Association between Type A blaZ Gene Polymorphism and Cefazolin Inoculum Effect in Methicillin-Susceptible Staphylococcus aureus

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Some proportion of type A blaZ gene-positive methicillin-susceptible Staphylococcus aureus strains exhibit the cefazolin inoculum effect (CIE). The type A blaZ gene was divided into two groups by single nucleotide polymorphisms (SNPs) at Ser226Pro and Cys229Tyr. The median cefazolin MICs at a high inoculum concentration were 5.69 µg/ml for the Ser-Cys group and 40.32 µg/ml for the Pro-Tyr group (P = 0.01). The SNPs at codons 226 and 229 in the amino acid sequence encoded by the blaZ gene were closely associated with the CIE.

Cefazolin is an antibiotic commonly used for methicillin-susceptible Staphylococcus aureus (MSSA) bacteremia and infections, and it is also recommended as an alternative to antistaphylococcal penicillins for MSSA endocarditis because of its convenient dosing and tolerability (1). However, there is concern that cefazolin use for severe MSSA infections such as endocarditis, osteomyelitis, septic arthritis, pneumonia, and large abscesses is associated with treatment failure because some MSSA strains can exhibit the cefazolin inoculum effect (CIE) (2–4). The CIE is associated with β-lactamase in S. aureus, and approximately 90% of clinical S. aureus isolates produce β-lactamase (5). There are four β-lactamase variants (A, B, C, and D), and the hydrolytic kinetics of various β-lactam antibiotics differ in the presence of each of the four variants (6–10). The classification of the β-lactamase type of each bacterial strain can also be identified by examining the amino acids present at positions 128 and 216 in the sequence encoded by the blaZ gene. Among the MSSA strains with the four variants of β-lactamase, type A β-lactamase-producing MSSA most rapidly hydrolyzes cefazolin (2, 6, 7). These isolates, and to a lesser extent, MSSA with type C β-lactamase, have been associated with the CIE in vitro.

Therefore, the sequence encoded by the type A blaZ gene, with a threonine at position 128 encoded as ACA and a serine at position 216 encoded as AGC, could be a predictable marker of the CIE. However, it is not uncommon to find MSSA isolates with the type A blaZ gene that do not show a CIE (3, 11, 12). Although some experts suggest that variation of the CIE among MSSA isolates with the type A blaZ gene is the result of differences in the amount of β-lactamase produced, the exact cause of this variation remains unclear.

Methods and bacterial isolates. MSSA blood isolates were collected from 2004 to 2013 at three Korean teaching hospitals. Of the 113 MSSA isolates collected, 17 (15%) possessing the type A blaZ gene were included in our analysis. The protocol of this study was approved by the Institutional Review Board at Pusan National University Hospital (IRB no. E-2016031). The cefazolin MICs of MSSA strains were determined by a broth microdilution method with cation-adjusted Mueller-Hinton II broth (Becton, Dickinson and Company, Sparks, MD) according to Clinical and Laboratory Standards Institute guidelines, except for the inoculum size used (13). The MICs for cultures with a high inoculum concentration (HI, ~5 × 10⁷ CFU/ml) were compared to the MICs for cultures with a standard inoculum concentration (SI, ~5 × 10⁵ CFU/ml) to identify strains with the CIE. We repeated MIC tests three times per isolate and calculated the geometric mean (GM) MIC for each isolate. We defined CIE positivity as an increase in GM MICs to ≥16 µg/ml at HI from the susceptible range of MICs at SI. S. aureus strain TX0117 (a highly active type A β-lactamase producer) (2), S. aureus ATCC 29213 (a low-activity type A β-lactamase producer), and S. aureus ATCC 25923 (a β-lactamase-negative strain) were used as controls (6).

Genomic DNA was extracted from the isolates by a spin column-based extraction method with commercially available kits (Qiagen, Hilden, Germany). PCRs and sequence analysis were performed as previously described with primers 5’-CAAGATGCATATGTGGTCTTTTCGCA-3’ and 5’-CATATGTTATGCTTGCA-3’ (3). Strains possessing the type A blaZ gene were selected on the basis of the amino acids present at residues 128 and 216 of the sequence encoded by the blaZ gene (position 128, threonine; position 216, serine) (9). Phylogenetic analyses of MSSA with the type A blaZ gene were generated with the neighbor-joining algorithm by the p-distance method with MEGA 6.06 (14). The sequence of the S. aureus PC1 β-lactamase gene was used as the reference sequence for the type A blaZ gene (NCBI Gene ID 13874570) (15).

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To measure the amount of BlaZ produced, we performed a Western blot analysis of BlaZ. *S. aureus* overnight cultures were diluted 100-fold in fresh tryptic soy broth and cultured for 20 h at 37°C. *S. aureus* cells were collected by centrifugation, suspended in lysis buffer, and incubated at 37°C for 30 min. The sample was sonicated and centrifuged at 10,000 × g for 10 min. The amount of protein in the supernatant was measured by the Bradford method. Proteins were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred from the gel to a membrane (Immobilon-P; Millipore). The membrane was treated with a blocking buffer containing 1:1,000 anti-BlaZ IgG (USBiological Life Sciences, Salem, MA, USA) at 4°C with gentle agitation overnight, followed by 1 h of incubation with 5% skim milk containing 1:2,000 anti-mouse IgG. Band intensity was measured by densitometry scanning (16).

SPSS software, version 22.0 (SPSS, Inc., an IBM Company, Chicago, IL, USA), was used for all statistical analyses. All tests of significance were two tailed; *P* ≤ 0.05 was considered to be significant.

**Results and discussion.** Seventeen clinical MSSA strains positive for the type A *blaZ* gene were analyzed. The GM MICs for the study strains were 1.57 (range, 1 to 3.17) μg/ml at SI and 17.36 (range, 4 to 101.59) μg/ml at HI. The mean MIC at HI was significantly higher than that at SI (Fig. 1). The mean increase in the HI MIC was 11.09-fold, and the greatest HI MIC increase was 50.80-fold. The study strains can be divided into two groups according to their HI MICs; 10 (58.8%) strains showed a CIE, and 7 strains did not (Fig. 1).

To investigate the association between hyperproduction of β-lactamase and the CIE, a Western blot assay of BlaZ, the β-lactamase protein, was conducted. We identified the maximal BlaZ-inducing concentration of cefazolin as 0.025 μg/ml in both the CIE-positive and CIE-negative groups, testing BlaZ production by Western blot assay and testing BlaZ production at serially diluted concentrations of cefazolin (0.005 to 1 μg/ml). The relative intensity of BlaZ was highest in the exponential growth phase (4 h). The three strains with the highest HI MICs were assigned to the CIE-positive group, and the three strains with the lowest HI MICs were assigned to the CIE-negative group. The mean HI MICs for the CIE-positive and CIE-negative groups were 67.73 ± 16.93 and 4.51 ± 0.95 μg/ml, respectively (*P* = 0.02). The relative intensity of BlaZ in the CIE-positive group was not significantly different from that in the CIE-negative group in the exponential growth phase (4 h, Fig. 2A); however, BlaZ production in the CIE-positive group was significantly higher than in the CIE-negative group in the stationary growth phase (24 h, Fig. 2B) under cefazolin-free conditions. Under cefazolin-treated conditions, BlaZ production was markedly greater in both groups during the exponential growth phase than under cefazolin-free conditions, but there was no significant difference in BlaZ production between the two groups (Fig. 2A). Under the conditions tested, the increase in BlaZ
production from the basal condition to the cefazolin-treated condition was significantly greater in the CIE-negative group than in the CIE-positive group. Therefore, BlaZ production was not significantly associated with the CIE.

The type A blaZ genes of the 17 MSSA strains isolated from blood were evaluated by phylogenetic analysis of the amino acids at positions 116 to 230. The strains were divided into two groups according to sequence similarity. There were three single nucleotide polymorphisms (SNPs) at amino acid residues 144, 226, and 229 that differentiated the two groups. The SNP affecting residue 144 was synonymous, and the SNPs at residues 226 and 229 were nonsynonymous, causing amino acid changes from serine to proline at position 226 and cysteine to tyrosine at position 229 (Table 1). To evaluate the association between these blaZ gene polymorphisms and the CIE, we assigned type A blaZ-positive MSSA to one of two groups (the serine 226-cysteine 229 [Ser-Cys] group or the proline 226-tyrosine 229 [Pro-Tyr] group) according to their SNP combinations. The median cefazolin MICs at HIs were 5.69 (interquartile range [IQR], 3.8 to 8.52) μg/ml in the Ser-Cys group and 40.32 (IQR, 25.40 to 50.80) μg/ml in the Pro-Tyr group (P < 0.01) (Fig. 3A). Of the 11 isolates in the Pro-Tyr group, 10 (91%) had a CIE (Fig. 3B) but all 6 isolates in the Ser-Cys group were CIE negative (P < 0.01). Polymorphisms at amino acid positions 226 and 229 of the sequence encoded by the type A blaZ gene were significantly associated with the CIE.

Recent retrospective studies have suggested that cefazolin had outcomes comparable to those of antistaphylococcal penicillins such as nafcillin and oxacillin (1, 17–19), even for serious infections. However, there are concerns about cefazolin therapeutic failures and relapses in the treatment of MSSA strains that demonstrate the CIE (2, 3, 20, 21). Previous studies have demonstrated that the CIE in MSSA is closely associated with the blaZ type, and type A blaZ-positive MSSA can present a CIE (3, 11, 12, 22, 23). However, the prevalence of CIE positivity among MSSA strains positive for type A blaZ is variable, and approximately 15 to 65% of these strains exhibited no CIE (3, 11, 12, 22, 23). Livorsi et al. (12) reported that only 17% of type A blaZ-positive MSSA isolates exhibited a CIE, whereas Lee et al. (11) reported that 65% did so.

Some experts assume that the variation of the CIE among type A blaZ-positive MSSA isolates may result from variation in the amount of β-lactamase produced. However, thus far, no study has addressed the cause of the variation of the CIE among MSSA strains possessing type A blaZ. Therefore, we planned this study to investigate the factors causing variation in the CIE among type A blaZ-positive MSSA strains, which are notable for their marked CIE expression.

First, we compared the amounts of β-lactamase protein produced by CIE-positive and -negative strains at a concentration of cefazolin determined to maximally induce BlaZ to assess the previous assumption that type A BlaZ hyperproducers express a CIE. Both CIE-positive and -negative MSSA strains exhibited a remarkable increase in BlaZ production after exposure to cefazolin. However, there was no correlation between the CIE and the amount of β-lactamase produced, although there were variations in β-lactamase production among the clinical strains. Therefore, we can conclude that hyperproduction of β-lactamase is not the main causative factor of CIE variation among type A blaZ-positive MSSA strains.

Second, we hypothesized that some variations in amino acids located close to active sites can cause variations in the CIE. We identified two amino acid polymorphisms at positions 226 (proline/serine) and 229 (tyrosine/cysteine) by comparing the sequences encoded by the type A blaZ genes of clinical strains. The CIE was markedly greater in the proline 226-tyrosine 229 group than in the serine 226-cysteine 229 group. Therefore, we have shown that polymorphisms in the type A blaZ gene that result in amino acid changes at positions 226 and 229 are strongly associated with the CIE in type A blaZ-positive MSSA strains.

This study is the first to identify the association between blaZ gene polymorphisms and the CIE in MSSA clinical isolates. Our findings provide novel knowledge about the CIE in MSSA and also provide some clinically useful information. Twenty to 50% of MSSA clinical isolates are type A β-lactamase producers (3, 6, 11, 24, 25). In animal studies, mortality rates were higher when infections with type A β-lactamase-producing S. aureus were treated with cefazolin (26, 27). Some investigators have suggested that cefazolin might be associated with treatment failure in serious infections such as nafcillin and oxacillin (1, 17–19), even for serious infections.

![FIG 3](http://aac.asm.org/) The association between blaZ polymorphisms and the CIE. (A) The median HI MIC for the proline 226-tyrosine 229 (Pro-Tyr) group was significantly higher than that for the serine 226-cysteine 229 (Ser-Cys) group. (B) More than 90% of the Pro-Tyr group type A blaZ-positive MSSA strains exhibited a CIE, but none in Ser-Cys group exhibited a CIE.
infections due to S. aureus with the CIE (2, 3). However, the presence of the type A blaZ gene is not a reliable marker of a CIE in MSSA infections because approximately 50% of type A blaZ-positive MSSA strains exhibited no CIE. However, 91% of the type A blaZ-positive MSSA strains with a proline at amino acid position 226 showed a CIE and 100% of those with a serine at position 226 showed no CIE. Therefore, our findings suggest that detection of the type A blaZ gene in combination with Pro-Tyr polymorphism detection at residues 226 and 229 can be a useful means of determining the CIE positivity of an MSSA infection.

Although our findings identified the association of the CIE with type A blaZ polymorphisms, we do not know the full story behind the mechanisms of the CIE. For example, it is not known how type C blaZ-positive MSSA, also associated with the CIE, can acquire CIE positivity. The clinical implications of blaZ polymorphisms and the CIE are also unclear. Further studies are warranted to determine the mechanisms of the CIE and their clinical implications.

In summary, SNPs of the blaZ gene causing variations of the amino acids at positions 226 and 229 were closely associated with the CIE in type A blaZ-positive MSSA clinical isolates.

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