Subinhibitory Concentrations of Antibiotics Exacerbate Staphylococcal Infection by Inducing Bacterial Virulence

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ABSTRACT

Antibiotics are widely used for the treatment of bacterial infections. However, injudicious use of antibiotics based on an empirical method may lead to the emergence of resistant strains. Despite appropriate administration of antibiotics, their concentrations may remain subinhibitory in the body, due to individual variations in tissue distribution and metabolism rates. This may promote bacterial virulence and complicate the treatment strategies. To investigate whether the administration of certain classes of antibiotics will induce bacterial virulence and worsen the infection under in vivo conditions. Different classes of antibiotics were tested in vitro for their ability to induce virulence in a methicillin-resistant S. aureus strain Mu3 and clinical isolates. Antibiotic-induced pathogenicity was assessed in vivo using mouse peritonitis and bacteremia models. In vitro, β-lactam antibiotics and tetracyclines induced the expression of multiple surface-associated virulence factors as well as the secretion of toxins. In peritonitis and bacteremia models, mice infected with MRSA and treated with ampicillin, ceftazidime, or tetracycline showed enhanced bacterial pathogenicity. The release of induced virulence factors in vivo was confirmed in a histological examination. Subinhibitory concentrations of antibiotics belonging to β-lactam and tetracycline aggravated infection by inducing staphylococcal virulence in vivo. Thus, when antibiotics are required, it is preferable to employ combination therapy and to initiate the appropriate treatment plan, following diagnosis. Our findings emphasize the risks associated with antibiotic-based therapy and underline the need for alternative therapeutic options.

IMPORTANCE

Antibiotics are widely applied to treat infectious diseases. Empirically treatment with incorrect antibiotics, or even correct antibiotics always falls into subinhibitory concentrations, due to dosing, distribution, or secretion. In this study, we have systematically evaluated in vitro virulence induction effect of antibiotics and in vivo exacerbated infection. The major highlight of this work is to prove the β-lactam and tetracyclines antibiotics exacerbated disease is due to their induction effect on staphylococcal virulence. This phenomenon is common and suggests that if β-lactam antibiotics remain the first line of defense during empirical therapy, we either need to increase patient reliability or the treatment approach may improve in the future when paired with anti-virulence drugs.

KEYWORDS

Staphylococcus aureus, subinhibitory concentration, virulence, antibiotics

The importance of timely administration of effective antibiotics in serious bacterial infections has been repeatedly emphasized (1). Therefore, antibiotics are often administered empirically to treat bacterial infections before antibiograms are available. Due to
these factors, the administered antibiotics may not be effective against multidrug-resistant pathogens (2, 3). The microbiological effects of antibiotics extend beyond antibacterial activities. In nature, antibiotics function as signaling molecules (4). At subinhibitory concentrations, they modulate gene expression and alter bacterial physiology (5). Despite using “appropriate” antibiotics and dosages, their availability in the body remains lower than the MIC. This happens due to differences in tissue distribution and metabolic rates among individuals. As a result, ineffective doses of antibiotics may stimulate bacterial virulence and worsen the disease outcome (6). Voluminous research has shown that antibiotics at subinhibitory concentrations can increase *S. aureus* virulence. Among several classes of antibiotics, β-lactams have been reported to induce a plethora of virulence factors such as alpha-toxin, Panton–Valentine leukocidin (PVL), phenol-soluble modulins (PSMs), and capsule production in vitro (6–11). Although a recent in vivo study discovered *S. aureus* lipoprotein as a major factor in β-lactam induced pathogenesis, their findings mainly focused on hypercytokinemia (10). In contrast to these findings, the present study focused on the antibiotic-induced virulence expression in *S. aureus* and replicated the actual infections elicited by antibiotics in vivo.

We first employed ampicillin as an example to confirm its induction effect on *S. aureus* virulence factors expression and production in vitro at subinhibitory concentrations. Later, we used mouse models to show that β-lactam and tetracycline antibiotics, when used at clinically relevant concentrations, may worsen bacterial infection in vivo. From these findings we demonstrated that antibiotics at subinhibitory concentrations may enhance *S. aureus* pathogenicity and put forward the need for alternative therapeutics.

**RESULTS**

Subinhibitory concentrations of ampicillin enhanced *S. aureus* virulence factors expression and production in vitro. Since ampicillin is still in the guideline for empirical treatment for Methicillin susceptible *S. aureus* (MSSA) (12), the enhancement of virulence gene expression by antibiotics in vitro was first tested using ampicillin. Increased expression of virulence in the presence of ampicillin was assessed using luminescence reporter assay, q-PCR, Western blot, hemolysis, leukotoxic and intracellular survival assays. The MIC of ampicillin against *S. aureus* Mu3 (MRSA strain) was 64 mg/L and it started to induce *hla* expression from 32 mg/L (Fig. 1a). Based on Western blot analysis, we noticed that ampicillin dramatically enhanced protein A and alpha-toxin production in Mu3 at concentrations ranging from 0.06 mg/L to 64 mg/L (Fig. 1b). It is well known that hemolysins produced by *S. aureus* cause hemolysis of erythrocytes. Hence, we tested the hemolytic activity of culture supernatant grown in the presence of a subinhibitory concentration of ampicillin (16 mg/L). A 35-fold increase in hemolytic activity of human erythrocytes was observed at this concentration (Fig. 1c). Genes related to *S. aureus* surface protein and toxins such as *spa, hla, fnbB, lukF-PV* and *clfA* showed heightened expression at subinhibitory concentration of ampicillin (Fig. 1d). Leucotoxicity assay was performed using Mu3 culture on J774.1 macrophage cells. Compared to the PBS-treated control, ampicillin (32 mg/L) treated samples showed 8-fold increase in leukotoxicity and this observation validated the induction of virulence by ampicillin (Fig. 1c). Microscopic examination of macrophages (Fig. 1e) indicated that bacterial cultures treated with ampicillin at subinhibitory concentrations markedly enhanced the lysis of macrophages.

The intracellular survival of *S. aureus* in macrophages is dependent on multiple virulence factors such as alpha-toxin, adhesins, and aureolysin (13). Increased expression of alpha-toxin and adhesins (mainly protein A, *clfA* and *fnbA*) (Fig. 1d) at subinhibitory concentrations of ampicillin led to the hypothesis that this antibiotic may induce the intracellular survival of *S. aureus* in macrophages. In the absence of ampicillin, after 24h of incubation, 90% of intracellular *S. aureus* were cleared by macrophages. In contrast, after infection with *S. aureus*, ampicillin treatment at concentrations ranging from 2 to 32 mg/L increased intracellular bacterial survival by 2- to 3-fold (Fig. 1f). This observation indicates that ampicillin at subinhibitory concentrations may impair the clearance of *S. aureus* in macrophages, and we anticipated that similar effects could
also be observed in vivo. Hence, the aggravation of S. aureus virulence in the presence of ampicillin and other antibiotics was further tested in mice infection models.

**Exacerbated S. aureus infection in mice is not associated with the side effects of ampicillin.** Studies have shown that after the administration of one dosage of ampicillin (40 mg/kg) subcutaneously, its highest serum concentration was around 30 mg/L in the mice and serum concentration retained higher than 3 mg/L for more than 2 h

FIG 1 Subinhibitory concentrations of ampicillin induce the production of virulence factors in vitro in S. aureus Mu3. (a) Strain Mu3 harboring pGhla was treated with various concentrations (mg/L) of ampicillin. Luminescence signals were monitored every 1 h, and the curve was plotted for hla gene expression. (b) Western blot analysis of S. aureus culture supernatant showing protein A and alpha-toxin production. A dose dependent increase in the production of these virulence factors was observed until 32 mg/L of ampicillin. (c) Leukotoxicity (macrophage J774.1 cells) and hemolytic (human red blood cell) activity of Mu3 culture supernatants grown at different concentrations of ampicillin. (d) qPCR analysis of virulence genes expression at subinhibitory concentrations of ampicillin. (e), Microscopic analysis of J774.1 macrophage cells treated with bacterial culture and observed under the microscope (400×). Culture supernatants of bacteria treated with 8 mg/L ampicillin or water control. (f) Intracellular bacteria recovered from ampicillin treated J774.1 macrophages. After bacteria internalized in macrophage J774.1, different concentrations of ampicillin were applied for 22 h and intracellular S. aureus survival were measured by viable count. One-way ANOVA was used to analyze the luminescence signals on agar plates by multiple comparisons of different groups with control group. Data represent mean values ± SD (*, \( P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 \)). AMP: ampicillin.
In our Western blot analysis, we found that 0.06 mg/L of ampicillin was sufficient to induce virulence. Hence, to attain a clinically relevant concentration, ampicillin at 40 mg/kg/dose was applied subcutaneously in mice and bacteremia was established by infecting with *S. aureus*. On day 3, in contrast to the treatment group, mice from the control group showed early recovery. From day 4 to day 10, a significant difference in the loss of body weight was observed between the two groups (Fig. 2a). However, the noninfected mice, treated with same amount of ampicillin alone, did not lose body weight. Thus, ampicillin treatment at subinhibitory dosages (comparable to clinical dosage) remarkably prolonged the period of convalescence. This confirmed that the worsened outcome in mice is not associated with the side effects of ampicillin treatment (Fig. 2a).

With respect to bacterial load in kidneys, liver and spleen, on day 3, no significant differences were observed between the two groups. (Fig. 2b). However, on day 6, in the ampicillin treated group, more bacteria were recovered from the liver ($P = 0.0156$) and spleen ($P = 0.0021$) (Fig. 2c and d). There were a 1-log and 2-log bacterial load differences in the liver and spleen, respectively. The increased bacterial load further confirmed that the ampicillin treatment at subinhibitory concentrations may enhance *S. aureus* virulence and pathogenesis in mice. These data indicate that the worsened outcomes of infection were solely caused by the introduction of ampicillin, but not due to its side effects.

**Antibiotics induced virulence in Mu3 and clinical strains.** The enhanced *S. aureus* virulence caused by ampicillin prompted us to test similar effects in other strains with different classes of antibiotics. Using a panel of clinical isolates, we clearly observed...
the production of protein A induced by different concentrations of ampicillin (Fig. 3a, Fig. S1). Later, in these isolates, we tested whether other classes of antibiotics will also show similar induction effect. Interestingly, along with β-lactam antibiotics, tetracyclines also induced virulence in most of the clinical isolates (Fig. 3b, Table 1). Using antibiotics belonging to these two classes, we identified all selected antibiotics heightening the activity of hla promoter, (Fig. 3d to g, Table 1) which plays a pivotal role in virulence. In bacteremia model, similar to ampicillin, tested antibiotics in these two classes at clinical dosage, worsened the infection (Fig. 3h, Fig. S2 a-h). However,
### TABLE 1

Inhibition zone and modulating effects of different antibiotics against different clinical isolates

| Abbr. | Generic name | Class | Isolate 14 | Isolate 15 | Isolate 22 | Isolate 24 | Isolate 25 | Isolate 34 | Isolate 42 | Isolate 43 | Isolate 44 | Isolate 45 | Isolate 46 | Isolate 63 | Isolate 64 | Isolate 65 |
|-------|--------------|-------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| ERY   | Erythromycin | Macrolides(B) | 23 | 26 | 8 | 23 | 27 | 24 | 25 | 25 | 7 | 25 | 22 | 25 | 25 | 22 |
| LEV   | Levofloxacin | Quinolones | 25 | 29 | 15 | 16 | 16 | 16 | 9 | 18 | 26 | 8 | 28 | 22 | 28 | 29 |
| GEN   | Gentamicin | Aminoglycosides | 16 | 19 | / | 17 | 7 | 18 | 17 | 18 | 8 | 18 | 17 | 20 | 21 |
| NTF   | Nifurtimox | / | 7 | 8 | 7 | 8 | 7 | 8 | 7 | 7 | 7 | 7 | 7 | 7 | 9 |
| POB   | Polymyxin B | Polypeptides | 7 | 7 | 7 | 7 | 7 | / | / | / | / | / | / | / | 8 | 7 |
| MER   | Meropenem | Carbapenems | 25 | 28 | 25 | 22 | 24 | 26 | 21 | 24 | 24 | 33 | 32 | 35 | 42 |
| CFT   | Ceftazidime | Cephalosporins | 20 | 27 | 19 | 14 | 17 | 16 | 16 | 26 | 32 | 31 | 17 | 30 | 28 | 31 |
| CFX   | Cefotaxime | Cephalosporins | 17 | 15 | 15 | 14 | 17 | 17 | 21 | 27 | 15 | 26 | 25 | 24 | 24 |
| TMT   | Trimethoprim | Sulfonamides | 23 | 22 | 24 | 20 | / | 21 | 17 | 20 | 20 | 22 | / | 22 | 18 | 22 |
| MIN   | Minocycline | Tetracyclines | 24 | 24 | 22 | 22 | 22 | 23 | 22 | 24 | 23 | 22 | 26 | 23 | 26 | 29 |
| CLI   | Clindamycin | Lincosamides | 24 | 28 | 16 | 24 | 27 | 28 | 30 | 27 | 28 | 27 | 28 | 27 | 29 | 33 |
| DAP   | Daptomycin | Lipopeptides | 18 | 18 | 17 | 16 | 18 | 16 | 18 | 16 | 16 | 19 | 17 | 17 | 17 | 20 |
| VAN   | Vancomycin | Glycopeptides | 15 | 18 | 17 | 15 | 15 | 16 | 15 | 15 | 15 | 16 | 15 | 16 | 16 | 17 |
| NAF   | Nafcillin | β-lactam | 16 | 30 | 12 | 12 | 18 | 14 | 11 | 28 | 32 | 13 | 30 | 25 | N | 20 |
| CEF   | Ceftriaxone | β-lactam | 11 | 24 | 15 | 13 | 19 | 15 | 13 | 27 | 25 | 18 | 25 | 24 | N | 24 |
| CAB   | Carbenicillin | β-lactam | / | 10 | / | / | / | / | 27 | 23 | / | / | 22 | 27 | N | 10 |
| CFZ   | Ceftazidime | β-lactam | 10 | 22 | 13 | 10 | 12 | 10 | 19 | 23 | 12 | 20 | 16 | N | 17 |
| IMI   | Imipenem | Carbapenems | 23 | 35 | 28 | 27 | 23 | 27 | 28 | 44 | 40 | 42 | 45 | N | 45 |
| OXA   | Oxacillin | β-lactam | 10 | 23 | 10 | 8 | 17 | 8 | 10 | 30 | 32 | 10 | 28 | 25 | N | 28 |
| CFL   | Cefaclor | β-lactam | / | 15 | / | / | / | / | 24 | 24 | / | 25 | 24 | N | 15 |
| AMP   | Ampicillin | β-lactam | / | / | / | / | / | / | 15 | 16 | / | 11 | 15 | N | / |
| CLT   | Chloramphenicol | Tetacyclines | 28 | 34 | 11 | 31 | 9 | 12 | 29 | 29 | 9 | 27 | 28 | 29 | 29 | 11 |
| TET   | Tetracycline | Tetacyclines | 29 | 34 | 8 | 31 | 8 | 10 | 28 | 28 | 8 | 26 | 29 | 28 | 29 | 8 |
| OXT   | Oxytetracycline | Tetacyclines | 29 | 36 | 7 | / | 27 | 27 | / | 28 | 27 | 27 | 28 | 31 | / |
| DMC   | Demeclocycline | Tetacyclines | 28 | 35 | 9 | 32 | 9 | 12 | 28 | 29 | 9 | 28 | 29 | 29 | 29 | 9 |
| MTC   | Methacycline | Tetacyclines | 29 | 36 | 9 | 32 | 9 | 12 | 28 | 28 | 8 | 28 | 28 | 28 | 30 | 10 |
| DMCX  | Doxycycline | Tetacyclines | 30 | 37 | 22 | 32 | 16 | 30 | 30 | 17 | 24 | 30 | 30 | 29 | 14 |
| MIN   | Minocycline | Tetacyclines | 27 | 30 | 29 | 28 | 26 | 26 | 27 | 28 | 27 | 26 | 26 | 19 | 21 |
| TIG   | Tigecycline | Tetacyclines | 25 | 27 | 28 | 29 | 23 | 31 | 22 | 23 | 23 | 22 | 22 | 23 | 24 | 21 |

(Continued on next page)
### TABLE 1 (Continued)

| abbr. | Generic name | Class | Isolate 66 | Isolate 72 | Isolate 73 | Isolate 76 | Isolate 83 | Isolate 84 | Isolate 85 | Isolate 86 | Isolate 509 | Isolate 513 | Mu3 USA300 |
|-------|--------------|-------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| CLT   | Chlorotetracycline | Tetracyclines | 11 | 29 | 9 | 25 | 26 | 28 | 11 | 29 | 20 | / | 7 | 11 |
| TET   | Tetracycline | Tetracyclines | 8 | 29 | 8 | 29 | 26 | 28 | 9 | 30 | 20 | / | / | 7 |
| OXT   | Oxytetracycline | Tetracyclines | / | 29 | 8 | 25 | 25 | 28 | 8 | 28 | 18 | / | 7 | 8 |
| DMC   | Demeclocycline | Tetracyclines | 9 | 28 | 8 | 27 | 25 | 29 | 10 | 28 | 20 | / | 7 | 8 |
| MTC   | Methacycline | Tetracyclines | 10 | 30 | 9 | 28 | 27 | 29 | 11 | 31 | 20 | / | 8 | 8 |
| DXY   | Doxycycline | Tetracyclines | 14 | 31 | 17 | 28 | 26 | 29 | 16 | 30 | 20 | 7 | 13 | 14 |
| MIN   | Minocycline | Tetracyclines | 24 | 27 | 27 | 26 | 23 | 27 | 27 | 28 | 18 | 10 | 14 | 24 |
| TIG   | Tigecycline | Tetracyclines | 20 | 23 | 23 | 22 | 22 | 24 | 22 | 17 | 17 | 17 | 17 | 21 |

### Induction or repression against different strains (1 to 9: induction; 2 to 1: repression)

| abbr. | Generic name | Class | Isolate 66 | Isolate 72 | Isolate 73 | Isolate 76 | Isolate 83 | Isolate 84 | Isolate 85 | Isolate 86 | Isolate 509 | Isolate 513 | Mu3 USA300 |
|-------|--------------|-------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| ERY   | Erythromycin | Macrolides(Bs) | 1 | 4 | -1 | -5 | 0 | -3/2 | 1 | 6 | 5 | 1 | 2 | -5 | 2 | -5 |
| LEV   | Levofloxacin | Quinolones | 3 | 4 | 1 | 1 | 1 | 2 | 3 | 3 | 1 | 1 | 1 | 1 | -1 | -3 | -3 |
| GEN   | Gentamicin | Aminoglycosides | 1 | 3 | 0 | -3 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| NTF   | Nitrofurazone | Nitrofurans | 2 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| POB   | Polymyxin B | Polypeptides | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AMP   | Ampicillin | Penicillins | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 3 | 2 | 3 | 2 | 9 | 2 |
| MER   | Meropenem | Carbapenems | 5 | 4 | 6 | 7 | 5 | 5 | 5 | 6 | 5 | 6 | 6 | 6 | 6 | 7 | 7 |
| CFT   | Cefotaxime | Cephalosporins | 6 | 4 | 4 | 7 | 5 | 5 | 4 | 5 | 4 | 5 | 5 | 5 | 6 | 6 | 7 |
| CFX   | Cefoxitin | Cephalosporins | 8 | 6 | 9 | 5 | 7 | 7 | 6 | 4 | 7 | 5 | 6 | 5 | 9 |
| TMT   | Trimethoprim | Sulfonamides | 2 | 5 | 1 | 3 | 0 | 2 | 2 | 6 | 3 | 3 | 3 | 3 | 2 | -1 | 6 |
| MIN   | Minocycline | Tetracyclines | 5 | 7 | 5 | 4 | 5 | 6 | 8 | 8 | 4 | 9 | 6 | 6 | 1 |
| CLI   | Clindamycin | Lincosamides | -5 | -5 | -7 | -7 | 0 | -7 | -8 | -5 | -5 | -7 | -5 | -7 | -6 | -5 |
| DAP   | Daptomycin | Lincosamides | 2 | 4 | 1 | 4 | 3 | 4 | 4 | 5 | 4 | 0 | 4 | 1 | 2 | 4 |
| VAN   | Vancomycin | Glycopeptides | 2 | 4 | 3 | 6 | 4 | 5 | 5 | 6 | 4 | 2 | 4 | 2 | 4 |
| NAF   | Nafcillin | β-lactam | 9 | 8 | 9 | 8 | 9 | 9 | 9 | 6 | 6 | 7 | 6 | 7 | N | 9 |
| CEF   | Cefazoline | β-lactam | 7 | 3 | 7 | 4 | 3 | 3 | 3 | 6 | 6 | 4 | 6 | 5 | N | 6 |
| CAR   | Carbenicillin | β-lactam | 0 | 3 | 2 | 1 | 1 | 2 | 0 | 6 | 4 | 0 | 5 | 7 | N | 2 |
| CFA   | Cefadroxil | β-lactam | 5 | 7 | 5 | 6 | 7 | 6 | 6 | 6 | 6 | 5 | 6 | 5 | N | 4 |
| IMI   | Imipenem | β-lactam | 5 | 0 | 3 | 1 | 0 | 7 | 4 | 1 | 5 | 8 | 1 |
| OXA   | Oxacillin | β-lactam | 7 | 9 | 9 | 8 | 7 | 8 | 7 | 5 | 8 | 7 | 5 |
| CFL   | Cefclidin | β-lactam | 3 | 5 | 1 | 1 | 4 | 5 | 2 | 9 | 9 | 2 | 9 | 9 | N | 8 |
| AMP   | Ampicillin | β-lactam | 1 | 1 | 1 | 1 | 1 | 0 | 7 | 4 | 1 | 5 | 8 | N |

### Induction or repression against different strains (1 to 9: induction; 9 to -1: repression)

| abbr. | Generic name | Class | Isolate 66 | Isolate 72 | Isolate 73 | Isolate 76 | Isolate 83 | Isolate 84 | Isolate 85 | Isolate 86 | Isolate 509 | Isolate 513 | Mu3 USA300 |
|-------|--------------|-------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| ERY   | Erythromycin | Macrolides(Bs) | -5 | 1 | 0 | 1 | -5 | -5 | -5 | -5 | -5 | 0 | 0 | 0 | -5 |
| LEV   | Levofloxacin | Quinolones | -3 | 2 | -3 | -2 | -2 | -2 | -3 | -2 | -4 | 5 | 5 | 4 | -5 |
| GEN   | Gentamicin | Aminoglycosides | -2 | 1 | 0 | -2 | -2 | -2 | -4 | 5 | 5 | 4 | 1 |
| NTF   | Nitrofurazone | Nitrofurans | 0 | 2 | 3 | 3 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| POB   | Polymyxin B | Polypeptides | 2 | 0 | 1 | -2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AMP   | Ampicillin | Penicillins | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| MER   | Meropenem | Carbapenems | 7 | 9 | 9 | 9 | 3 | 3 | 4 | 5 | 2 | 3 | 6 |
| CFT   | Cefotaxime | Cephalosporins | 5 | 8 | 9 | 8 | 7 | 5 | 5 | 5 | 7 | 3 | 3 | 8 |
| CFX   | Cefoxitin | Cephalosporins | 6 | 9 | 8 | 8 | 8 | 6 | 1 | 6 | 8 | 4 | 8 |
| TMT   | Trimethoprim | Sulfonamides | 0 | 5 | -2 | 4 | 2 | -2 | -2 | -4 | 4 | 3 | 3 |
| MIN   | Minocycline | Tetracyclines | 6 | 6 | 6 | 7 | 6 | 1 | -5 | -2 | -5 | 6 | 4 | 4 | 6 |

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| abbr. | Generic name | Class            | Isolate 66 | Isolate 72 | Isolate 73 | Isolate 76 | Isolate 83 | Isolate 84 | Isolate 85 | Isolate 86 | Isolate 509 | Isolate 513 | Muß | USA300 |
|-------|--------------|------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|-----|--------|
| CLI   | Clindamycin  | Lincosamides    | 2          | 2          | −7         | 0          | 5          | −7         | −7         | −7         | −5         | −8         | 0   | 0      |
| DAP   | Daptomycin   | Lincosamides    | 2          | 6          | 2          | 4          | 2          | 1          | 2          | 1          | 3          | 6           | 5   | 6      |
| VAN   | Vancomycin   | Glycopeptides    | 3          | 5          | 4          | 5          | 3          | 3          | 4          | 3          | 3          | 8           | 6   | 6      |
| NAF   | Nafcillin    | β-lactam         | N          | 9          | 9          | 9          | 9          | 9          | 7          | 8          | 9          | 9           | 6   | 6      |
| CEF   | Ceftriaxone  | β-lactam         | N          | 8          | 8          | 8          | 6          | 7          | 7          | 7          | 5          | 4           | 3   | 4      |
| CAB   | Carbenicillin| β-lactam         | N          | 1          | 0          | 0          | 0          | 2          | 1          | 0          | 0           | 7           | 6   |        |
| CFZ   | Cefazidime   | β-lactam         | N          | 6          | 7          | 7          | 5          | 6          | 4          | 6          | 4           | 5           | 5   | 4      |
| IMI   | Imipenem     | β-lactam         | N          | 4          | 3          | 5          | 5          | 5          | 5          | 0          | 7           | 4           | 3   | 4      |
| OXA   | Oxacillin    | β-lactam         | N          | 6          | 4          | 7          | 5          | 4          | 5          | 7          | 3           | 5           | 5   | 5      |
| CFS   | Cefaclor     | β-lactam         | N          | 5          | 3          | 5          | 0          | 3          | 4          | 3          | 0           | 5           | 5   | 7      |
| AMP   | Ampicillin   | β-lactam         | N          | 1          | 1          | 0          | 0          | 2          | 0          | 2          | 0           | 2           | 4   | 7      |
| CLT   | Chloramphenicol| Tetracyclines   | 5          | 5          | 8          | 6          | 5          | 5          | 5          | 5          | 5           | 0           | 6   | 6      |
| TET   | Tetracycline | Tetracyclines    | 3          | 3          | 8          | 4          | 5          | 5          | 4          | 5          | 5           | 0           | 1   | 2      |
| OXT   | Oxytetracycline| Tetracyclines  | 0          | 0          | 8          | 1          | 5          | 4          | 3          | 5          | 4           | 0           | 6   | 3      |
| DMC   | Demeclocycline| Tetracyclines    | 4          | 4          | 8          | 6          | 5          | 5          | 4          | 5          | 0           | 4           | 1   |        |
| MTC   | Methacycline | Tetracyclines    | 3          | 4          | 6          | 5          | 5          | 4          | 4          | 3          | 4           | −4           | 3   | 1      |
| DXC   | Doxycycline  | Tetracyclines    | 6          | 5          | 8          | 7          | 4          | 6          | 6          | 5          | 4           | 0           | 6   | 4      |
| MIN   | Minocycline  | Tetracyclines    | 7          | 7          | 7          | 8          | 5          | 4          | 5          | 4          | 4           | 6           | 3   | 3      |
| TIG   | Tetracycline | Tetracyclines    | 5          | 6          | 5          | 6          | 2          | 3          | 6          | 2          | 4           | 6           | 2   | 3      |

\(^a/\) means no inhibition.
when these antibiotics at same doses were administered on uninfected mice, they did not affect the body weight (Fig. S2i). Thus, our findings suggested that the tested antibiotics had a virulence induction effect, and clinical S. aureus isolates may have comparable responses to these antibiotics.

**Ceftazidime, ampicillin, and tetracycline induced virulence in vivo.** Histological examination of mice kidneys infected with S. aureus and treated with ampicillin and ceftazidime revealed more abscesses than those for vehicle group (ceftazidime: 15 ± 4.2; Ampicillin: 9.6 ± 4.9; Control: 4.2 ± 1.8). Whereas tetracycline treated mice displayed small and numerous abscesses (54.6 ± 21.2) (Fig. 4). Infected mice treated with these antibiotics also showed higher production of protein A and alpha-toxin in their kidneys than those for vehicle group (Fig. 4). Although ceftazidime and tetracycline showed remarkable variation in abscess formation, their bacterial load in kidneys were identical on day 2, (Fig. S2j). This indicates that virulence in MRSA strain Mu3 is produced entirely by the treated antibiotics and is unaffected by bacterial load. These observations substantiate that when antibiotics such as ceftazidime, ampicillin and tetracycline were used at subinhibitory concentrations, not only would they fail to save the infected mice, but they may also induce S. aureus virulence leading to aggravated infection.

**DISCUSSION**

In this study, we showed that antibiotics can increase S. aureus pathogenicity in vitro and in vivo. The induction of virulence factors by antibiotics in laboratory and clinical S. aureus strains was identified using a luminescence reporter system, cell invasion investigations, protein-based tests and in mouse peritonitis model and bacteremia model.

Numerous studies have shown that the antibiotic ampicillin can induce virulence in S. aureus (16, 17). Like prior studies, ampicillin at subinhibitory concentrations enhanced S. aureus hla expression in vitro. Despite this evidence, ampicillin is still used alone or in combination with macrolides or tetracycline during empirical treatment (12, 18). In some
clinical settings, MRSA patients are treated with vancomycin and β-lactam antibiotics (19–21). Since ampicillin reaches the infection site earlier than vancomycin (22, 23), the bacterial virulence would already have been induced by ampicillin before vancomycin could kill the bacteria. Due to this potent virulence induction activity of ampicillin, we anticipated that β-lactam antibiotics may adversely affect clinical outcomes. We found that ampicillin, ceftazidime, and tetracycline increased *S. aureus* virulence genes expression and their production *in vitro*, and increased pathogenesis *in vivo*.

The induction of virulence by antibiotics was tested using multiple strains. Off note, we specifically used MRSA strain Mu3 for testing the virulence expression *in vivo*. Compared to other MRSA strains, the basic expression level of alpha-toxin in Mu3 is lower. Meanwhile, the concentration window for the response to ampicillin is broad ranging from 0.06 μg/mL to 32 μg/mL. These properties allowed us to demonstrate observable virulence induction effect *in vivo* using Mu3 strain and ampicillin.

Since ampicillin is not the strongest antibiotic in term of induction of *hla* expression as observed by disc diffusion assay, we wanted to test other antibiotics for virulence induction under *in vitro* and *in vivo* conditions. Antibiotics which show stronger induction effect on virulence expression *in vitro* may substantially worsen the infection *in vivo*. Hence, ceftazidime and tetracycline were assessed for virulence induction *in vivo*. It is noteworthy to mention that the antibiotics cephalosporins and tetracycline aggravated the disease outcome during the therapy, indicating that virulence induction is common for different antibiotics. Although the majority of the tested strains showed antibiotic-induced virulence, some did not respond to subinhibitory concentrations of ampicillin. Thus, the antibiotic-induced virulence is strain dependent.

Another limitation of this study is the use of mouse models. Mice and rats are widely used to assess antibiotic effectiveness or to investigate *S. aureus* pathogenesis *in vivo*. However, the mouse neutrophil is resistant to many *S. aureus* toxins, such as PVL. Hence, mouse may not be the optimal model for studying staphylococcal pathogenesis. Apart from mouse, the rabbit is an optimum model for studying *S. aureus* pathogenesis. Compared to human cells, rabbit cells are more susceptible to *S. aureus* toxins. For example, rabbit erythrocytes and neutrophils are more susceptible to alpha-toxin and PVL, respectively (24, 25). Some infection models such as osteomyelitis, employ larger animals like dogs, ovine, goats, and pigs. Despite the advantages of using rabbits and larger animals, we could not use these animals in our study due to their body size and special requirements by animal facility. Additionally, the need for higher sample size of these animals for infection studies may pose more challenges. Replicating these experiments in rabbits or in clinical settings may result in even worse outcomes because several generated *S. aureus* toxins cannot be represented in mice models. Despite these limitations, we believe that our investigation has provided an insight about the complications associated with antibiotics and may direct us to use nonantibiotic therapies against pathogenic bacteria.

Peritonitis and bacteremia are two life-threatening infections caused by *S. aureus*. Our mice peritonitis and bacteremia models have successfully illustrated that *S. aureus* infections were severely exacerbated after treatment with selected antibiotics at subinhibitory concentrations. Apart from the three antibiotics (AMP, CFZ, and TET) examined in our animal studies, there were other antibiotics that exhibited higher virulence-inducing properties in the paper disc experiment (Fig. 3), suggesting that similar or even worse outcomes may occur in real clinical scenarios. In clinical practice, clinicians are required to provide treatment plans prior to the identification of the pathogens or the availability of the antibiogram, which means that patients may receive erroneous antibiotics or subinhibitory concentrations of antibiotics. Even though the antibiotics that we tested are not first line antibiotics for confirmed MRSA cases, these antibiotics are nonetheless included in the guideline for empirical therapy (12, 18).

We believe that additional research on the antibiotic-induced virulence in combinatorial therapy is essential. For example, *S. aureus* pathogenesis during combinatorial treatment with β-lactam and other antibiotics could be evaluated in animal models. Antibiotics could be chosen based on local epidemiology and national guidelines and
except in severe necrotic cases, combined therapy is rarely necessary. There may be a theoretical rationale for combining two or three antibiotics in severe infections with signs of toxic shock, necrotizing fasciitis, or purpura fulminans (26). When antibiotics are used in combination, subinhibitory concentrations are feasible, particularly for some deep infections. However, this may lead to virulence induction and emergence of multidrug-resistant isolates (27). Due to the broad-spectrum activity and relatively less side effects, β-lactam antibiotics are widely used in the antimicrobial therapy. Since clindamycin at subinhibitory concentrations displays anti-virulence property (16), combination of β-lactam antibiotics with clindamycin has been widely used in antimicrobial therapy. But S. aureus rapidly develops inducible clindamycin resistance, particularly in CA-MRSA, limiting the utility of clindamycin as an empirical treatment (28, 29). In these strains, clindamycin may fail to display anti-virulence property. Off note, in our study clindamycin did not show any anti-virulence effect in clindamycin resistant clinical isolates (Table 1).

Collectively, our findings provided compelling evidence that the worsened infections resulted from induced staphylococcal virulence by subinhibitory antibiotic doses. Our in vivo findings comprehended that antibiotic-induced S. aureus infections are extremely serious and judicious use of antibiotics is essential. Based on our findings, we emphasize on the restricted use of antibiotics not only for empirical treatment but also for clinical and retrospective studies. Apart from animal experiments, population-based epidemiological studies are needed to explore the marked influence of antibiotics in clinical outcomes of patients during empirical treatment.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 2. Brain heart infusion (BHI) broth and BHI agar plates were used throughout to grow S. aureus. Chloramphenicol was used at 10 μg/mL. Unless otherwise stated, all cultures were grown aerobically at 37°C with shaking, and growth was monitored at 600 nm with a HITACHI U-2800 spectrophotometer.

MIC tests. MIC was determined by inoculating 5 × 10⁴ S. aureus cells in 100 μL BHI medium on 96-well plates with a serial dilution of antibiotics. The MIC was defined as the minimum concentration resulting in a cell density less than 0.01 OD at 620 nm (16, 30), which corresponded to no visible growth, after incubating for 18 h at 37°C.

Measurement of gene expression by bacterial cultures. Using the published protocol, different bacterial strains transformed with plasmid pGLhla or pGLspa (Table 2) were subjected to bioluminescence assay (7). In brief, 100 μL (10⁸ CFU/mL) of S. aureus samples in triplicate were dispensed into clear-bottom 96-well microtiter plates and incubated at 37°C. The bacterial growth was assessed by measuring the optical density at 620 nm (OD₆₂₀). For bioluminescence, lux reading was taken every 30 min using DTX 800/880 multimode plate reader (Beckman).

Disk diffusion and lux assays. A single colony of bioluminescent S. aureus from BHI agar was resuspended in 200 μL of sterile water. Immediately, this suspension was added to 75 mL of 0.7% (wt/vol) soft agar (375-fold dilution of original suspension), mixed thoroughly and overlaid onto BHI agar plates. Five μL of antibiotics at 4 mM concentration were added to each paper disc and placed on the plates overlaid with bacterial soft agar. The plates were incubated at 37°C, and after 20 h, inhibition zones were measured, and luminescence was detected with a PE IVIS Spectrum in vivo imaging system (PerkinElmer).

Real-time PCR to verify expression levels. Using RNeasy kit (Qiagen, Germany), and by following the manufacturer’s protocol, RNA from S. aureus strains was extracted (7). Contaminating chromosomal DNA was removed by DNase treatment (Life Technologies, Hong Kong). Purified S. aureus RNA was reverse transcribed into cDNA by PrimeScript RT reagent kit (TaKaRa, Japan) and then subjected to real-time PCR analysis using a Viia 7 thermocycler (life technologies, Hong Kong) and SYBR Premix Ex taq (TaKaRa, Japan). A relative quantification of S. aureus transcripts was determined by measuring the ratio of expression of target transcripts to expression of gyrB (housekeeping or calibration gene). The sequence of primers used in real-time PCR experiments are mentioned in Table 3.

Western blot. S. aureus strains were cultured in BHI broth and supernatant was collected at different time intervals. For ampicillin treated samples, after 24h of incubation, the OD₆₀₀ of the culture was adjusted to 6 and the supernatant was collected by centrifugation. The collected supernatant was subjected to boiling in loading buffer. After this step, 5 μL of the culture supernatant was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel. The Western blot protocol was performed as described in the product guide of Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, United Kingdom). Alpha-hemolysin was detected by using rabbit anti-staphyloccocal α-hemolysin antibody (1:20,000) (Sigma-Aldrich) and goat Horseradish Peroxidase (HRP)-conjugated anti-rabbit IgG (1:5,000) (Sigma-Aldrich). Protein A was visualized with HRP-conjugated Rabbit anti-staphylococcal Spa antibody (1:20,000) (Abcam).

Leucotoxic assay. Leukotoxic assay was performed as previously described (31). In brief, J774.1 mouse macrophage cells (ATCC TIB-67) were seeded in 96-well plates with a density of 5.0 × 10⁴ cells per well.
Staphylococcal culture supernatant (grown in presence or absence of antibiotics) was diluted to 10 times in DMEM and 100 μL/well of this mixture was added in triplicate to the cultured J774.1 cells. Following incubation at 37°C for 1 h, cell viability was measured by performing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

**Hemolysis assay on human red blood cells.** Isolation of human erythrocytes and hemolysis assay were performed using the published protocols (31, 32). Briefly, 50 μL of washed human erythrocytes (5 × 10⁶ cells/mL) were added to microtiter plates (Cellstar TC; Greiner, Germany). Wells were treated with human red blood cells in triplicate and incubated for 1 h at 37°C. The resulting hemoglobin concentration was measured by the determination of absorbance at 540 nm.

**TABLE 2** Strains and plasmids used in this study

| Strain | Phenotype | Spa typing | Source          |
|--------|-----------|------------|-----------------|
| Lab strains |          |            |                 |
| RN6390 | MSSA, Agr− | Lab stock |                 |
| Newman | MSSA, Agr+ | Lab stock |                 |
| COL    | MRSA, Agr− | Lab stock |                 |
| USA300 FPR 3757 | CA-MRSA, Agr+ | ATCC ABB1776 |                 |
| Mu3    | MRSA, Agr+ | ATCC700698 |                 |
| Clinical isolates |          |            |                 |
| AE052  | CA-MRSA, Agr+ | This study |                 |
| ST45   | MRSA, Agr+ | This study |                 |
| ST239A | MRSA, Agr+, isolate 509 | This study |                 |
| ST239AH| MRSA, Agr+, isolate 513 | This study |                 |
| Isolate 14 | Clinical isolate | T1170 | This study |
| Isolate 15 | Clinical isolate | T1081 | This study |
| Isolate 22 | Clinical isolate | T1081 | This study |
| Isolate 24 | Clinical isolate | T1081 | This study |
| Isolate 25 | Clinical isolate | T062 | This study |
| Isolate 34 | Clinical isolate | T081 | This study |
| Isolate 42 | Clinical isolate | T081 | This study |
| Isolate 43 | Clinical isolate | T211 | This study |
| Isolate 44 | Clinical isolate | T211 | This study |
| Isolate 45 | Clinical isolate | T1081 | This study |
| Isolate 46 | Clinical isolate | T211 | This study |
| Isolate 63 | Clinical isolate | T211 | This study |
| Isolate 64 | Clinical isolate | T065 | This study |
| Isolate 65 | Clinical isolate | T4398 | This study |
| Isolate 66 | Clinical isolate | T091 | This study |
| Isolate 72 | Clinical isolate | T091 | This study |
| Isolate 73 | Clinical isolate | T034 | This study |
| Isolate 83 | Clinical isolate | T548 | This study |
| Isolate 84 | Clinical isolate | T127 | This study |
| Isolate 85 | Clinical isolate | T189 | This study |
| Isolate 86 | Clinical isolate | T002 | This study |
| Plasmid |          |            |                 |
| pGL    | gfp-luxABCDE dual reporter | Lab stock |                 |
| pGLhla | gfp-luxABCDE dual reporter driven by hla promoter | Lab stock |                 |
| pGLspa | gfp-luxABCDE dual reporter driven by spa promoter | Lab stock |                 |

Staphylococcal culture supernatant (grown in presence or absence of antibiotics) was diluted to 10 times in DMEM and 100 μL/well of this mixture was added in triplicate to the cultured J774.1 cells. Following incubation at 37°C for 1 h, cell viability was measured by performing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

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**TABLE 3** Primers used in this study

| Gene | Primer for Real-time PCR |
|------|--------------------------|
| rt-hla-f | AAAAAACTGCTAGTATTAGAACGAAAGG |
| rt-hla-r | GGCCAGGGCTAAAACACTTTTG |
| rt-spa-f | CAGCAAAACCAGTCAGGCTA |
| rt-spa-r | GCTAATGTAAATCCCAACGATC |
| rt-fnbA-f | ACAAGTTGAAFAGGCAAGCC |
| rt-fnbA-r | GCCCTACATCTGTGGAGCTG |
| rt-clfA-f | ATGGCACAGTGGTTTTGACCTG |
| rt-clfA-r | TAGGCACTGAAAAACCAATTCCG |
| rt-lukF-f | TTTAAGCTTGAAGAGTTTCACCA |
| rt-lukF-r | CCAACCGATTGCAATAATTATG |
| rt-gyrB-f | CAAATGATCGACGTTCCTG |
| rt-gyrB-r | CGGCATGATCGACGTTCCTG |
with 50 μL of serially diluted bacterial culture supernatant and incubated for 60 min at 37°C. ddH2O and PBS were used as positive and negative control, respectively, in each assay. Following centrifugation, the absorption at 450 nm (A450) of the resulting supernatants was determined with a Multimode detection DTX plate reader (Beckman, Germany). All experiments were performed in triplicates and three independent assays were performed to draw the conclusion.

Intracellular survival assay. The ability of S. aureus to persist in J774.1 mouse macrophage cells was assessed by measuring the intracellular viable count of bacteria. Briefly, prior to bacterial inoculation, wells containing J774.1 were rinsed twice with warm PBS. The overnight bacterial culture grown on BHI agar was resuspended in DMEM medium (supplemented with 1% FBS) and added to J774.1 cells with a density of ~5 × 10⁶ CFU/mL (33). After 1 h, 5 μg/mL of lyostaphin was added to remove the extracellular bacteria (34). The J774.1 cells infected with intracellular S. aureus was treated with different concentrations of ampicillin and incubated for 22 h. Following incubation, total bacteria in each well were determined by CFU analysis. Uninfected control wells which underwent the same washes were processed in parallel and served as negative controls. Wells containing medium only were used for background correction. The levels of intracellular bacterial survival in control and antibiotic treated samples were calculated by the formula: final CFU of experimental well/CFU of initial intracellular bacteria. Results were assessed by repeating the experiment three times with triplicate samples in each trial.

Mouse peritonitis model. As previously described (35), we kept the 6- to 8-week-old BALB/c female mice in biosafety level 2 animal facility. Mice were housed in microisolator cages, and they received food and water ad libitum. Standard operating procedure was followed for the ethically approved protocols (CULATR 3055-13 and 3678-15). The experiments were conducted in biosafety level 2 animal facility.

Animals were daily monitored for symptoms of disease (body weight drop, inactivity, ruffled fur and labored breath) and death. During infection, animals showing severe disease symptoms (such as loss of mobility) and loss of over 20% of body weight were euthanized by i.p. injection of 100 mg/kg pentobarbital.

To establish peritonitis model, mid-exponential phase of S. aureus culture was washed twice with sterilized PBS and resuspended in PBS to obtain 1 × 10⁸ CFU/100 μL. Mice were i.p. injected with 4 × 10⁸ S. aureus. Six hours later, mice were randomized into two groups (n = 12). Each group received a dose of either 100 μL PBS or 100 μL of 8 mg/mL ampicillin in PBS subcutaneously (s.c.) twice daily (12-h interval). A third group of mice (n = 6) (as a control) were treated only with ampicillin without bacterial infection. To determine the postinfection viable bacterial count, 6 mice from each group were euthanized on day 3 and 12. The experiment was repeated once.

For bacterial load on day 6, the third trial of experiment was conducted and 15 mice from each group were euthanized. Kidneys, livers and spleens were harvested, homogenized in PBS, and plated on BHI agar.

Mouse bacteremia model. S. aureus strain Mu3 was cultured to attain the early-exponential phase, washed twice with sterilized PBS, and resuspended in PBS to obtain a cell density of 1 × 10⁸ CFU/100 μL. The female BALB/c mice, 6-8-week-old, were infected through tail vein (i.v.) with S. aureus (1 × 10⁸ CFU/mouse) and randomized into 16 groups consisting of 5 mice per group. One hour after infection, mice were treated with designated concentrations of antibiotics (s.c.) or PBS (serving as control). Antibiotics used in this study are listed in Table 4 and were administered twice per day. Treatments were performed twice per day at 12-h interval. The survival was monitored according to the body condition scoring system. After selection of antibiotics, experiment was repeated twice with selected antibiotics, ampicillin (0.8 mg/dose), tetracycline (0.15 mg/dose) and ceftazidime (0.33 mg/dose).

For histological studies, experiment was repeated again, and samples were obtained from mice which had undergone survival experiments. On day 2, mice from each group were euthanized and kidneys were collected. One kidney from each mouse was fixed in formalin for histological examination. The other kidney from each mouse was homogenized in PBS and plated on BHA to determine the bacterial viable count.

Histology. Kidney samples collected from i.v. lethal infection model were stored in 10% formalin for 48h and rinsed with 70% ethanol. Tissues were embedded in paraffin, thin-sectioned, stained with hematoxylin and eosin (H&E) or immunohistochemistry (IHC) and examined by microscopy (36). For IHC, antibodies which were used for Western blot analysis, were applied at different dilutions. α-Toxin was detected with rabbit anti-staphylococcal alpha-toxin antibody (1:10,000) (Sigma-Aldrich) and goat Horseradish Peroxidase (HRP)-conjugated anti-rabbit IgG (1:10,000) (Sigma-Aldrich).

### Table 4 List of antibiotics used for animal bacteremia models

| Antibiotics | Dose per mouse | Relative to human dose | Dose (mg/kg) |
|-------------|----------------|-----------------------|--------------|
| Nafcillin   | 0.33 mg and 0.66 mg | 1 g and 2 g | 16 mg/kg/dose and 33 mg/kg/dose |
| Meropenem   | 0.15 mg and 0.33 mg | 0.5 g and 1 g | 8 mg/kg/dose and 16 mg/kg/dose |
| Cefazidime  | 0.33 mg and 0.66 mg | 1 g and 2 g | 16 mg/kg/dose and 33 mg/kg/dose |
| Gentamicin  | 0.04 mg and 0.08 mg | 0.125 g and 0.25 g | 2 mg/kg/dose and 4 mg/kg/dose |
| Erythromycin| 0.33 mg and 0.66 mg | 1 g and 2 g | 16 mg/kg/dose and 33 mg/kg/dose |
| Tetracycline| 0.06 mg and 0.15 mg | 0.25 g and 0.5 g | 4 mg/kg/dose and 8 mg/kg/dose |
| Chloramphenicol | 0.15 mg and 0.33 mg | 0.5 g and 1 g | 8 mg/kg/dose and 16 mg/kg/dose |
| Vancomycin  | 0.15 mg          | 0.5 g          | 8 mg/kg/dose |

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P.G. and R.Y.T.K. conceived the work, designed the study, and wrote the manuscript. P.G. conducted all the experiments. Y.W. and P.G. did the paper disc assays and animal experiments. R.E.W. and P.G. did the western blotting. K.W.W., H.T.V.I., and P.G. did the histology. Y.L., S.S.C.T., and P.H.P. contribute to the draft of manuscript. P.H.P., K.Y.Y., J.D., and R.Y.T.K. supervised the study and helped interpretation of experimental results. All authors were involved in data analysis and assisted in editing the manuscript.

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