Tracking the Evolution of *Staphylococcus aureus* under Vancomycin Selective Pressure: The Role of the Small Colony Variant Phenotype

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Running Title: The SCV’s Niche in Vancomycin Resistance in *S. aureus*

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Abstract

*Staphylococcus aureus* small colony variants (SCV) often persist despite antibiotic therapy. Against a $10^8$ CFU/ml MRSA (COL) population containing 0%, 1%, 10%, 50%, or 100% of an isogenic *hemB* knockout (la48) sub-population displaying the SCV phenotype, vancomycin achieved maximal reductions of 4.99, 5.39, 4.50, 3.28, and 1.66 $\log_{10}$CFU/ml over 48h. Vancomycin $\geq 16$mg/L shifted a population from 50% SCV at 0h to 100% SCV at 48h, which was well characterized by a Hill-type model ($R^2 > 0.90$).
Staphylococcus aureus is a virulent pathogen responsible for a myriad of infections ranging from minor community acquired skin and soft tissue infections to severe nosocomial infections. While the current IDSA guidelines recommend vancomycin as the primary agent for treatment of methicillin resistant S. aureus (MRSA) infections, the utility of the drug has been brought into question due to increasing reports of heterogeneous resistance, treatment failure, and nephrotoxicity. Despite the global decrease in vancomycin susceptibility, the exact mechanism by which S. aureus develops resistance is not well understood. It has been suggested that S. aureus adapts by utilizing an array of genotypic alterations that arise stepwise during the selective pressure of antimicrobial therapy.

One pathway that S. aureus may exploit during the evolution of antimicrobial resistance is the development of small colony variants (SCVs) that grow slowly relative to the normal phenotype (NP). In vitro testing and macrophage models have confirmed that the SCV phenotype is less susceptible to vancomycin. Studies with other antibiotics also suggest that SCV subpopulations may cooperate with NP S. aureus to attenuate antimicrobial activity. At present, it is unknown whether SCVs alter vancomycin pharmacodynamics through interactions with NP S. aureus or how the selection of a vancomycin regimen influences the relationship between the two phenotypes. The objective of the current study was, therefore, to utilize reconstructive population biology to determine how the interplay of both phenotypes alters vancomycin pharmacodynamics.
The MRSA strain COL (NP) and its isogenic *hemB* knockout Ia48 (COL *hemB::ermB*, a stable SCV phenotype) were utilized. The creation of the mutant strain and its features were previously characterized. (14) Prior to each experiment, a solution of vancomycin was prepared using analytical grade powder (Sigma Chemical, St. Louis, MO) in the following concentrations: 0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128mg/L. Brain heart infusion (BHI) broth supplemented with magnesium (12.5mg/L) and calcium (25mg/L) was used for every experiment. SCV and NP suspensions were volumetrically titrated to achieve different starting compositions with a total bacterial load of $10^8$ CFU/ml. Two experiments were conducted exclusively investigating the NP or the SCV phenotype (0% SCV/100% NP and 100% SCV/0% NP), and three mixed populations were investigated with starting inoculums of approximately 1% SCV/99% NP, 10% SCV/90% NP, and 50% SCV/50% NP. Time-killing experiments were conducted over 48 hours as previously described. (11) In mixed population experiments, bacteria were plated on both plain BHI agar and onto BHI containing gentamicin (2.0mg/L) to select for the SCV phenotype.

An integrated pharmacokinetic/pharmacodynamics (PK/PD) approach was utilized as defined previously. (11) Using the linear trapezoid rule, the area under the CFU curve in a 48-hour time span (AUCFU$_{0-48}$) was calculated for each concentration and a growth control. The Log Ratio Area was then calculated as the logarithm of the AUCFU$_{0-48}$ of drug divided by the AUCFU$_{0-48}$ growth control (Equation 1).
A four-parameter concentration-effect Hill-type model was fit to the effect parameter using Systat (Version 12, Systat Software Inc., San Jose, CA). Using Equation 2, $E$ (dependent variable) represents the Log Ratio Area, $E_0$ is the effect at a vancomycin concentration of 0, $E_{\text{max}}$ is the drug’s maximum effect, $C$ is the concentration of vancomycin, $EC_{50}$ is the vancomycin concentration displaying 50% of the maximum effect, and $H$ is the sigmoidicity constant. The overall model fits were assessed based on coefficients of determination ($R^2$).

The antibiotic activity and pharmacodynamics of vancomycin against each population are depicted in Figure 1. Vancomycin concentrations >16mg/L achieved bactericidal activity against the exclusively NP population by 24 hours (4.99 log$_{10}$CFU/ml reduction by 48 hours) compared to a maximal reduction of 1.66 log$_{10}$CFU/ml against the exclusively SCV population by 48 hours. Despite the presence of SCVs in the mixed populations, at 48 hours vancomycin concentrations >16mg/L achieved maximal reductions of 5.39, 4.50, and 3.28 log$_{10}$CFU/ml in the experiments initially containing 1%, 10%, and 50% SCVs, respectively.

Overall the Hill-type model displayed excellent fits to the Log Ratio Areas with $R^2$ values exceeding 0.99 for each experiment. The model-fitted parameters $E_{\text{max}}$, $EC_{50}$, and $R^2$ are listed for each time-killing experiment in Figure 1. When comparing the exclusively
NP and SCV populations, the $E_{\text{max}}$ for the NP population was nearly double the $E_{\text{max}}$ of the SCV population ($E_{\text{max}} \text{NP}=2.17$, $E_{\text{max}} \text{SCV}=1.15$). In the mixed population experiments, the $E_{\text{max}}$ for the three populations were 1.96, 1.92, and 1.67, which corresponded to starting inoculum s containing 1%, 10%, and 50% SCV subpopulations, respectively. As the proportion of the SCV in the starting inoculum increased, the activity of vancomycin decreased in a manner that was consistent with the trend observed in colony counts.

In addition to the PK/PD analysis, the maximum percentages of SCVs observed for each vancomycin concentration were plotted for the three mixed populations and are depicted in Figure 2. The concentration plots were also fitted with a Hill-type function to reveal the relationship between drug concentration and proportion of SCVs. In all three experiments, vancomycin concentrations $>8\text{mg/L}$ resulted in a decrease in the total bacterial population with a concurrent rise in the ratio of SCVs to NP $S. \text{aureus}$. After exposure to vancomycin $>16\text{mg/L}$ for 48 hours, the inoculum initially containing 50% SCVs was completely dominated by 100% SCVs. Similarly, vancomycin concentrations $>16\text{mg/L}$ raised a $10^6 \text{CFU/ml}$ SCV subpopulation from about 1% of the starting inoculum to approximately 33% of the total population.

$S. \text{aureus}$ has a remarkable ability to evolve in the face of antimicrobial therapy. One mechanism by which $S. \text{aureus}$ may persist in difficult-to-treat, deep seated infections is through the formation of SCVs. Here, we sought to determine the interaction of the SCV phenotype with the NP under vancomycin pressure. Clinically isolated SCVs typically...
have an inability to synthesize menadione, haemin, or thymidine, resulting in a disrupted metabolism that confers a slow growth rate.(10) Due to the high rate of reversion from the SCV phenotype back to the NP, clinical SCV isolates of *S. aureus* are often difficult to study in vitro.(14) However, insertion of an *ermB* cassette into the haemin biosynthesis gene *hemB* generates a stable SCV phenotype that mimics clinically isolated electron-transport-defective SCVs that are auxotrophic for haemin – making *hemB* mutants the ideal SCVs for the current investigation.

Building upon previous studies, we determined that vancomycin activity against the SCV was diminished relative to the NP against a high bacterial density.(11) Moreover, in all of the mixed population experiments involving an initial inoculum of $10^8$ CFU/ml, attenuation of vancomycin activity was greater among *S. aureus* populations containing a larger proportion of the SCV phenotype. Findings by Massey and Peacock suggest the SCV phenotype of *S. aureus* may interact with the NP to augment the survival of the parent phenotype during antimicrobial treatment(13); in the presence of gentamicin, they observed that the SCV acidified its environment, thereby inhibiting gentamicin activity and protecting NP gentamicin-sensitive bacteria. In the current study, vancomycin retained bactericidal activity regardless of the proportion of the SCV in the starting inoculum, which contrasts with the collaborative survival observed by Massey and Peacock during gentamicin exposure. These findings suggest that the two phenotypes do not cooperate during vancomycin therapy.
It is also noteworthy that high concentrations of vancomycin selected for the SCVs in mixed population experiments. It has been postulated that *S. aureus* SCVs exist in a dynamic equilibrium with the NP, and the emergence of SCVs in persistent infections is attributed to the survival advantage of pre-existing SCVs compared to the NP during vancomycin exposure.\(^{(15, 16)}\) In the current work, the equilibrium was likely shifted in favor of the SCVs through a similar pathway – vancomycin was more effective at eradicating the fast-growing NP of *S. aureus*, thus allowing the slower growing but more resistant SCVs to expand in the overall population.

Although SCVs have been isolated from persistent infections and subsequently analyzed, the population dynamics of *S. aureus* have yet to be characterized in severe infections such as endocarditis.\(^{(17-19)}\) In lieu of in vivo data revealing the interplay between *S. aureus* phenotypes during antibiotic treatment, our observations suggest that vancomycin will preferentially select for the amplification of SCV subpopulations. Increasing vancomycin exposure against a MRSA population that is not fully eradicated may be counterproductive, as more exposure may result in a bacterial shift towards a variant phenotype that is adept at evading antimicrobial effects and persisting intracellularly.\(^{(20, 21)}\) Upon the discontinuation of antibiotics, the SCVs may revert to the NP and perpetuate a cycle of treatment failure and recurrent infection.\(^{(22, 23)}\)

Thus, clinicians should consider vancomycin combination regimens or alternative antimicrobials in patients with severe persistent MRSA infections in which the SCV
phenotype may play a role. In vitro analyses have identified fluoroquinolones and oritavancin as retaining high levels of activity against *S. aureus* SCVs.(24, 25) Daptomycin has also been shown to retain more activity against SCVs relative to vancomycin in vitro (26), and β-lactam combinations with daptomycin may offer a new option for combating SCVs.(27, 28) Macrophage models have not only revealed that oritavancin, rifampicin, moxifloxacin, and quinupristin-dalfopristin are active against intracellular SCVs(12), but SCVs may be more susceptible to β-lactams while in the intracellular domain as well.(29) Combination therapy including either rifampicin or oritavancin appears to be particularly effective at eradicating intracellular SCVs.(12) In the clinical setting, combinations integrating either rifampin or fluoroquinolones with other antibiotics have been frequently utilized following the identification of SCVs, and in the case of a device-related infection, surgical debridement is frequently necessary.(10) Further investigations exploring intramacrophage persistence, the host immune response, and the extended time course of SCV emergence will help elucidate counter-strategies for eliminating *S. aureus* SCVs.
Figure 1. Vancomycin time-killing experiments involving exclusively the NP: 0% SCV/100% NP (A) and exclusively the SCV: 100% SCV/0% NP (B), as well as three mixed population experiments consisting of 1% SCV/99% NP (C), 10% SCV/90% NP (D), and 50% SCV/50% NP (E). The pharmacodynamic relationship between Log Ratio Area and vancomycin concentration are also displayed for 0% SCV/100% NP (F), 100% SCV/0% NP (G), 1% SCV/99% NP (H), 10% SCV/90% NP (I), and 50% SCV/50% NP (J). $R^2$, $E_{\text{max}}$, and $EC_{50}$ values are listed next to the corresponding Hill-plots (percent standard errors are listed parenthetically).
Table 1. Summary of Parameters for Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Emax (95% CI)</th>
<th>EC50 (95% CI)</th>
<th>R²</th>
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</thead>
<tbody>
<tr>
<td>A. 0% SCV 100% NP</td>
<td>2.17 (0.99)</td>
<td>13.7 (1.50)</td>
<td>0.999</td>
</tr>
<tr>
<td>B. 100% SCV 0% NP</td>
<td>1.15 (2.02)</td>
<td>10.1 (1.50)</td>
<td>0.997</td>
</tr>
<tr>
<td>C. 1% SCV 99% NP</td>
<td>1.96 (2.10)</td>
<td>15.9 (80.5)</td>
<td>0.997</td>
</tr>
<tr>
<td>D. 10% SCV 90% NP</td>
<td>1.92 (3.09)</td>
<td>15.5 (3.82)</td>
<td>0.994</td>
</tr>
<tr>
<td>E. 50% SCV 50% NP</td>
<td>1.67 (2.18)</td>
<td>15.0 (2.66)</td>
<td>0.997</td>
</tr>
</tbody>
</table>

Key to Symbols
- ○: 0 mg/L  
- ●: 0.5 mg/L  
- □: 1 mg/L  
- ▲: 2 mg/L  
- ▼: 4 mg/L  
- ■: 8 mg/L  
- □: 16 mg/L  
- ●: 32 mg/L  
- ▲: 64 mg/L  
- ○: 128 mg/L
Figure 2. Colony counts used to track SCV subpopulations (A,B,C). In mixed culture experiments, samples were plated on plain BHI agar as well as BHI containing 2.0mg/L gentamicin. Gentamicin plates only permitted the growth of SCVs and the corresponding CFU plots derived from the drug plates are displayed for 1% SCV/99% NP (A), 10% SCV/90% NP (B), and 50% SCV/50% NP (C). Additionally, the maximum percentages of the SCV detected over 48 hours for each vancomycin concentration are plotted and fit to a Hill-type function for 1% SCV/99% NP (D), 10% SCV/90% NP (E), and 50% SCV/50% NP (F).
References:


3. Lodise TP, Lomaestro B, Graves J, Drusano GL. 2008. Larger vancomycin doses (at least four grams per day) are associated with an increased incidence of nephrotoxicity. Antimicrobial agents and chemotherapy 52:1330-1336.


