AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

Adjunctive Clavulanic Acid Abolishes the Cefazolin Inoculum Effect in an Experimental Rat Model of Methicillin-Sensitive *Staphylococcus aureus* Endocarditis

William R. Miller,[a,b] Kavindra V. Singh,[a,b] Cesar A. Arias,[a,b,c,d,e] Barbara E. Murray[a,b,c]

[a]Center for Antimicrobial Resistance and Microbial Genomics, UTHealth, McGovern Medical School, Houston, Texas, USA

[b]Department of Internal Medicine, Division of Infectious Diseases, UTHealth, McGovern Medical School, Houston, Texas, USA

[c]Department of Microbiology and Molecular Genetics, UTHealth, McGovern Medical School, Houston, Texas, USA

[d]Center for Infectious Diseases, UTHealth, School of Public Health, Houston, Texas, USA

[e]Molecular Genetics and Antimicrobial Resistance Unit, International Center for Microbial Genomics, Universidad El Bosque, Bogota, Colombia

ABSTRACT We tested the ability of clavulanic acid to restore the efficacy of cefazolin against *Staphylococcus aureus* TX0117, which exhibits the cefazolin inoculum effect (CzIE). In the rat infective endocarditis model, the coadministration of cefazolin plus clavulanic acid resulted in a significant reduction of bacterial counts (7.1 ± 0.5 $\log_{10}$ CFU/g) compared to that with cefazolin alone (2 ± 0.6 $\log_{10}$ CFU/g; $P < 0.0001$). The addition of a β-lactamase inhibitor may be a viable strategy for overcoming the CzIE.

KEYWORDS *Staphylococcus aureus*, animal models, cefazolin, clavulanic acid, infective endocarditis, inoculum effect

Staphylococcus aureus is a major pathogen in both community and health care settings, with a significant burden of morbidity and mortality (1). In many areas, a resurgence of methicillin-susceptible *S. aureus* (MSSA) infections has occurred, (2, 3) and despite *in vitro* susceptibility to a variety of antimicrobials, the treatment of invasive infections remains a challenge. Semisynthetic penicillins, such as nafcillin, have traditionally been used as a first-line therapy owing to their stability to hydrolysis by staphylococcal β-lactamases (4–6). However, recent retrospective clinical data have led some clinicians to favor cefazolin for definitive therapy, due to a favorable side effect profile, advantageous dosing schedule, and, possibly, an improved efficacy (4, 7).

The clinical failures of cefazolin in deep-seated MSSA infections, including infective endocarditis, have been described in association with isolates producing a β-lactamase (most commonly types A and C) (8–10) and showing an inoculum effect with cefazolin (CzIE). *In vitro*, the CzIE is defined as a significant increase in the MIC to 16 μg/ml or greater of cefazolin at a high inoculum (10^7 or more CFU/ml compared to the MIC at the standard inoculum [10^5 CFU/ml]) (11). It is assumed that clinical failures occur as a consequence of strains possessing the CzIE at a high bacterial burden at the site of infection. In a previously described experimental model of endocarditis in rats, an MSSA isolate positive for the CzIE was associated with significantly higher bacterial counts in vegetations when treated with cefazolin compared to those with nafcillin, ceftaroline, or daptomycin (12, 13). Furthermore, prospective observational data on the use of cefazolin in patients with MSSA bacteremia where the presence of the CzIE was assessed demonstrated that CzIE-positive isolates were associated with a significant

Received 31 May 2018 **Returned for modification** 25 June 2018 **Accepted** 17 August 2018

Accepted manuscript posted online 27 August 2018

Citation Miller WR, Singh KV, Arias CA, Murray BE. 2018. Adjunctive clavulanic acid abolishes the cefazolin inoculum effect in an experimental rat model of methicillin-sensitive *Staphylococcus aureus* endocarditis. Antimicrob Agents Chemother 62:e01158-18. https://doi.org/10.1128/AAC.01158-18.

Address correspondence to William R. Miller, William.R.Miller@uth.tmc.edu.

TABLE 1 *S. aureus* MICs at standard and high inocula

Treatment[a]	MIC (μg/ml)		
	ATCC 25923 (βla⁻, CIE⁻)	ATCC 29213 (βla⁺, CIE⁻)	TX0117 (βla⁺, CIE⁺)
Standard inoculum (10^5 CFU/ml)			
AMC	0.125/0.031	0.25/0.063	0.5/0.125
SAM	ND[b]	0.125/0.063	0.5/0.25
TZP	ND	1/0.125	1/0.125
Cefazolin	0.5	0.5	1
High inoculum (10^7 CFU/ml)			
AMC	0.125/0.031	1/0.25	8/2
SAM	ND	2/1	4/2
TZP	ND	1/0.125	4/0.5
Cefazolin	0.5	4	128
Cefazolin combinations (μg/ml)[c]			
Cefazolin plus AMP (10)	ND	4	64
Cefazolin plus AMC (10/2.5)	ND	<0.125	<0.125
Cefazolin plus AMC (1/0.25)	ND	<0.125	0.5
Cefazolin plus AMC (0.5/0.125)	ND	0.5	1
Cefazolin plus AMC (0.25/0.063)	ND	0.5	1
Cefazolin plus SAM (0.5/0.25)	ND	ND	2
Cefazolin plus TZP (1/0.125)	ND	ND	1

[a]AMC, amoxicillin-clavulanic acid; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; AMP, ampicillin.
[b]ND, not done.
[c]MICs for cefazolin combinations reported as MIC of cefazolin in the presence of specified concentrations of AMP, SAM, TZP, or AMC.

increase in therapeutic failure and mortality (14, 15). Thus, strategies to preserve the usefulness of cefazolin in the setting of the CzlE are needed. In this study, we sought to examine whether the use of clavulanic acid, a commonly available β-lactamase inhibitor, could restore the efficacy of cefazolin against an MSSA isolate exhibiting the CzlE *in vitro* and in a rat model of endocarditis. We postulated that the inhibition of the staphylococcal β-lactamase, by impairing the degradation of cefazolin, would prevent the inoculum effect *in vivo*.

We initially sought to test this hypothesis *in vitro* by determining broth microdilution MICs at standard and high inocula. Three MSSA strains were tested: (i) ATCC 29213, a BlaZ-producing isolate lacking the CzlE, (ii) TX0117, a prototypical strain exhibiting the CzlE, and (iii) ATCC 25923, an MSSA strain lacking β-lactamase (Table 1). At a standard inoculum, there was minimal difference between the MICs of amoxicillin-clavulanate (AMC) and cefazolin for each of the strains. At a high inoculum, ATCC 25923 (without β-lactamase activity) had no change in cefazolin MIC and ATCC 29213 displayed an increase from 0.5 to 4 μg/ml (below the 16 μg/ml cutoff for the CzlE), while the cefazolin MIC for TX0117 was 128 μg/ml, consistent with prior studies (10). When tested in combination, even low concentrations of AMC restored the cefazolin MIC of TX0117 to below the 16 μg/ml cutoff in a dose-dependent manner (cefazolin MICs were reduced to 1, 0.5, and <0.125 μg/ml in the presence of 0.125, 0.5, and 2.5 μg/ml of the clavulanic acid component, respectively). Of note, for *in vitro* susceptibilities, AMC was tested together, as clavulanic acid is available clinically only as a coformulation with amoxicillin. The dual β-lactam combination of cefazolin plus ampicillin (at a fixed ampicillin concentration of 10 μg/ml, corresponding to the highest concentration of amoxicillin used with the coformulation) produced only a 1-fold dilution change in the MIC, showing that clavulanic acid and not a synergistic interaction between the two β-lactams was responsible for the observed shift in the MIC. To test whether β-lactamase inhibitors available for the intravenous (i.v.) route were also effective, MICs of cefazolin were performed in the presence of fixed subinhibitory concentrations of both ampicillin-sulbactam (SAM; 0.5/0.25 μg/ml) and piperacillin-tazobactam (TZP; 1/0.125 μg/ml). The sulbactam combination resulted in a 64-fold reduction (128 μg/ml to 2 μg/ml) in cefazolin MIC at a high inoculum, and the combination with tazobactam resulted in a 128-fold reduction (128 μg/ml to 1 μg/ml) (Table 1).

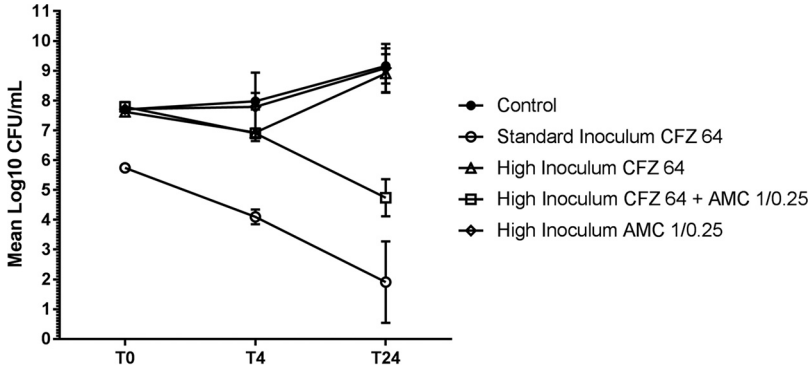

FIG 1 Time-kill curve of *S. aureus* strain TX0117 at standard and high inocula. At a standard inoculum, growth in the presence of CFZ 64 μg/ml resulted in an approximately (3.5 $\pm$ 0.8)-$\log_{10}$ reduction in CFU/ml at 24 h. At a high inoculum, growth in the presence of CFZ 64 μg/ml or AMC 1/0.25 μg/ml showed similar colony counts to the antibiotic-free control. The combination of CFZ 64 μg/ml plus AMC 1/0.25 μg/ml resulted in a reduction of 3 $\pm$ 0.4 $\log_{10}$ CFU/ml at 24 h. These results are geometric means from three independent experiments; error bars represent the standard deviations. AMC, amoxicillin-clavulanate; CFZ, cefazolin.

Using time-kill curves, we confirmed that clavulanic acid restored the efficacy of cefazolin at a high inoculum to levels comparable to that for cefazolin with a standard inoculum against TX0117 (Fig. 1). At a standard inoculum, a (3.5 $\pm$ 0.8)-$\log_{10}$ decrease in CFU/ml was observed in the presence of 64 μg/ml of cefazolin. At a high inoculum, cefazolin alone at 64 μg/ml resulted in only a slight decrease of CFU/ml at 4 h, with regrowth to 8.2 $\times$ 10^8 CFU/ml by 24 h, similar to that for TX0117 grown without antibiotics. The addition of AMC (1/0.25 μg/ml) to cefazolin restored the killing activity of cefazolin, with a (3.1 $\pm$ 0.4)-$\log_{10}$ reduction in CFU/ml at 24 h. AMC alone (1/0.25 μg/ml) showed no inhibition of growth.

Using the rat endocarditis model (12), we sought to examine whether the combination of cefazolin plus clavulanic acid would be effective *in vivo* at doses comparable to those achieved in human serum with i.v. administration of cefazolin (6-g total daily dose) and oral administration of clavulanic acid (125-mg dose) (16, 17). This protocol was approved by the Animal Welfare Committee at UTHealth. Rats infected with *S. aureus* strain TX0117 were treated beginning 30 h after inoculation with intramuscular injection of either cefazolin alone (50 mg/kg every 8 h) or cefazolin plus clavulanic acid (50 mg/kg and 4 mg/kg, respectively, given together every 8 h) for 72 h. The strain TX0117c, a derivative of TX0117 lacking β-lactamase activity (12), was used as a control for the cefazolin-only treatment arm. The differences in CFU between groups were compared using the Mann-Whitney Wilcoxon unpaired test, with significance at a *P* value of $<$0.05 using two-tailed significance levels. There was no significant difference in mean ($\pm$ standard deviation [SD]) bacterial counts between TX0117 (7.3 $\pm$ 1.3 $\log_{10}$ CFU/g vegetation) and TX0117c (7.9 $\pm$ 0.8 $\log_{10}$ CFU/g) in the infected control animals prior to starting treatment ($T = 0$). Figure 2A shows that rats infected with TX0117 and treated with cefazolin alone showed a reduction of 2 $\pm$ 0.6 $\log_{10}$ CFU/g after 72 h of antibiotic therapy. When cefazolin was given in combination with clavulanic acid, however, there was a (7.1 $\pm$ 0.5)-$\log_{10}$ CFU/g reduction in bacterial colony counts (mean difference between arms of 5 $\pm$ 0.7 $\log_{10}$ CFU/g, $P < 0.0001$). Importantly, 6 of the 7 vegetations from the combination treatment group were sterile, while all of the 11 vegetations from the cefazolin monotherapy group were infected. Furthermore, the efficacy of the combination of cefazolin plus clavulanic acid in this work is comparable to that of nafcillin (2.0 $\pm$ 2.9 $\log_{10}$ CFU/g) as assessed in a previous study of rat endocarditis using *S. aureus* strain TX0117 published by our group (12). The effect of combination therapy with cefazolin plus clavulanic acid was comparable to that using cefazolin alone against TX0117 lacking β-lactamase (TX0117c) (Fig. 2B), with a mean reduction of 6.5 $\pm$ 0.6 $\log_{10}$ CFU/g compared to that of untreated TX0117c controls ($P < 0.0001$).

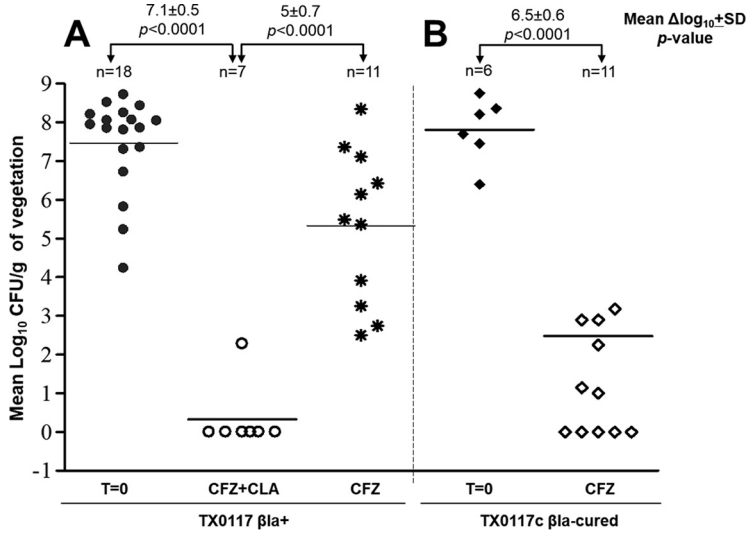

FIG 2 Clavulanic acid abolishes the cefazolin inoculum effect in a rat endocarditis model. (A) *S. aureus* TX0117 treated with CFZ plus CLA, CFZ alone, or untreated control animals ($T = 0$). Animals treated with CFZ plus CLA showed an additional mean reduction of 5 ± 0.7 $\log_{10}$ in CFU/g of vegetation compared to CFZ alone after 72 h of therapy. (B) *S. aureus* TX0117c without BlaZ activity, treated with cefazolin alone showed a mean reduction of 6.5 ± 0.6 $\log_{10}$ CFU/g compared to that of the untreated Bla-cured infected controls ($T = 0$), a decrease comparable to the parental strain treated with the cefazolin plus clavulanic acid combination (decrease of 7.1 ± 0.5 CFU/g). CLA, clavulanic acid; CFZ, cefazolin; βla+, β-lactamase positive; βla-cured, no β-lactamase activity; SD, standard deviation.

On the basis of the available clinical data, the use of cefazolin as a primary therapy for the treatment of serious MSSA infections is likely to increase. Clinical isolates of *S. aureus* possessing the *blaZ* gene are common (73% to 87% isolates) (11, 18), and studies have shown that the presence of the inoculum effect can range from 3% in low-prevalence settings to as much as 55% in settings where cefazolin is used as the front line agent for MSSA (15, 18). Rapid and efficient diagnostics for the detection of the CzIE in the clinical microbiology laboratory are not currently available, and although the investigation of rapid detection is ongoing, broth microdilution using a high inoculum remains the gold standard. Recent data from a prospective clinical study in Argentina where cephalosporins were used as a first-line therapy showed that patients infected with MSSA isolates exhibiting the CzIE were significantly more likely to have higher 30-day all-cause mortality (risk ratio [RR], 2.65; 95% confidence interval [CI], 1.10 to 6.42; $P = 0.03$) than those without the effect (15). A study in Korea supported these observations, as patients with CzIE-positive isolates treated with cefazolin were noted to have increased rates of treatment failure (61.5% versus 28.9%, $P = 0.049$) or increased all-cause mortality at 1 month (15.4% versus none, $P = 0.047$) compared to those with CzIE-negative isolates (14). This effect was not seen in those patients who received nafcillin, suggesting that the CzIE itself, and not the increased virulence of these strains, drives treatment failure. These observations suggest that effective therapeutic interventions to counter the CzIE are important when cefazolin is used as a first-line therapy. In the present study, we show that the addition of the β-lactamase inhibitor clavulanic acid is sufficient to restore the efficacy of cefazolin against the β-lactamase-producing isolate *S. aureus* TX0117 *in vivo*. This provides a proof of concept for the use of β-lactamase inhibitors in situations where the CzIE may be of clinical concern. Our observations, both *in vitro* and *in vivo*, suggest that even small concentrations of the β-lactamase inhibitor are sufficient to reverse the CzIE. While we administered clavulanic acid with each cefazolin dose, with the rationale of having the β-lactamase inhibitor at effective levels during the peak exposure to the drug, the ideal dosing regimen and duration of the combination therapy remain to be established. While the established treatment for MSSA suspected of possess-

ing the inoculum effect is still an antistaphylococcal penicillin (i.e., nafcillin) when available, our data lend support to the addition of a β-lactamase inhibitor to cefazolin in certain scenarios (such as the intolerance of nafcillin, a need for less frequent dosing intervals, or in those areas where isoxazolyl penicillins are not readily available).

In summary, the expanding use of cefazolin as a first-line treatment for serious MSSA infections may increase the risk of therapeutic failures associated with the CzIE. We demonstrate that the addition of clavulanic acid to cefazolin abolishes the inoculum effect in a rat model of endocarditis with *S. aureus* TX0117, and this combination may serve as an interesting therapeutic strategy in the future in select situations.

(Parts of this work were presented at the American Society for Microbiology Microbe 2016 meeting and at IDWeek 2017.)

ACKNOWLEDGMENTS

This work was supported in part by NIH-NIAID grants R21/R33 AI121519 and K24 AI121296, a UTHealth Presidential award, and a University of Texas System STARS award to C.A.A. and UTHealth Center for Antimicrobial Resistance and Microbial Genomics (CARMiG) seed funds to W.R.M.

The funding agency had no role in experimental design, data collection, or interpretation of this work. W.R.M. has received grants and/or honoraria from Achaogen and Merck. K.V.S. has received grants from Paratek Pharmaceuticals and Merck. C.A.A. has received grants from Merck and MeMed. B.E.M. has received grants from Paratek Pharmaceuticals and Merck and served on the advisory board for Cempra and Paratek.

REFERENCES

1. Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev 28:603–661. https://doi.org/10.1128/CMR.00134-14.
2. Dantes R, Mu Y, Belflower R, Aragon D, Dumyati G, Harrison LH, Lessa FC, Lynfield R, Nadle J, Petit S, Ray SM, Schaffner W, Townes J, Fridkin S, Emerging Infections Program–Active Bacterial Core Surveillance MRSA Surveillance Investigators. 2013. National burden of invasive methicillin-resistant *Staphylococcus aureus* infections, United States, 2011. JAMA Intern Med 173:1970–1978. https://doi.org/10.1001/jamainternmed.2013.10423.
3. Hadler JL, Petit S, Mandour M, Cartter ML. 2012. Trends in invasive infection with methicillin-resistant *Staphylococcus aureus*, Connecticut, USA, 2001–2010. Emerg Infect Dis 18:917–924. https://doi.org/10.3201/eid1806.120182.
4. McDanel JS, Roghmann MC, Perencevich EN, Ohl ME, Goto M, Livorsi DJ, Jones M, Albertson JP, Nair R, O'Shea AMJ, Schweizer ML. 2017. Comparative effectiveness of cefazolin versus nafcillin or oxacillin for treatment of methicillin-susceptible *Staphylococcus aureus* infections complicated by bacteremia: a nationwide cohort study. Clin Infect Dis 65:100–106. https://doi.org/10.1093/cid/cix287.
5. Lee S, Kwon K, Kim H, Chang H, Lee J, Choe P, Park W, Kim N, Oh M, Song D, Kim S. 2014. Clinical implications of cefazolin inoculum effect and beta-lactamase type on methicillin-susceptible *Staphylococcus aureus* bacteremia. Microb Drug Resist 20:568–574. https://doi.org/10.1089/mdr.2013.0229.
6. Vardakas KZ, Apiranthiti KN, Falagas ME. 2014. Antistaphylococcal penicillins versus cephalosporins for definitive treatment of methicillin-susceptible *Staphylococcus aureus* bacteraemia: a systematic review and meta-analysis. Int J Antimicrob Agents 44:486–492. https://doi.org/10.1016/j.ijantimicag.2014.09.002.
7. Pollett S, Baxi SM, Rutherford GW, Doernberg SB, Bacchetti P, Chambers HF. 2016. Cefazolin versus nafcillin for methicillin-sensitive *Staphylococcus aureus* bloodstream infection in a California tertiary medical center. Antimicrob Agents Chemother 60:4684–4689. https://doi.org/10.1128/AAC.00243-16.
8. Bryant RE, Alford RH. 1977. Unsuccessful treatment of staphylococcal endocarditis with cefazolin. JAMA 237:569–570. https://doi.org/10.1001/jama.1977.03270330059022.
9. Kaye D, Hewitt W, Remington J, Turck M. 1977. Cefazolin and *Staphylococcus aureus* endocarditis. JAMA 237:2601. https://doi.org/10.1001/jama.1977.03270510023006.
10. Nannini EC, Singh KV, Murray BE. 2003. Relapse of type A beta-lactamase-producing *Staphylococcus aureus* native valve endocarditis during cefazolin therapy: revisiting the issue. Clin Infect Dis 37:1194–1198. https://doi.org/10.1086/379021.
11. Nannini EC, Stryjewski ME, Singh KV, Bourgogne A, Rude TH, Corey GR, Fowler VG, Murray BE. 2009. Inoculum effect with cefazolin among clinical isolates of methicillin-susceptible *Staphylococcus aureus*: frequency and possible cause of cefazolin treatment failure. Antimicrob Agents Chemother 53:3437–3441. https://doi.org/10.1128/AAC.00317-09.
12. Nannini EC, Singh KV, Arias CA, Murray BE. 2013. *In vivo* effects of cefazolin, daptomycin, and nafcillin in experimental endocarditis with a methicillin-susceptible *Staphylococcus aureus* strain showing an inoculum effect against cefazolin. Antimicrob Agents Chemother 57:4276–4281. https://doi.org/10.1128/AAC.00856-13.
13. Singh KV, Tran TT, Nannini EC, Tam VH, Arias CA, Murray BE. 2017. Efficacy of ceftaroline in a rat model of endocarditis against methicillin-susceptible *Staphylococcus aureus* exhibiting the cefazolin high inoculum effect. Antimicrob Agents Chemother 61: e00324-17. https://doi.org/10.1128/AAC.00324-17.
14. Lee S, Song K, Jung S, Park W, Lee S, Kim Y, Kwak Y, Kim Y, Kiem S, Kim H, Kim E, Park K, Kim N, Jang H, Kim H. 2018. Comparative outcomes of cefazolin versus nafcillin for methicillin-susceptible *Staphylococcus aureus* bacteraemia: a prospective multicentre cohort study in Korea. Clin Microbiol Infect 24:152–158. https://doi.org/10.1016/j.cmi.2017.07.001.
15. Miller WR, Seas C, Carvajal LP, Diaz L, Echeverri AM, Ferro C, Rios R, Porras P, Luna C, Gotuzzo E, Munita JM, Nannini EC, Carcamo C, Reyes J, Arias CA. 2018. The cefazolin inoculum effect is associated with increased mortality in methicillin-susceptible *Staphylococcus aureus* bacteremia. Open Forum Infect Dis 23:ofy123. https://doi.org/10.1093/ofid/ofy123.
16. Xiong YQ, Willard J, Kadurugamuwa JL, Francis KP, Bayer AS, Yu J. 2005.

Real-time *in vivo* bioluminescent imaging for evaluating the efficacy of antibiotics in a rat *Staphylococcus aureus* endocarditis model. Antimicrob Agents Chemother 49:380–387. https://doi.org/10.1128/AAC.49.1.380-387.2005.

17. Adam D, De Visser I, Koeppe P. 1982. Pharmacokinetics of amoxicillin and clavulanic acid administered alone and in combination. Antimicrob Agents Chemother 22:353–357. https://doi.org/10.1128/AAC.22.3.353.
18. Wang SK, Gilchrist A, Loukitcheva A, Plotkin BJ, Sigar IM, Gross AE, O'Donnell JN, Pettit N, Buros A, O'Driscoll T, Rhodes NJ, Bethel C, Segreti J, Charnot-Katsikas A, Singh K, Scheetz MH. 2018. Prevalence of a cefazolin inoculum effect associated with *blaZ* gene types among methicillin-susceptible *Staphylococcus aureus* isolates from four major medical centers in Chicago. Antimicrob Agents Chemother 62:e00382-18. https://doi.org/10.1128/AAC.00382-18.