β-Lactamase Production Diminishes the Prophylactic Efficacy of Ampicillin and Cefazolin in a Guinea Pig Model of Staphylococcus aureus Wound Infection

Douglas S. Kernodle, Rama K. R. Voladri, and Allen B. Kaiser

Clinical trials in surgery suggest that some failures of antibiotic prophylaxis are related to the in vivo degradation of β-lactams by Staphylococcus aureus β-lactamase. To explore this issue further, isogeneic isolates of S. aureus differing only in whether they contained the structural gene for type A staphylococcal β-lactamase were constructed and compared for their ability to establish an abscess in a guinea pig model. With ampicillin prophylaxis, the ID₅₀ was 870 cfu for the β-lactamase-negative isolate VK7114 and 240 cfu for the β-lactamase-producing isolate VK7115 (P < .001). Similarly, the ID₅₀ was greater for the β-lactamase-negative isolate when cefazolin prophylaxis was administered (599 vs. 128 cfu, VK7114 and VK7115; P < .001). In the setting of prophylaxis with β-lactamase-susceptible antibiotics, β-lactamase contributes to the pathogenesis of S. aureus wound infections.

An estimated 325,000–920,000 postsurgical wound infections occur annually in the United States [1–3]. Staphylococcus aureus is the prototypic clean wound pathogen and causes more wound infections than any other organism [4]. Under conditions of an accompanying foreign body or in the presence of devitalized tissue, it can establish a wound infection with an extremely small inoculum, possibly as low as 1 cfu [5–9].

Although concepts of the pathogenesis of infection are commonly described in terms of the interactions between microbial virulence factors and host defenses, this model is probably inadequate for understanding the pathogenesis of surgical wound infections. As prophylactic antibiotics are administered routinely with many surgical procedures to reduce the risk of infection, the interactions between microbial resistance factors and antibiotics may be just as important as those between microbial virulence factors and host defenses in determining whether a wound infection complicates the operation. Clinical trials and animal models have shown that antibiotics do not prevent wound infection by antibiotic-resistant pathogens [7–10]. Partial antibiotic resistance, such as that exhibited by some β-lactamase-producing isolates of S. aureus to a relatively β-lactamase-labile antibiotic such as cefazolin, has also been implicated as a cause of some failures of antibiotic prophylaxis [11].

Materials and Methods

Construction of isogeneic S. aureus isolates. Isolate DK2076 is a clinical isolate recovered during surveillance cultures of the nares of operating room personnel at Saint Thomas Hospital in Nashville in 1986. It is a phage group 94/96 isolate, yet is remarkable in that it does not possess plasmids or produce β-lactamase in contrast to the majority of phage group 94/96 isolates, which contain a 17.2-kb plasmid, produce large amounts of type A staphylococcal β-lactamase, and exhibit borderline susceptibility to the anti-staphylococcal penicillins [19–21].

An E. coli–S. aureus shuttle vector, pVK101, was constructed with erythromycin as a selectable marker in S. aureus. First, the S. aureus plasmid pE194 was digested with SalI and cloned into the SacI site of pBC SK+ (Stratagene, La Jolla, CA) to create pVK100 as described previously [22]. Next, the genetic loci associated with β-lactamase inducibility were added by ligating a 4-kb HinIII-EcoRI fragment from S. aureus plasmid p3796 [23] containing blaR1 and blal into pVK100 to produce pVK101. The gene encoding the type A β-lactamase structural gene, blaz, was recovered as a 4-kb HindIII fragment from pS1 [23] and cloned into the phagemid pTZ18R (BioRad Laboratories, Fullerton, CA).
to create pVK102. In this clone, the 3’ end of the blaZ-containing insert is close to a HindIII site in the polylinker region of pTZ18R vector. Subsequently, HindIII digestion of pVK102 enabled the type A blaZ to be mobilized on a 1.1-kb fragment, which was cloned into pVK101 to produce pVK103 (figure 1).

*S. aureus* isolate VK7114 was produced by introducing pVK101 into DK2076 by using protoplast transformation with RN4220 (from RP Novick, Public Health Research Institute, New York) as an intermediate host [24]. *S. aureus* isolate VK7115 was produced by protoplast transformation of pVK103 into DK2076.

**In vitro characterization of *S. aureus* isolates.** Determinations of β-lactamase type and quantity were done using whole-cell suspensions of bacteria and previously described methods [11, 25]. Methicillin at a subinhibitory concentration of 0.5 μg/mL was used to induce β-lactamase production. Microdilution broth MICs were determined by use of standard techniques [26], except that the inoculum size was varied over a range from 10^3 to 10^6 cfu/mL. Assays were done in 2% salt-supplemented Mueller-Hinton broth (Difco, Detroit), and results were determined after 24 h of incubation at 35°C.

**In vivo procedures.** Details of this low-inoculum prophylaxis model have been described previously [6–9]. *S. aureus* colonies were harvested after overnight growth on tryptic soy agar and suspended in PBS to achieve a standard turbidity. Serial 10-fold and 2-fold dilutions were done to prepare a range of inocula that produced an abscess from 0 to 100% of the time. Each dilution was mixed in a 1/1 (vol/vol) ratio with sterile dextran microbeads (Cytodex; Sigma, St. Louis). Backcounts were done in triplicate and averaged to determine the precise bacterial inoculum.

On the day of in vivo experimentation, the dorsal hair was removed from albino Hartley guinea pigs of either sex, weighing 500 ± 50 g (Harlan Sprague-Dawley, Indianapolis), and a grid was drawn designating 12 areas for intermuscular inoculation. Each site was inoculated with 0.2 mL of one of the bacteria-bead suspensions. For animals receiving ampicillin or cefazolin prophylaxis, 100 mg/kg was administered subcutaneously at the time of bacterial inoculation. Control (placebo) guinea pigs received prophylaxis with saline at the time of bacterial inoculation. After the administration of antibiotic prophylaxis and inoculation with the bacteria-bead suspensions, the guinea pigs were returned to their cages. Two poorly absorbable antibiotics, gentamicin (80 μg/mL) and polymyxin B (50 μg/mL), were added to their drinking water to reduce the risk of mortality from prophylactic antibiotic-associated colitis [6, 27].

After 3–4 days of confinement, the guinea pigs were sacrificed and new growth dorsal hair was removed by depilation. By sterile technique, microbeads, with or without adherent abscess material, were removed from each of the 12 sites and inoculated onto sheep blood agar plates. The plates were incubated at 35°C for 24 h, and the presence or absence of bacterial growth was recorded.

**Data analysis and statistical determinations.** Logistic regression was used to assess the effect of different prophylactic regimens to induce *S. aureus* to produce β-lactamase; blaZ refers to structural gene encoding type A staphylococcal β-lactamase. Plasmid pVK101 is identical to pVK103 except it does not contain blaZ.
mens on the probability of infection. Adjustment was made for the amount of the inoculum in these regression models, by use of the log of the backcount. The ID$_{50}$ was calculated from the results of the logistic regression equation using data for each prophylactic regimen. The number of guinea pigs and data points used in constructing the logistic regression curves, ID$_{50}$ calculations, and statistical significance determinations for each prophylaxis–$S. aureus$ isolate regimen combination were as follows: placebo-DK2076, 6 guinea pigs and 66 data points; placebo-VK7114, 6 guinea pigs and 65 data points; ampicillin-VK7115, 11 guinea pigs and 117 data points; ampicillin-VK7115, 9 guinea pigs and 95 data points; cefazolin-VK7114, 22 guinea pigs and 248 data points; and cefazolin-VK7115, 22 guinea pigs and 249 data points. All analyses used PC-SAS, release 6.04 (SAS Institute, Cary, NC).

Results

Construction and in vitro characterization of isogeneic $S. aureus$ isolates. Isogeneic isolates of $S. aureus$ that differ only in whether they produce type A staphylococcal $\beta$-lactamase were constructed by transforming the $E. coli$–$S. aureus$ shuttle vectors pVK101 and pVK103 into the plasmid-free, $\beta$-lactamase–negative $S. aureus$ isolate DK2076 to produce isolates VK7114 and VK7115, respectively. As pVK101 and pVK103 (figure 1) are identical except for the presence of the type A $\beta$-lactamase structural gene, $blaZ$, in pVK103, VK7114 and VK7115 were identical except that only VK7115 produced $\beta$-lactamase. When induced with subinhibitory concentrations of methicillin, VK7115 hydrolyzed ampicillin and cefazolin at rates of 225 and 1.5 $\mu$g/min/10$^8$ cfu, respectively. This is comparable to hydrolysis rates exhibited by most phage group 94/96 isolates, which generally produce large amounts of type A $\beta$-lactamase and exhibit borderline susceptibility to the anti-staphylococcal penicillins [19–21]. During in vitro susceptibility testing, only VK7115 demonstrated a marked inoculum effect with ampicillin and cefazolin (table 1).

In vivo prophylaxis studies. To test directly the contribution of $\beta$-lactamase production to failures of prophylaxis in a low-inoculum guinea pig model, we determined the ability of placebo (saline), ampicillin, and cefazolin to prevent infection by the paired isogeneic isolates. Significant direct correlations between the inoculum size and the infection rate were observed for each isolate–prophylactic regimen combination (figure 2). Differences in ID$_{50}$s of isolates DK2076, VK7114, and VK7115 after prophylaxis with placebo were not statistically significant. In contrast, when prophylaxis with 100 mg/kg ampicillin was administered, the ID$_{50}$ of VK7115 was lower than that exhibited by VK7114, demonstrating that $\beta$-lactamase production enabled a smaller inoculum of bacteria to establish infection ($P < .001$ for the curves representing VK7114 and VK7115). Similarly, there was a 5-fold reduction in the ID$_{50}$ of VK7115 compared with that of VK7114 when prophylaxis with 100 mg/kg cefazolin was administered ($P < .001$).

Discussion

We have shown that the inoculum of bacteria required to establish a soft tissue infection in the setting of prophylaxis with ampicillin or cefazolin is smaller for a $\beta$-lactamase–producing isolate of $S. aureus$ than for its paired isogeneic isolate lacking $\beta$-lactamase activity. This observation supports the hypothesis that the in vivo hydrolysis of a relatively $\beta$-lactam–labile antibiotic by $\beta$-lactamase enables an $S. aureus$ isolate to survive prophylaxis and contributes to the pathogenesis of type A $\beta$-lactamase structural gene, $blaZ$, in pVK103, VK7114 and VK7115 were identical except that only VK7115 produced a clean surgical wound infection [11]. Furthermore, $S. aureus$ resistance is demonstrable in vivo with inocula in the 10$^2$–10$^3$-cfu/mL range, whereas the in vitro demonstration of a two- to 4-fold difference in the MIC of VK7114 and VK7115 required inocula of $>10^5$ cfu/mL (table 1).

Why $S. aureus$ resistance to $\beta$-lactams that can be reliably identified only with large inocula in vitro can be demonstrated with small inocula in vivo might be related to differences in the diffusion capabilities of antibiotics under these conditions. In vitro susceptibility testing is done in media that allow rapid diffusion of antibiotics, with the result that new molecules of $\beta$-lactam can replace those that the bacteria degrade. As gram-positive bacteria lack an outer cell membrane, single bacteria

### Table 1. MICs of ampicillin and cefazolin for isogeneic $S. aureus$ isolates.

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<th>Antibiotic/isolate</th>
<th>$10^1$</th>
<th>$10^2$</th>
<th>$10^3$</th>
<th>$10^4$</th>
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<th>$10^6$</th>
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<td>DK2076</td>
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<td>VK7114</td>
<td>0.12</td>
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<td>0.12</td>
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<tr>
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<td>4</td>
<td>32</td>
<td>$&gt;128$</td>
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are highly susceptible to β-lactams in vitro, and only when a large number of staphylococci are present is β-lactamase-mediated resistance observed in vitro [28]. In contrast, the wound environment includes areas of devitalized tissue and inflammation that likely restrict the ability of antibiotic in the blood and tissues to replace what has been inactivated by a β-lactamase-producing staphylococcal isolate. This might give a small inoculum of β-lactamase-producing bacteria a survival advantage over a β-lactamase-negative isolate.

The β-lactamase instability of some cephalosporins also has been implicated as a cause of treatment failure of serious S. aureus infections. Reports of failures of cephaloridine in endocarditis [29] along with its susceptibility to inactivation by S. aureus β-lactamase [30, 31] prompted the recommendation that more stable β-lactams should be used [32]. Similarly, the introduction of cefazolin into clinical practice in the mid-1970s was followed by reports of its susceptibility to β-lactamase-mediated degradation [12–18]. Sporadic reports of clinical treatment failure with cefazolin in endocarditis raised questions about whether cefazolin-hydrolyzing β-lactamases adversely influenced the outcome of therapy [33, 34]. Others interpreted these few reports of therapeutic failure as reflecting the inherent difficulties in managing endocarditis and cautioned against rejecting the use of cefazolin in staphylococcal infections on the basis of uncontrolled studies [35].

Using in vivo models, several investigators have addressed the role of β-lactamase-mediated hydrolysis of cefazolin in treatment failure. Chapman and Steigbigel [36] found that S. aureus isolates that exhibit relative resistance to cefazolin (using high-inoculum MICs) responded less favorably to therapy with cefazolin than did other, more β-lactamase-stable antibiotics in a murine model of peritonitis. Furthermore, S. aureus isolates that lacked an inoculum effect to cefazolin were effectively treated with cefazolin in vivo. Conclusions derived from animal models of endocarditis have been mixed, with some studies supporting [37, 38] and others disputing [39, 40] the importance of β-lactamase-mediated cefazolin hydrolysis in influencing the response to therapy. Fields et al. [41] concluded that cefazolin was inactivated in vivo by an isolate of S. aureus producing type A β-lactamase on the basis of their recovery of less bioactive cefazolin than cefmetazole from tissue cages in rabbits that had been infected with this isolate, despite favorable serum pharmacokinetics of cefazolin and no differences in the concentrations of the two drugs in uninfected tissue cages. In each of these studies, the activity of cefazolin was compared with that of a more β-lactamase-stable drug.

Because of differences in the pharmacokinetics of different antibiotics as well as other determinants of antimicrobial activity, such as the affinity of binding to vital penicillin-binding proteins, it is difficult to make inferences about the importance of β-lactamase stability to antibiotic efficacy on the basis of comparisons of different antibiotics. A more direct way to determine the effect of β-lactamase on antibiotic efficacy is to
use the strategy that we used in this investigation, that is, to compare isogeneic isolates of *S. aureus* that differ only in the production of β-lactamase. The isolates constructed in this study could be useful in resolving issues concerning the effect of β-lactamase on antibiotic efficacy in the treatment of other serious *S. aureus* infections.

In summary, we conclude that β-lactamase contributes to the pathogenesis of *S. aureus* wound infections in the setting of prophylaxis with ampicillin or cefazolin. Furthermore, *S. aureus* resistance is demonstrable with lower inocula in vivo than in vitro. This difference may be related to greater restriction of antibiotic diffusion in the wound environment.

Acknowledgment

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References

14. Fong IW, Engelking ER, Kirby WMM. Relative inactivation by *Staphylo-


