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Inoculum Effect with Cefazolin among Clinical Isolates of Methicillin-Susceptible *Staphylococcus aureus*: Frequency and Possible Cause of Cefazolin Treatment Failure▽

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Methicillin (meticillin)-susceptible *Staphylococcus aureus* (MSSA) strains producing large amounts of type A β-lactamase (Bla) have been associated with cefazolin failures, but the frequency and impact of these strains have not been well studied. Here we examined 98 MSSA clinical isolates and found that 26% produced type A Bla, 15% type B, 46% type C, and none type D and that 13% lacked *blaZ*. The cefazolin MIC_{90} was 2 μg/ml for a standard inoculum and 32 μg/ml for a high inoculum, with 19% of isolates displaying a pronounced inoculum effect (MICs of ≥16 μg/ml with 10^7 CFU/ml) (9 type A and 10 type C Bla producers). At the high inoculum, type A producers displayed higher cefazolin MICs than type B or C producers, while type B and C producers displayed higher cefamandole MICs. Among isolates from hemodialysis patients with MSSA bacteremia, three from the six patients who experienced cefazolin failure showed a cefazolin inoculum effect, while none from the six patients successfully treated with cefazolin showed an inoculum effect, suggesting an association between these strains and cefazolin failure ($P = 0.09$ by Fisher's exact test). In summary, 19% of MSSA clinical isolates showed a pronounced inoculum effect with cefazolin, a phenomenon that could explain the cases of cefazolin failure previously reported for hemodialysis patients with MSSA bacteremia. These results suggest that for serious MSSA infections, the presence of a significant inoculum effect with cefazolin could be associated with clinical failure in patients treated with this cephalosporin, particularly when it is used at low doses.

Even though the rate of methicillin **(meticillin)** resistance in *Staphylococcus aureus* is rising worldwide, a recent international prospective study found that 85.2% of *S. aureus* strains producing native valve endocarditis were susceptible to methicillin (methicillin-susceptible *S. aureus* [MSSA]) (13). Cephalosporins (e.g., cefazolin) are recommended for patients with MSSA endocarditis who have non-immediate-type hypersensitivity to penicillin (1, 29), and in addition, patients are often switched to cefazolin because of a more favorable dosing schedule.

Approximately 90% of *S. aureus* isolates produce β-lactamase(s) (Bla) (17). To date, four variants of Bla have been identified (A, B, C, and D) (10, 11, 16, 26), and each of these has a different substrate profile (25). Type A Bla efficiently hydrolyzes cefazolin (30). Strains that produce a large amount of this enzyme have been associated with cefazolin failures in patients suffering from high-inoculum staphylococcal disease (e.g., endocarditis) (3, 5, 14, 15). Unlike low-level Bla producers, high-level producer strains typically show high cefazolin MICs when a large inoculum is used (14, 15). In high-inoculum, deep-seated infections, the Bla produced by these strains of *S. aureus* might inactivate the susceptible β-lactam (i.e., cefazolin) at a rate high enough to overcome its antibacterial effect. Therefore, the presence of these strains may have significant clinical implications in infections where the bacterial burden is high, such as endocarditis, osteomyelitis, septic arthritis, pneumonia, and/or large abscesses that have not been drained.

The goals of this study were to determine the frequency of an inoculum effect with cefazolin as a measure of high-level production of Bla and to determine the type of Bla present among clinical isolates of MSSA. The relationship between an inoculum effect with cefazolin and failure in a subgroup of patients with *S. aureus* bacteremia was also assessed.

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MATERIALS AND METHODS

Strains. Eighty-six MSSA strains were randomly selected from repositories of isolates from patients evaluated for complicated skin/soft tissue infections (cSSTI) (ATLAS phase III trials), hospital-acquired pneumonia (HAP) (ATTAIN phase III trials), and endocarditis (ICE cohort) (13, 18, 21, 22). These clinical studies were conducted over the last several years in multiple countries around the world. *S. aureus* strain TX0117 (high-level producer of type A Bla)

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(14), *S. aureus* ATCC 29213 (known to produce small amounts of type A Bla) (10), and *S. aureus* ATCC 25923 (Bla-negative strain) were used as controls.

Twenty-four strains from a prospective observational study of MSSA bacteremia in patients on hemodialysis were also analyzed (23); these isolates were specifically selected to include equal numbers of patients receiving either vancomycin or cefazolin considered to have achieved cure or failure (six strains from each group). The 12 strains from patients who received vancomycin as their primary therapy were added to the 86 strains mentioned above, leading to 98 MSSA strains for the main analysis of the study. Investigators performing the laboratory experiments were blinded to any clinical data related to the strains.

Susceptibility tests. MICs were determined by a broth microdilution method using cation-adjusted Mueller-Hinton II broth, per Clinical and Laboratory Standards Institute (CLSI) guidelines (4), with the following exceptions. Cefazolin MICs were determined using low (~5×10^4 CFU/ml), standard (~5×10^5 CFU/ml) (SI), intermediate (~5×10^6 CFU/ml), and high (~5×10^7 CFU/ml) (HI) inocula and were read at 24 h. Inocula were estimated by optical density measurements, with random determinations of CFU/ml to confirm the inoculum concentrations. Our definition of a pronounced inoculum effect was an MIC of ≥16 μg/ml with the HI, which is the breakpoint for cefazolin nonsusceptibility with SI, based on the CLSI recommendations. The Bla-negative strains were also confirmed to be nitrocefin disk test negative after oxacillin induction (4). Cefazolin and cefamandole were obtained from Sigma Chemicals (St. Louis, MO).

Sequence analysis. All strains underwent genomic DNA extraction by the hexadecyltrimethyl ammonium bromide method, as described previously (12, 28), except for the addition of 10 units/ml of lysostaphin. PCRs were performed using the following primers designed to amplify a 355-bp region within the structural *blaZ* gene: F, 5′-CAAAGATGATATAGTTGCTTATTC-3′; and R, 5′-CATATGTTATTGCTTGCACCAC-3′. The size of PCR amplification products was verified by gel electrophoresis for the majority of the strains. PCR products were then analyzed by DNA sequencing. Sequence analysis was performed using the BLAST network service of the NCBI and DNASTAR software (DNASTAR Inc.) to compare similarities among other sequences. The classification of the Bla type of each strain was based on the amino acids at residues 128 and 216 encoded by the *blaZ* gene. For Bla type A, threonine is found at position 128 and serine is at position 216; for type B, lysine is at position 128 and asparagine is at position 216; for type C, threonine is at position 128 and asparagine is at position 216; and for type D, alanine is at position 128 and serine is at position 216 (26). Control strains were *S. aureus* ATCC 29213, ATCC 25923, and TX0117. Strains negative by PCR were also tested by high-stringency colony lysate hybridization using our previously described method (20), with the minor modification of using 40 units/ml of lysostaphin (Sigma, St. Louis, MO) in the lysis solution. The PCR-amplified intragenic *blaZ* fragment from TX0117 (type A) was used as a DNA probe for hybridization.

Statistical analyses. Comparisons between geometric mean (GM) MICs were performed by the nonparametric Mann-Whitney U test. Analysis of the distribution of the various types of Bla-producing strains was done by Fisher's exact test. *P* values of <0.05 were considered statistically significant. All statistical comparisons were performed using the NCSS/PASS Dawson edition program (Kaysville). In order to avoid selection bias, MSSA strains treated with cefazolin in the bacteremia study were not included for the analysis of prevalence. This study was reviewed and approved by the Duke University Institutional Review Board.

RESULTS

Bla typing and cefazolin and cefamandole MICs. Among the 98 isolates, sequence analysis of a 355-bp region within the *blaZ* gene containing residues 128 and 216 showed that 25.5% of strains encoded type A Bla, 15.3% type B, 45.9% type C, and none type D and that 13.2% were *blaZ* negative (confirmed by hybridization). Similar rates were seen for the different groups of strains included (strains from patients with cSSTI, HAP, and endocarditis), except among MSSA bloodstream isolates from hemodialysis patients, where type A strains were slightly more common than type C strains.

In the initial analysis of 98 isolates from patients with endocarditis ($n = 29$), HAP ($n = 29$), cSSTI ($n = 28$), and bacteremia ($n = 12$), the cefamandole and cefazolin MIC_{50}s and MIC_{90}s increased two- to fourfold for SI versus HI MICs (Table 1). At HI, type A producers had higher cefazolin MICs than cefamandole MICs, while type C strains displayed the opposite effect. The type B Bla producers were similar to type C Bla producers, but with a higher cefamandole MIC_{90} and a lower cefazolin MIC_{90}. Cefazolin GM MICs were significantly higher among type A strains than among type C and type B strains, while no significant difference was observed between type B and C strains. No effect on the cefazolin and cefamandole MICs at HI was seen among Bla-negative strains. The control strains ATCC 29213 (weak Bla producer) and TX0117 (high-level type A Bla producer) (14) were both confirmed to be type A producers; a PCR product was not amplified from the ATCC 25923 strain (Bla negative). The Bla-negative strains were also confirmed to be nitrocefin disk test negative after oxacillin induction (4). As previously reported (14), no increases in the cefazolin MIC at HI were observed with the Bla-negative strain ATCC 25923, while a slight increase (to 4 μg/ml) was seen with the weak type A Bla producer ATCC 29213.

TABLE 1. Cefazolin and cefamandole MIC_{50}s, MIC_{90}s, and GM MICs at HI and SI by type of Bla produced[a]

Strain group (*n*)	Parameter	Cefazolin MIC (μg/ml)		Cefamandole MIC (μg/ml)	
		SI	HI	SI	HI
Type A Bla producers (25)	GM MIC	1.3	11.2[b]	1	5.7
	MIC_{50}	1	8	1	4
	MIC_{90}	4	64	2	32
Type B Bla producers (15)	GM MIC	0.9	2.8[b]	1.3	13.6
	MIC_{50}	1	2	1	8
	MIC_{90}	1	8	4	64
Type C Bla producers (45)	GM MIC	0.9	5.6[b]	1.7	12.7
	MIC_{50}	1	4	2	16
	MIC_{90}	1	16	4	32
Bla-negative strains (13)	GM MIC	0.6	1[b]	0.6	0.4
	MIC_{50}	1	1	1	1
	MIC_{90}	1	1	1	1
All strains (98)	GM MIC	1	4.8	1.2	6.7
	MIC_{50}	1	4	1	8
	MIC_{90}	2	32	4	32

[a] HI, ~5×10^7 CFU/ml; SI, ~5×10^5 CFU/ml.
[b] The following *P* values were determined by the Mann-Whitney U test: for type A versus type B producers, 0.002; for type A versus type C producers, 0.04; for type B versus type C producers, 0.17; and for type A, B, and C producers versus Bla-negative strains, <0.0001 for all.

Further analysis of the inoculum effect with cefazolin. We next looked further at the interaction between cefazolin and the 98 MSSA isolates, using four different inocula. When a low inoculum was tested, all isolates were inhibited at ≤1 μg/ml, and with SI, all had an MIC of ≤4 μg/ml (Table 2). With increasing inoculum concentrations, the percentage of strains inhibited at each concentration was reduced. The cefazolin GM MIC for each inoculum was consistent with this observation, with a result of 0.5, 1, 2, and 5 μg/ml with low, standard, intermediate, and high inocula, respectively.

As shown in Table 2 for the HI, 19 of 98 MSSA strains (19.2%) showed a pronounced inoculum effect with a cefazolin MIC of ≥16 μg/ml (the CLSI breakpoint for nonsusceptibility using the standard inoculum), with two strains still growing at 64 μg/ml. Sequencing analysis of these 19 isolates (from patients with bacteremia [$n = 3$], endocarditis [$n = 2$], HAP [$n =$

TABLE 2. Correlation between cefazolin MIC and inoculum size for 98 strains

Inoculum size	% of strains inhibited at cefazolin concn (μg/ml)							
	≤1	2	4	8	16[a]	32	64	≥128
Low	100	0	0	0	0	0	0	0
Standard	89	5	6	0	0	0	0	0
Intermediate	33	50	8	2	2	1	3	0
High	23	12	20	24	5	9	3	2

[a] CLSI breakpoint for nonsusceptibility.

8], and cSSTI [$n = 6$]) showed that 9 had type A *blaZ* and 10 had type C *blaZ*, while none had the type B *blaZ* gene. Therefore, approximately 9% of all strains and 36% of type A strains were hyperproducers of Bla type A. Analysis of these 19 strains showed that the HI GM MIC of cefazolin was significantly higher for the Bla type A strains than for the Bla type C strains (Table 3).

Cefazolin inoculum effect with *S. aureus* bacteremia strains from hemodialysis patients. Among the 24 strains isolated from patients with MSSA bacteremia who were undergoing hemodialysis, 9 were Bla type A, 3 type B, and 7 type C and 5 were Bla negative. Six (29%) of these 24 strains showed an inoculum effect with cefazolin (MICs of 16 to 32 μg/ml). Of these six strains, five produced type A Bla (all with cefazolin MICs of 32 μg/ml), and one was a type C Bla producer. Two of these strains were isolated from the six patients successfully treated with vancomycin, one was from the six patients who had failure with vancomycin, and three were from the six patients who failed cefazolin treatment. None of the strains showing an inoculum effect with cefazolin were isolated from patients successfully treated with cefazolin (Table 4). The three strains obtained from patients failing therapy with cefazolin (two relapses and one death as preestablished causes of cefazolin failure in the bacteremia study [23]) carried the type A Bla gene and had a cefazolin MIC of 32 μg/ml at HI. The cefazolin GM MIC for the six strains belonging to the cefazolin failure group was higher than the GM MIC for the six strains from the cefazolin success group, although the difference was not statistically significant (8.9 μg/ml versus 2.2 μg/ml; $P = 0.138$).

DISCUSSION

Cefazolin has been associated with clinical failures in patients with endocarditis (3, 5, 14, 15) caused by MSSA strains displaying increased cefazolin MICs at HI (14, 15). However, the frequency of these strains and the relationship between them and clinical outcomes have not been clearly established. Therefore, we determined the frequency of such strains among clinical MSSA isolates, exploring also their presumptive role as a cause of failure in patients with *S. aureus* bacteremia. As a result, several key findings from the current study should be underscored.

TABLE 3. Analysis of strains with cefazolin MICs of ≥16 μg/ml ($n = 19$) at HI

Strain group	Cefazolin GM MIC (range) (μg/ml)		Cefamandole GM MIC (range) (μg/ml)	
	SI	HI	SI	HI
Bla A-producing strains ($n = 9$)	2.3 (1–4)	54.9[a] (32 –≥128)	1.7 (0.5–2)	13.7[b] (8–32)
Bla C-producing strains ($n = 10$)	1.1 (0.5–2)	22.7[a] (16–32)	2.3 (1–8)	34.3[b] (32–64)

[a] P value = 0.002 by Mann-Whitney U test.
[b] P value = 0.01 by Mann-Whitney U test.

TABLE 4. Cefazolin MICs at HI and distribution of strains with a cefazolin inoculum effect in MSSA bacteremia substudy

Treatment arm (n)	No. of strains with pronounced inoculum effect[a]	Cefazolin GM MIC at HI (μg/ml)
Vancomycin (12)		
Cure (6)	2	5
Failure (6)	1	4
Cefazolin (12)		
Cure (6)	0[b]	2.2[c]
Failure (6)	3[b]	8.9[c]

[a] Cefazolin MIC of ≥16 μg/ml (five type A and one type C Bla producers).
[b] P value = 0.090 (cefazolin failure versus cefazolin cure) by Fisher's exact test.
[c] P value = 0.138 (cefazolin GM MIC for cefazolin failure group versus that for cefazolin cure group) by Mann-Whitney U test.

First, although the majority of clinical MSSA strains carry Bla, there is significant variability between prevalence rates for each type of this enzyme in published studies. Among clinical MSSA strains studied in the present report, we found that type C *blaZ* predominated (~46%), followed by type A, type B, and no *blaZ* gene. Importantly, the strains analyzed in this study were from diverse populations enrolled in contemporary clinical studies conducted worldwide, and some of them may have been recovered from patients on prior antibiotics, possibly affecting the type of strains isolated (8, 9); however, this fact reflects what is seen in clinical practice. Different frequencies of each type of staphylococcal Bla have been reported. Kernodle et al., using the whole-cell extract method, reported that 54%, 15%, 30%, and 1% of strains were type A-, B-, C-, and D-producing strains, respectively (10). More in agreement with our findings, higher rates of type B/C strains (74%) than type A strains (20%) have been reported in Japan (24). Unfortunately, both of these comparator studies were conducted in the 1990s, making comparisons to current isolates of uncertain significance.

Second, a pronounced inoculum effect against cefazolin was present in approximately one-fifth of the clinical MSSA strains. We found that 19 isolates (19.2%) showed a pronounced rise in the cefazolin MIC, to ≥16 μg/ml, with HI; 9 were Bla type A and 10 were Bla type C producers. This rise in cefazolin MIC may be a significant factor in serious MSSA infections and could be the underlying cause of reported cases of cefazolin failure in treatment of MSSA strains (3, 5, 14, 15). It should be noted, however, that only 9 of 25 (36%) type A and 10 of 45 (24%) type C Bla producer strains displayed a significant cefazolin inoculum effect. This highlights the fact that it is not only the type of Bla that determines the cefazolin inoculum effect but also the amount of Bla produced. None of the type B Bla-producing strains showed a significant inoculum effect on the cefazolin MIC, and as expected, no inoculum effect was observed among Bla-negative isolates. As a screening procedure, a semiquantitative nitrocefin disk reaction using a con-

centration of cells from an overnight culture could identify most type A Bla-producing strains with a significant cefazolin inoculum effect but failed to detect type C ones (our personal observations).

Third, the presence of MSSA bloodstream isolates producing Bla type A with a pronounced inoculum effect may be associated with failures of cefazolin. Overall, we found three strains with high-level production of Bla A (cefazolin MIC of 32 μg/ml at HI) among the six subjects who failed cefazolin therapy, while none of the six MSSA strains isolated from the six patients successfully treated with cefazolin showed a cefazolin inoculum effect. While these findings did not reach statistical significance, they show a suggestive trend. Moreover, a numerically higher cefazolin GM MIC was found for the cefazolin failure group than for the cefazolin success group. These results suggest a possible role of high-level production of type A Bla as a cause of cefazolin failure. Interestingly, a recent retrospective study reported favorable outcomes for 12 patients with prosthetic joint infection caused by type A Bla-producing MSSA strains who were treated with cefazolin plus an antibiotic-impregnated spacer (19). However, it was not determined if these strains were high-level producers of type A Bla, which is likely the critical factor. Furthermore, these patients had an appropriate surgical procedure, which would decrease the number of remaining bacteria.

Deep-seated staphylococcal infections are difficult to treat, frequently requiring surgical intervention. The number of organisms inside an infected vegetation may be as high as 10^{11} CFU/g of tissue (6). Considering that up to one-half of the Bla can be excreted (2), the concentration of enzyme inside vegetations might be substantial. If the infecting MSSA strain produces large amounts of an active Bla, the enzyme may inactivate its target at a rate high enough to overcome its antibacterial effect. In the case of cefazolin, serum concentrations are still ~17 μg/ml 4 h after 1 g is given intravenously (7). However, concentrations inside infected tissue are considerably lower (e.g., 6.9 μg/ml at 60 min in bone [27]), and concentrations within a vegetation or a deep-seated abscess are also likely to be lower than those in serum. As with other β-lactams, the time that the cephalosporin concentration is maintained above the MIC is critical for bacterial suppression. However, if a strain produces sufficient amounts of Bla with a high hydrolysis rate against the cephalosporin, then the MIC inside the HI-infected site might be increased significantly, leading to a reduction in the time that the cephalosporin concentration is above the MIC. It is likely that increasing the antibiotic concentration at the infection site, up to a point, could overcome the Bla effect.

In conclusion, a pronounced inoculum effect against cefazolin, with elevation of the MIC to ≥16 μg/ml, was seen with almost 20% of strains studied, with about half of them being type A Bla producers. Our findings also suggest an association between the presence of a cefazolin inoculum effect and cefazolin failure in patients with MSSA bacteremia, consistent with previous reports (3, 5, 14, 15). Therefore, in serious and HI infections caused by MSSA, particularly those which have not undergone debridement and/or device removal, the presence of an inoculum effect with cefazolin could be associated with clinical failure, especially if cefazolin is administered at low doses.

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