blood</td>
<td>0</td>
<td>Vancomycin (agar) 1</td>
</tr>
<tr>
<td>A9636</td>
<td>Bone</td>
<td>3</td>
<td>Vancomycin (agar) 0.25</td>
</tr>
<tr>
<td>A9637</td>
<td>Bone</td>
<td>9</td>
<td>Vancomycin (agar) 0.25</td>
</tr>
<tr>
<td>A9638</td>
<td>Blood</td>
<td>12</td>
<td>Vancomycin (agar) 0.25</td>
</tr>
<tr>
<td>A9639</td>
<td>Blood</td>
<td>12</td>
<td>Vancomycin (agar) 0.25</td>
</tr>
</tbody>
</table>

Daptomycin (broth) 0.5

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<tr>
<th>Isolate</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A9635</td>
<td>Blood</td>
<td>0</td>
<td>Oxacillin (agar) 0.25</td>
</tr>
<tr>
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<td>Bone</td>
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<td>9</td>
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</tr>
<tr>
<td>A9638</td>
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<td>12</td>
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</tr>
</tbody>
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Daptomycin (broth) 1

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>A9635</td>
<td>Blood</td>
<td>0</td>
<td>Daptomycin (broth) 0.5</td>
</tr>
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<td>Bone</td>
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</tr>
</tbody>
</table>

Daptomycin (broth) 2
S. aureus trol strain ATCC 29213. Neither ATCC 29213 grew on the brain-heart infusion agar with 4 g/mL vancomycin. Although S. aureus A9636 was vancomycin susceptible by agar dilution, it demonstrated growth on brain-heart infusion agar containing 4 μg/mL of vancomycin, which is consistent with hetero-VISA [25, 6]. The population profiles of S. aureus A9637 and S. aureus A9639 revealed a continued rightward shift and increasingly resembled the profile of S. aureus PC-3, a known VISA (vancomycin MIC, 8 μg/mL) [20].

Polymerase chain reaction failed to identify the meca genetic element in S. aureus A9635. All study isolates (S. aureus A9635-A9639) were indistinguishable by pulsed field gel electrophoresis and lacked delta-lysin activity, suggesting impaired agr function (data not shown).

Triton X-100–induced autolysis was reduced in S. aureus A9639, compared with S. aureus A9535 (Figure 2). In addition, killing of VISA A9639 was attenuated, compared with S. aureus A9635, at one or both of the time points in the daptomycin, vancomycin, and oxacillin time kill experiments. At the fixed, uniform drug concentrations used in these studies, significantly reduced killing of S. aureus A9639 relative to S. aureus A9635 was observed for daptomycin at 4 h, for vancomycin at 24 h, and for oxacillin at both at 4 h and 24 h (Table 2).

**DISCUSSION**

The majority of cases of RVS in S. aureus reported in the literature have involved MRSA, with few well described cases of RVS occurring in MSSA [15–17]. It is possible that RVS may remain undetected in MSSA with hetero-VISA characteristics, because these isolates appear to be vancomycin susceptible by current testing guidelines [4, 6]. Because hetero-VISA appears to be an intermediary step toward the development of full-fledged VISA [10] in the absence of vancomycin selective pressure, it is possible that the true burden of RVS among MSSA is higher than is currently appreciated. The data presented in this study reaffirms the concept that RVS (hetero-VISA and VISA) can arise in MSSA. Unlike in other reports [15, 16], evolution of RVS occurred in a meca-negative background.

**RESULTS**

For the series of S. aureus isolates described in this study, vancomycin MICs, as assessed by agar dilution, demonstrated a progressive increase over time (Table 1), with S. aureus A9638 and S. aureus A9639 being vancomycin nonsusceptible according to current Clinical Laboratory Standards Institute break points (susceptible defined as an MIC ≤2 μg/mL) [4]. This increase in vancomycin MIC was accompanied by an increase in daptomycin MICs, and the final 2 isolates in the series were daptomycin nonsusceptible (MIC, >1 μg/mL) [4]. All isolates were oxacillin susceptible. Figure 1 demonstrates that the population profile of S. aureus A9635 paralleled that of the MSSA control strain ATCC 29213. Neither S. aureus A9635 nor S. aureus ATCC 29213 grew on the brain-heart infusion agar with 4 μg/mL vancomycin. Although S. aureus A9636 was vancomycin susceptible by agar dilution, it demonstrated growth on brain-heart infusion agar containing 4 μg/mL of vancomycin, which is consistent with hetero-VISA [25, 6]. The population profiles of S. aureus A9637 and S. aureus A9639 revealed a continued rightward shift and increasingly resembled the profile of S. aureus PC-3, a known VISA (vancomycin MIC, 8 μg/mL) [20].

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S. aureus MIC observed in S. aureus MIC. In addition, this observation is supported by clinical data progenitor, is not unexpected given its elevated vancomycin VISA isolate at 24 h, relative to the vancomycin-susceptible 
methicillin MIC in S. aureus. Interestingly, loss of PBP4 activity has been associated with decreased cell wall content of penicillin binding protein 4 (PBP4). Inter-
mycin was associated with reduction in both autolysis and the VISA in a clinical series of MRSA isolates exposed to vanco-
tomy. Both the loss of agr function and development of VISA have been associated with decreased autolysis [26, 13]. Autolysins have been implicated in penicillin- and vancomycin-mediated staphylococcal killing. Therefore, the reduced rates of Triton X-100-induced autolysis observed in S. aureus A9639 (VISA), relative to S. aureus A9635, raised the possibility that vanco-
mycin and ß-lactam killing would also be decreased.

The significantly attenuated vancomycin killing seen in the VISA isolate at 24 h, relative to the vancomycin-susceptible progenitor, is not unexpected given its elevated vancomycin MIC. In addition, this observation is supported by clinical data noting reduced vancomycin efficacy for the treatment of RVS S. aureus infections [2, 3, 14]. Despite the subtly lower oxacillin MIC observed in S. aureus A9639 (compared with S. aureus A9635), there was significantly reduced killing by oxacillin at both 4 h and 24 h in our time kill experiments. Of note, Sieradzki and Tomasz [12] reported that the development of VISA in a clinical series of MRSA isolates exposed to vanco-
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mycin was associated with reduction in both autolysis and the cell wall content of penicillin binding protein 4 (PBP4). Interest-

Table 2. Bacterial Killing of Staphylococcus aureus A9635 and S. aureus A9639 following 4 h and 24 h Exposure To Vancomycin, Oxacillin, and Daptomycin

<table>
<thead>
<tr>
<th>Antibiotic, duration of exposure</th>
<th>Bacterial killing, log CFU/mL ± SD</th>
<th>S. aureus A9635</th>
<th>S. aureus A9639</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>0.33 ± 0.14</td>
<td>0.17 ± 0.15</td>
<td>.13</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>2.86 ± 0.50</td>
<td>2.07 ± 0.23</td>
<td>.02</td>
<td></td>
</tr>
<tr>
<td>Oxacillin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>0.46 ± 0.21</td>
<td>−0.05 ± 0.15</td>
<td>.003</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>4.15 ± 1.14</td>
<td>1.98 ± 0.42</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td>Daptomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td>3.20 ± 0.38</td>
<td>0.90 ± 0.33</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>3.94 ± 1.55</td>
<td>2.05 ± 1.37</td>
<td>.08</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Bacterial killing was calculated as the log colony forming units (CFU)/mL at time 0 minus the log CFU/mL at time x. Five experiments were performed for each set of conditions. The P value was determined by t test comparing the killing of S. aureus A9635 to that of VISA A9639. SD, standard deviation.

* Vancomycin-intermediate S. aureus.

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lar, agr [7]. In all of the study isolates, agr function was altered (whether through mutation or down-regulation), poten-
tially providing 1 of the necessary genetic characteristics required for the subsequent emergence of RVS following vanco-
mycin exposure. Despite the reported positive correlation between RVS and daptomycin nonsusceptibility, other groups have reported that daptomycin retains bactericidal activity against hetero-
VISA and VISA in vitro [30–32], and against VISA in a rabbit endocarditis model [31]. In this study, the bactericidal activity of daptomycin was significantly greater at 4 h for the vanco-
mycin- and daptomycin-susceptible MSSA progenitor A9635, compared with VISA A9639; at 24 h, the mean daptomycin killing was higher for S. aureus A9635 than for VISA A9639, but the difference was not statistically significant (P = .08). Whereas daptomycin is thought to exert its antibacterial effect through cell membrane depolarization [33], recently it has been shown that daptomycin also induces the S. aureus cell wall stimulon, suggesting that it may also inhibit peptidoglycan synthesis [34]. Thus, the finding of reduced daptomycin, vanco-
mycin, and oxacillin killing (at 4 h, 24 h, or both time points) in the VISA isolate relative to its MSSA and VSSA progenitor may relate to ≥1 common targets involved in peptidoglycan synthesis.

In conclusion, clinicians should be aware that, in settings in which MSSA infections are treated with vancomycin, failure of antibiotic therapy may be attributable to development of RVS. Indeed, because hetero-VISA is not readily detected by routine

1172 • CID 2009:49 (15 October) • Pillai et al
susceptibility testing, the true burden of RVS (in either MSSA or MRSA backgrounds) remains unknown. Similar to the observations seen in RVS-MRSA, absence of agr function was noted in this series of MSSA isolates. In this series, development of VISA was associated with reduced autolysis and attenuated killing following in vitro exposure to several antibiotics of different classes.

Acknowledgments

Potential conflicts of interest. G.M.E. has received research funding from and has served as a consultant to Cubist Pharmaceuticals, Pfizer, and Astellas; R.C.M. has served as a consultant to Pfizer, Cubist, Astellas, and Wyeth; A.W.K. has received research funding from Cubist Pharmaceuticals, Pfizer, and Merck and has served as a consultant to Pfizer, Cubist, and Astellas. All other authors: no conflicts.

References


