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ORIGINAL ARTICLE

Inoculum effect of methicillin-susceptible *Staphylococcus aureus* against broad-spectrum beta-lactam antibiotics

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Abstract

Scarce information concerning the inoculum effect (InE) of methicillin-susceptible *Staphylococcus aureus* (MSSA) against broad-spectrum β-lactam antibiotics is available. We investigated the InE of MSSA against ceftriaxone, cefepime, meropenem, ampicillin/sulbactam and piperacillin/tazobactam. The bacteraemic MSSA isolates were collected at ten Korean general hospitals from Sep 2013 to Mar 2015. The InE was defined if MICs of antibiotics at high inoculum (HI, ~5×10^7 CFU/ml) increased beyond the susceptible range compared to those at standard inoculum (SI, ~5×10^5 CFU/ml). All isolates were sequenced for *blaZ* gene typing. Among 302 MSSA isolates, 254 (84.1%) were positive for *blaZ*; types A, B, C and D were 13.6%, 26.8%, 43.4% and 0.3%, respectively. Mean HI MICs of all tested antibiotics were significantly increased and increases in HI MIC of piperacillin/tazobactam (HI, 48.14 ± 4.08 vs. SI, 2.04 ± 0.08 mg/L, $p < 0.001$) and ampicillin/sulbactam (HI, 24.15 ± 1.27 vs. SI, 2.79 ± 0.11 mg/L, $p < 0.001$) were most prominent. No MSSA isolates exhibited meropenem InE, and few isolates exhibited cefepime (0.3%) and ceftriaxone (2.3%) InE, whereas 43.0% and 65.9% of MSSA isolates exhibited piperacillin/tazobactam and ampicillin/sulbactam InE, respectively. About 93% of type C *blaZ* versus 45% of non-type C exhibited ampicillin/sulbactam InE ($p < 0.001$) and 88% of type C *blaZ* versus 9% of non-type C exhibited piperacillin/tazobactam InE ($p < 0.001$). A large proportion of MSSA clinical isolates, especially those positive for type C *blaZ*, showed marked ampicillin/sulbactam InE and piperacillin/tazobactam.

Keywords *Staphylococcus aureus* · Beta-lactamase · Inhibitor · Inoculum effect · *blaZ* · Ampicillin-sulbactam

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Introduction

Methicillin-susceptible *Staphylococcus aureus* (MSSA) is an important pathogen in pneumonia and skin/soft tissue infections such as diabetic foot infection. Semisynthetic penicillins, such as nafcillin or oxacillin, are recommended as first-line antibiotics for serious MSSA infections and cefazolin as an alternative [1]. However, clinicians usually use empirical broad-spectrum β-lactam antibiotics such as ampicillin/sulbactam, ceftriaxone, cefepime and piperacillin/tazobactam for serious infections until culture and antimicrobial susceptibility results are reported [2–6]. Clinicians use broad-spectrum β-lactam antibiotics even after antimicrobial susceptibility results are reported if the probability of polymicrobial infection can be excluded.

Some MSSA isolates exhibit the inoculum effect against β-lactam antibiotics, i.e., the minimal inhibitory concentration of cefazolin is increased with a high inoculum to a certain degree [7, 8]. It is known that the inoculum effect (InE) of MSSA against cefazolin, one of the widely used antibiotics for MSSA infection, is not uncommon and that the cefazolin InE is closely related to the specific type of the *blaZ* gene. Types A and C *blaZ* gene-positive MSSA isolates usually exhibit the cefazolin InE [9–11]. In addition, recent studies have suggested that the InE of MSSA against cefazolin can contribute to failure in the treatment of high-burden MSSA infections [9, 10, 12].

It is also known that the InE of Gram-negative bacterial production of extended spectrum β-lactamase or AmpC type β-lactamase against broad-spectrum β-lactam antibiotics such as carbapenems, cefepime and β-lactam/β-lactamase inhibitors is not uncommon, and the resulting clinical implications can be significant [13–16]. On the other hand, there has been limited study concerning the InE of MSSA against broad-spectrum β-lactam antibiotics, although some studies have dealt with the inoculum effect of β-lactams other than cefazolin using a few isolates [17, 18].

Therefore, the purpose of this study was to determine the frequency of InE of MSSA against various broad-spectrum β-antibiotics and the association between the InE of MSSA and *blaZ* gene type. In addition, we tried to provide data to estimate the clinical implications of the InE of MSSA against broad-spectrum β-antibiotics.

Materials and methods

Study design and bacterial isolates

This was a post hoc study of the "Korea Infectious Diseases Study Group – *Staphylococcus aureus* Bacteraemia 2013" (KIND-SAB 2013) cohort study, which was performed at ten general hospitals from Sep 2013 to Mar 2015, wherein MSSA blood isolates were collected [11, 12]. We investigated the ceftriaxone, cefepime, meropenem, piperacillin/tazobactam and ampicillin/sulbactam InE of MSSA clinical isolates that were collected from a previous MSSA bacteraemia study. The KIND-SAB 2013 cohort study was approved by the institutional review boards at each participating hospital.

Sequence analysis

Genomic DNA of all isolates was extracted by spin-column-based extraction methods using commercially available kits (Qiagen, Hilden, Germany). Polymerase chain reactions (PCRs) were performed using the following primers designed to amplify a 355-bp region within the structural *blaZ* gene: 5′-CAAAGATGATATAGTTGCTTATTC-3′ and 5′-CATATGTTATTGCTTGCACCAC-3′ [9, 10]. The PCR products that were positive for the *blaZ* gene were analysed by DNA sequencing. Sequence analysis was performed using the NCBI BLAST network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The β-lactamase type of each strain was classified based on the amino acids at residues 128 and 216 encoded by the *blaZ* gene [19].

Susceptibility tests and measurement of the inoculum effect

The minimal inhibitory concentrations (MICs) of cefazolin were determined by a broth microdilution method using cation-adjusted Mueller-Hinton II broth (Becton, Dickinson and Company, Sparks, MD, USA) according to Clinical and Laboratory Standards Institute (CLSI) guidelines with the exception of the inoculum size of the strains [20]. Oxacillin, ceftriaxone, cefepime, meropenem, piperacillin/tazobactam and ampicillin/sulbactam were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The MICs of oxacillin, ceftriaxone, cefepime, meropenem, piperacillin/tazobactam and ampicillin/sulbactam obtained with a high inoculum (HI, ~5×10^7 CFU/ml) were compared with those obtained with a standard inoculum (SI, ~5×10^5 CFU/ml) to identify the strains with an InE. The MIC value of each isolate was determined by two different researchers. The InE of oxacillin, ceftriaxone, cefepime, meropenem, piperacillin/tazobactam and ampicillin/sulbactam was defined as increasing MIC to ≥ 4, ≥ 16, ≥ 16, ≥ 8, ≥ 16/4 and ≥ 16/8 mg/L at HI, respectively [20].

Clinical data and statistical analysis

We planned to provide preliminary clinical data for antibiotics that exhibited marked InE. Therefore, post hoc analysis was performed to analyse the association between outcomes and InE of β-lactam/β-lactamase inhibitors because a large proportion of MSSA exhibited InE against β-lactam/β-lactamase

inhibitors in our study. We identified MSSA bacteraemia patients who received β-lactam/β-lactamase inhibitors as empirical or definitive antibiotics and compared outcomes between InE-positive and InE-negative groups. Empirical antibiotic was defined as antibiotic administered before a report of antibiotic susceptibility of the isolates, and definitive antibiotic was defined as antibiotic administered after a report of antibiotic susceptibility of the isolates. SPSS software (version 23.0, SPSS, Inc., an IBM Company, Chicago, IL, USA) was used for all statistical analyses. Student's t test and the χ^2 or Fisher's exact test were used depending on the variable type. All significance tests were two-tailed, and $p \leq 0.05$ was considered significant.

Results

Among 302 MSSA isolates that were collected at ten general hospitals in Korea, 254 (84.1%) were positive for the *blaZ* gene; types A, B, C and D were 13.6%, 26.8%, 43.4% and 0.3%, respectively. All MSSA isolates exhibited MICs within the susceptible range against six tested antibiotics. Mean HI MICs of oxacillin (HI [mean ± SD] vs. SI [mean ± SD]; 2.28 ± 0.08 vs. 0.58 ± 0.02, $p < 0.001$), ceftriaxone (5.74 ± 0.20 vs. 4.65 ± 0.09 mg/L, $p < 0.001$), cefepime (4.05 ± 0.11 vs. 3.44 ± 0.09, $p < 0.001$), meropenem (0.32 ± 0.01 vs. 0.18 ± 0.01 μg/mL, $p < 0{,}001$), piperacillin/tazobactam (48.14 ± 4.08 vs. 2.04 ± 0.08 mg/L, $p < 0.001$) and ampicillin/sulbactam (24.15 ± 1.27 vs. 2.79 ± 0.11 mg/L, $p < 0.001$) were significantly increased (Fig. 1).

No isolates exhibited meropenem InE. Only 0.3 and 2.3% of MSSA isolates exhibited cefepime and ceftriaxone InE, respectively. Although oxacillin InE was found in 20% of MSSA isolates, the increase in oxacillin MIC at HI did not exceed 16 mg/L. However, MSSA isolates exhibited marked increases in MICs of piperacillin/tazobactam and ampicillin/sulbactam at HI, and 43.0 and 65.9% of MSSA isolates exhibited piperacillin/tazobactam and ampicillin/sulbactam InE (Fig. 2).

The geometric mean of HI MICs against ampicillin/sulbactam was significantly higher in type C *blaZ*-positive isolates (60.06 mg/L) than non-type C *blaZ* isolates (type A 4.35 mg/L, type B 6.46 mg/L, and *blaZ* negative 1.61 mg/L, $p < 0.001$). The geometric mean of HI MICs against piperacillin/tazobactam was also significantly higher in type C *blaZ*-positive isolates (33.21 mg/L) than non-type C *blaZ* isolates (type A 11.80 mg/L, type B 13.48 mg/L, and *blaZ* negative 1.34 mg/L, $p < 0.001$). The geometric means of HI MICs against oxacillin, ceftriaxone, cefepime and meropenem were not significantly different between type C and non-type C isolates (Fig. 3).

One hundred twenty-two isolates (93.1%) of 131 type C *blaZ*-positive MSSA isolates versus 77 (45.0%) of 171 non-type C isolates exhibited ampicillin/sulbactam InE ($p < 0.001$, Fig. 4A), and 115 (87.8%) of type C *blaZ*-positive isolates versus 15 (8.8%) of non-type C isolates exhibited piperacillin/tazobactam InE ($p < 0.001$, Fig. 4B). The InE of two β-lactam/β-lactamase inhibitors exhibited a strong positive correlation ($r = 0.818$), which was statistically significant ($p < 0.001$, Fig. 4C, D).

Sixty-four MSSA bacteraemia patients among 302 received β-lactam/β-lactamase inhibitors as an empirical antibiotic. Outcomes of the ampicillin/sulbactam InE-positive ($n = 27$) group who received "empirical" β-lactam/β-lactamase inhibitors were significantly higher than negative ($n = 23$) groups (Table 1, 3-month mortality of InE-positive vs. InE-negative; 50.0% vs.7.7%, $p = 0.01$). Outcomes of the ampicillin/sulbactam InE-positive ($n = 28$) group who received "definitive" β-lactam/β-lactamase inhibitors for the treatment of MSSA bacteraemia were not significantly different from negative ($n = 14$) groups, although there was a tendency toward higher mortality in the InE-positive group (Table 1, 3-month mortality 31.6% vs. 8.3%, $p = 0.20$). Outcomes of the piperacillin/tazobactam InE-positive group who received β-lactam/β-lactamase inhibitors "empirically" (3-month mortality of InE-positive [$n = 27$] vs. InE-negative [$n = 23$]; 38.9% vs. 34.8%, $p = 0.79$) and "definitively" (3-month mortality 38.5% vs. 11.1%, $p = 0.10$) were not significantly different from negative groups.

Discussion

In this study, we found that MICs against MSSA of most β-lactam antibiotics that we tested increased to some degree as inoculum of the organisms were increased. The MIC of β-lactam/β-lactamase inhibitors at HI was especially prominent, and more than half of MSSA isolates exhibited InE against β-lactam/β-lactamase inhibitors, whereas the MICs of other broad-spectrum β-lactam antibiotics at HI were not significant enough to exceed break points. Almost all MSSA isolates exhibited minimal InE against ceftriaxone and cefepime but not against meropenem. The β-lactamase of *S. aureus* is structurally a class A serine β-lactamase according to Bush's classification [21]. It is known that a main target of β-lactamase inhibitors such as sulbactam and tazobactam is the class A serine β-lactamase of Gram-negative organisms, and the enzyme is easily inhibited by these inhibitors [21, 22]. However, our study demonstrates that large proportions of MSSA clinical isolates producing β-lactamase exhibit increased MICs beyond the susceptible range if the inocula of organisms are increased.

We have demonstrated an association between the MIC increase of β-lactam/β-lactamase inhibitors at HI and the type of *blaZ* encoding β-lactamase in MSSA. Several previous studies have reported an association between the cefazolin

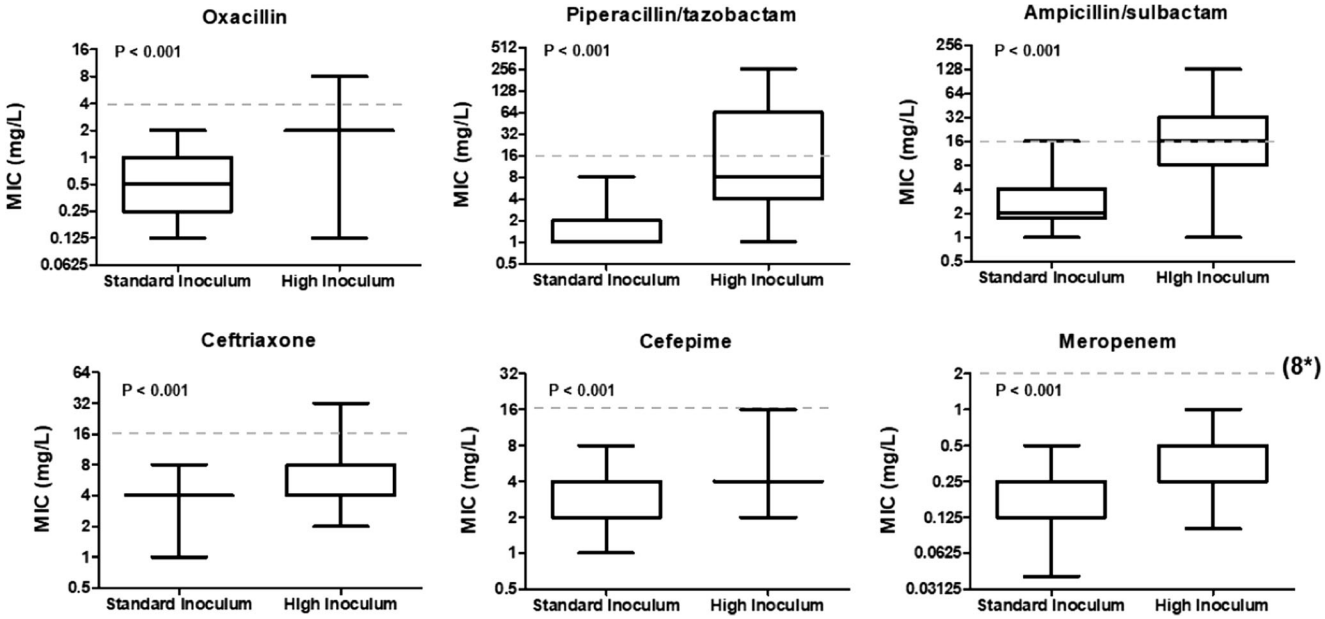

Fig. 1 Comparison of high inoculum versus standard inoculum MICs of 302 MSSA blood isolates; oxacillin, piperacillin/tazobactam, ampicillin/sulbactam, ceftriaxone, cefepime, and meropenem. Grey dashed lines indicate break points of each antibiotic, and the break point of meropenem* is ≥ 8 mg/L

InE and *blaZ* gene type in MSSA; most cefazolin InE-positive isolates possess type A *blaZ*, and some cefazolin InE-positive isolates possess type C [9, 11, 23–25]. Our study shows that pronounced InE of β-lactam/β-lactamase inhibitors has a close association with the type C *blaZ* gene. MSSA possessing type C *blaZ* was about 10 times more likely to express piperacillin/tazobactam InE than MSSA possessing non-type C *blaZ*. It is known that 20 to 50% of MSSA isolates had the type C *blaZ* gene and that the proportion of type C-possessing MSSA varies according to the geographic region

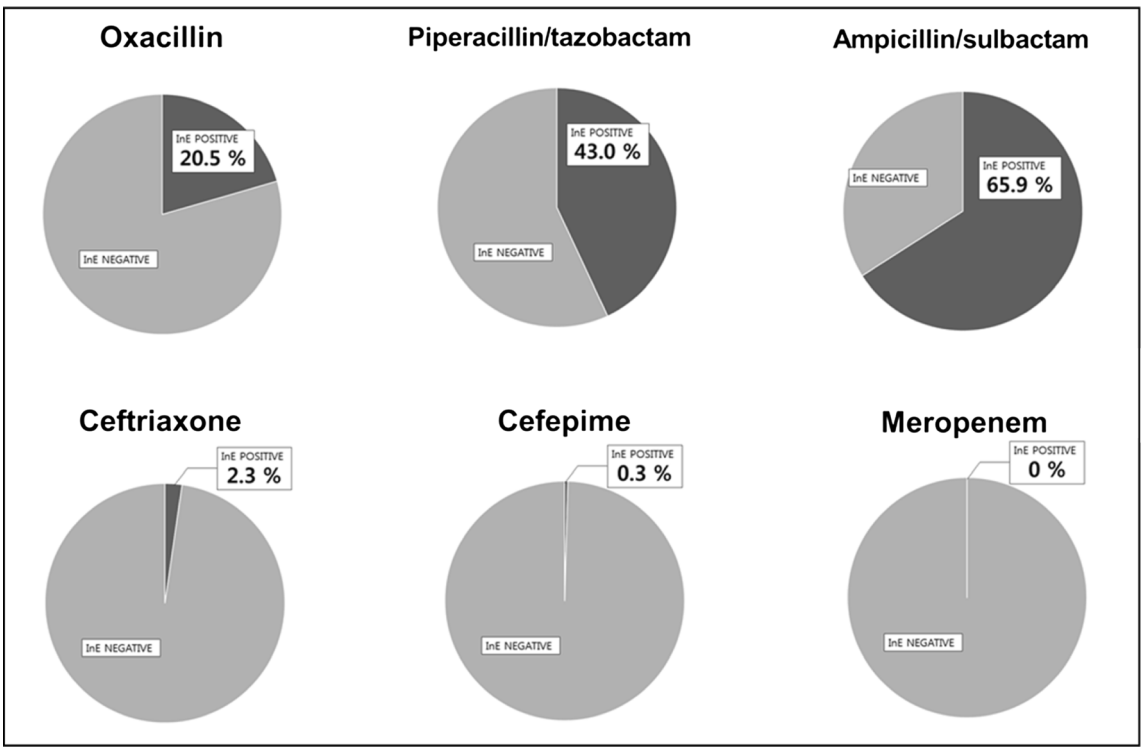

Fig. 2 Proportions of inoculum effect (InE)-positive isolates among 302 MSSA blood isolates against oxacillin, piperacillin/tazobactam, ampicillin/sulbactam, ceftriaxone, cefepime, and meropenem. Black colour indicates InE-positive, and grey colour indicates InE-negative

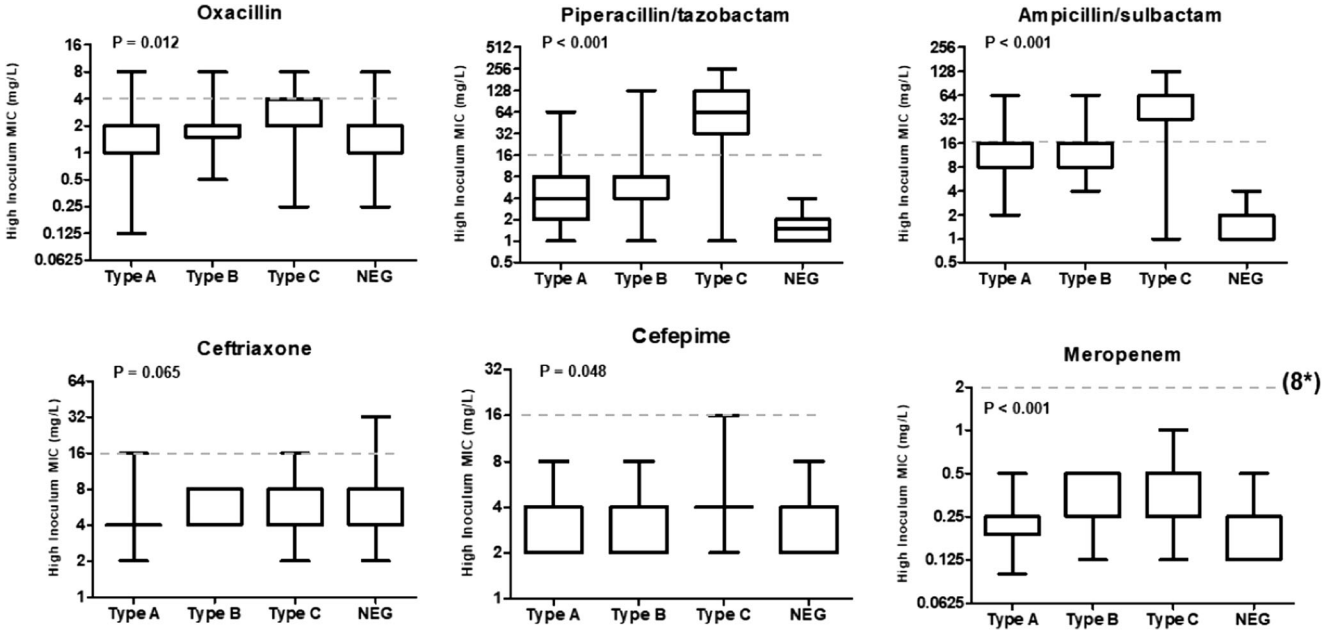

Fig. 3 Comparison of high inoculum MICs of 302 MSSA blood isolates according to *blaZ* type against oxacillin, piperacillin/tazobactam, ampicillin/sulbactam ceftriaxone, cefepime, and meropenem; 41 type A, 81 type B, 131 type C and 48 *blaZ*-negative MSSA excluding 1 type D-positive MSSA. Grey dashed lines indicate the break points of each antibiotic, and the break point of meropenem* is ≥ 8 mg/L

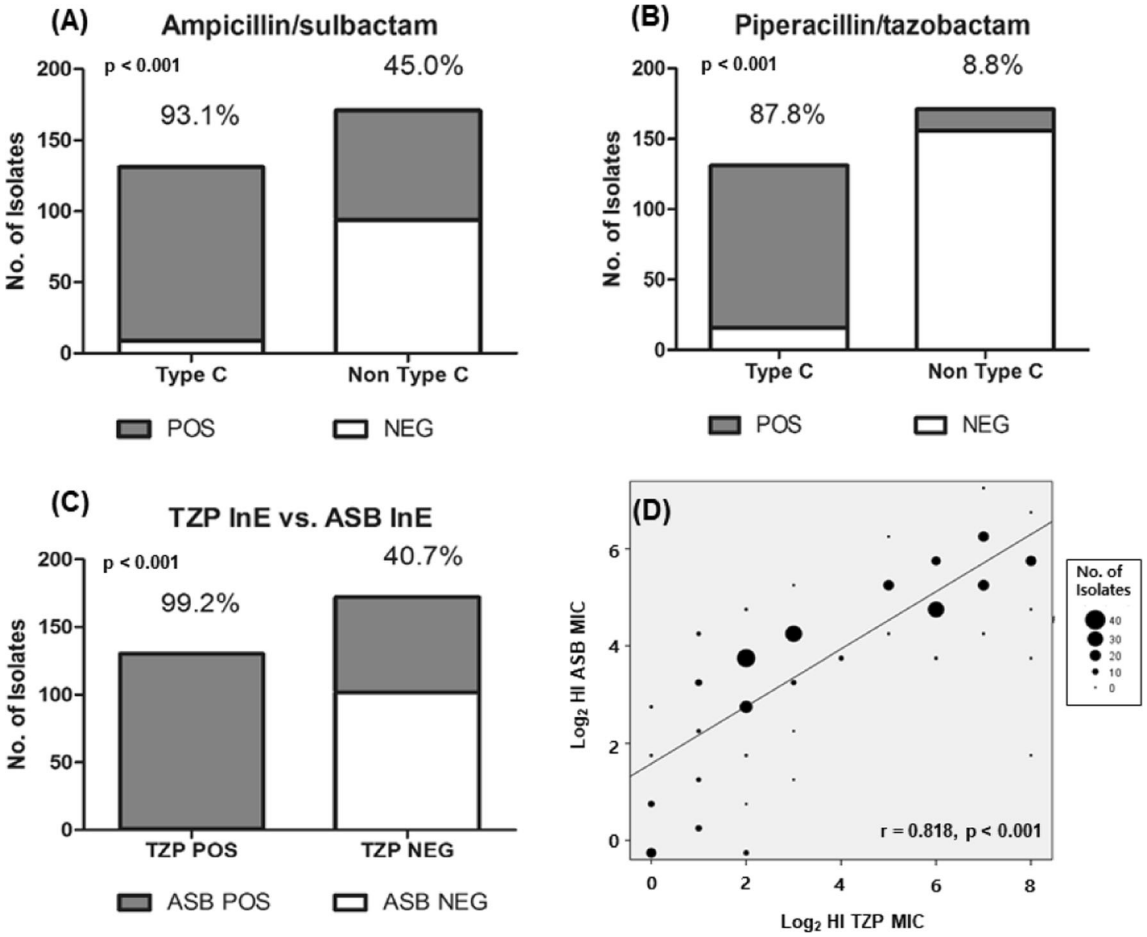

Fig. 4 Comparison of inoculum effect (InE) positive rates of ampicillin/sulbactam (A) and piperacillin/tazobactam (B) between 131 type C *blaZ*-positive MSSA and 171 non-type C MSSA. The correlation of ampicillin/sulbactam (ASB) InE versus piperacillin/tazobactam (TZP) InE was plotted (C), (D). HI: high inoculum, MIC: minimum inhibitory concentration

Table 1 Outcomes of MSSA bacteraemia treated by β-lactam/β-lactamase inhibitors (empirical and definitive use) compared between ampicillin/sulbactam inoculum effect (ASB InE)-positive and ASB InE-negative groups

Outcomes	ASB InE Positive (%)*	ASB InE Negative (%)*	Odds ratio (95%CI)	*P* value
	Empirical use (Positive, $n = 39$ vs. Negative, $n = 25$)			
7-day mortality	6 (15.4)	1 (4.0)	4.36 (0.49–38.65)	0.23
1-month mortality†	12 (32.4)	1 (5.6)	8.16 (0.97–68.74)	0.04
3-month mortality†	14 (50.0%)	1 (7.7)	12.00 (1.37–105.139)	0.01
	Definitive use (Positive, $n = 28$ vs. Negative, $n = 14$)			
7-day mortality	2 (7.1)	1 (7.1)	1.00 (0.08–12.07)	> 0.99
1-month mortality‡	6 (26.1)	1 (8.3)	3.88 (0.41–36.79)	0.38
3-month mortality‡	6 (31.6)	1 (8.3)	5.08 (0.53–48.86)	0.20

*Percentage based on patients with available data (excludes those with missing data)

†In analysis of empirical use, outcome data of 9 patients at 1 month and 33 patients at 3 months were not available

‡In analysis of definitive use, outcome data of 7 patients at 1 month and 11 patients at 3 months were not available

[9–11, 23–28]. Therefore, InE against β-lactam/β-lactamase inhibitors might have an influence on treatment outcomes and this could be extended in countries with a higher proportion of type C *blaZ*-expressing MSSA such as Korea [9, 12, 23, 24].

Our study also showed that the InE against ampicillin/sulbactam has a close association with the InE against piperacillin/tazobactam. These findings suggest that the InE of type C *blaZ*-expressing MSSA against β-lactam/β-lactamase inhibitors might be caused by a decreased inhibition of β-lactamase inhibitors rather than an increased hydrolytic effect of the type C variant of β-lactamase against its β-lactam partner. Therefore, if an organism exhibits InE against one β-lactam/β-lactamase inhibitor, it is likely to exhibit InE against other β-lactam/β-lactamase inhibitors. In addition, recently developed β-lactamase inhibitors also might be affected by the InE of MSSA because these new β-lactamase inhibitors have structural similarity to sulbactam or tazobactam [22].

This study can provide some clinically useful information. Our findings suggest that the utility of β-lactam/β-lactamase inhibitors for MSSA infection combined with suspicious polymicrobial infection can be affected by the InE rather than using other broad-spectrum β-lactam antibiotics, especially when the causative organism possesses the type C *blaZ* gene. First-line antibiotics for MSSA infections include nafcillin or oxacillin, and an alternative is cefazolin [1]. However, physicians use broad-spectrum β-lactam antibiotics against MSSA infections before the culture and antimicrobial susceptibility data are reported. Using broad-spectrum β-lactam antibiotics for MSSA infection is not uncommon even after culture results are reported if the patients are likely to have polymicrobial infections. Nannini et al. warned that the cefazolin inoculum effect could be associated with cefazolin treatment failure [10]. Our group previously evaluated the association between the InE against cefazolin and clinical outcomes of MSSA infections and demonstrated that the InE against cefazolin was associated with treatment failure of MSSA bacteraemia when the patients were treated with cefazolin [9, 11, 12]. However, the association between the InE of β-lactam antibiotics other than cefazolin and clinical outcomes of MSSA bacteraemia have never been studied, and it is also unclear whether pronounced InE against β-lactam/β-lactamase inhibitors has significant clinical implications.

Therefore, we attempted to provide useful information concerning the association between the InE of β-lactam/β-lactamase inhibitors and clinical outcomes and evaluated the association between the InE of β-lactam/β-lactamase inhibitors and clinical outcomes using clinical and demographic information. In our study, outcomes of the ampicillin/sulbactam InE-positive group exhibited worse outcomes than the InE-negative group if β-lactam/β-lactamase inhibitors were used empirically. Outcomes of the ampicillin/sulbactam InE-positive group had a tendency to be worse than the InE-negative group if β-lactam/β-lactamase inhibitors were used definitively, although this was not statistically significant. Our findings imply that using β-lactam/β-lactamase inhibitors for serious MSSA infections for a long duration perhaps is affected by the InE and cause treatment failure, especially when the causative organism possesses the type C *blaZ* gene. Further study is warranted to identify the association between the InE and clinical outcomes.

In summary, MSSA isolates exhibited increasing MICs at HI against most broad-spectrum β-lactam antibiotics. A large proportion of MSSA clinical isolates showed marked InE against β-lactam/β-lactamase inhibitors such as piperacillin/tazobactam and ampicillin/sulbactam among the broad-spectrum β-lactam antibiotics. Type C *blaZ* presence was associated with piperacillin/tazobactam and ampicillin/sulbactam InE. The inoculum effect of MSSAs against β-lactam/β-lactamase inhibitors may be associated with poor outcomes.

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Compliance with ethical standards

Ethical approval The study entitled "Establishment of Network for Clinical Research of *Staphylococcus aureus* Infection" had been approved by the IRB at each participating hospital, and the protocol used in this study using previously collected clinical information and corresponding isolates was approved by the IRB at Pusan National University Hospital (IRB No. E-2016032).

Transparency declarations Nothing to declare.

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