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Original Article

The Cefazolin Inoculum Effect and the Presence of type A *blaZ* Gene according to *agr* Genotype in Methicillin-Susceptible *Staphylococcus aureus* Bacteremia

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ABSTRACT

Background: Recent data suggests the inoculum effect of methicillin-susceptible *Staphylococcus aureus* (MSSA) against beta-lactam antibiotics and their association with functionality or genotypic variation of *agr* locus.
Methods: MSSA blood isolates were collected at a tertiary care hospital in Korea from June 2014 to December 2017. The functionality of the *agr* operon was measured by δ-hemolysin assays. Multiplex PCR was performed to determine the *agr* genotype. The cefazolin minimum inhibitory concentrations (MICs) at a high inoculum concentration (~5×10^7 CFU/ml) were compared to the MICs at a standard inoculum concentration (~5×10^5 CFU/ml) to identify strains with the cefazolin inoculum effect (CIE). The DNA sequencing of *blaZ* gene was performed to classify the *blaZ* genotype.
Results: Among the 195 MSSA blood isolates, *agr* genotype I was most common (68.2%), followed by type III (16.4%), type IV (9.2%), and type II (6.2%). Sixty-seven (34.3%) MSSA isolates had dysfunctional *agr*, but neither CIE nor *blaZ* genotype was associated with dysfunctional *agr*. The MSSA with *agr* type III genotype exhibited significantly higher CIE positivity (*agr* III 43.8% *vs.* non-*agr* III 5.5%, P <0.01) and erythromycin/clindamycin resistance. In the subgroup analysis of type A *blaZ* possessing MSSA, almost all of the *agr* III MSSA isolates exhibited CIE, while only 20% of non-*agr* III isolates had CIE (P <0.01).
Conclusion: In MSSA blood isolates, CIE might be associated with *agr* genotype rather than with dysfunctional *agr*.

Keywords: *Staphylococcus aureus*; Quorum sensing; Cefazolin; Inoculum effect

INTRODUCTION

Some strains of methicillin-susceptible *Staphylococcus aureus* (MSSA) exhibit the inoculum effect against cefazolin, an antibiotic widely used to treat bacterial infections [1, 2]. Cefazolin inoculum effect (CIE) is closely related to type A or C *blaZ* MSSA isolates [3, 4]. Hence, the use of cefazolin for treating severe infections or large burden infections caused by CIE positive

Conflict of Interest
No conflicts of interest.

Author Contributions
Conceptualization: SL, SOL. Data curation: SL, SOL. Formal analysis: SL, SOL. Funding acquisition: SL. Investigation: SL, SOL, SP. Methodology: SP. Supervision: JEL, SHL. Validation: JEL, SHL. Writing - original draft: SL, SOL. Writing - review & editing: SL, SOL.

MSSA can lead to treatment failure [1, 3-6]. Cefazolin is frequently used as the antibiotic of choice for severe MSSA infections such as osteomyelitis and septic arthritis, due to its high tolerability and favorable dosing schedule [7, 8].

The accessory gene regulator (*agr*) locus is a quorum-sensing (QS) gene cluster and virulence regulator of *S. aureus* and can play an important role in virulence [9, 10]. Many studies have demonstrated that *agr* genotype can affect the antibiotic response of methicillin-resistant *S. aureus* (MRSA) [11-14]. Several studies have demonstrated that infection caused by MSSA with reduced vancomycin susceptibility was associated with poor clinical outcomes [15-18]. Further, Kok EY et al. reported that the reduced vancomycin susceptibility was associated with dysfunctional *agr* in MSSA bacteremia [19]. Although a previous study suggested that CIE could be associated with dysfunctional *agr* locus in MSSA bacteremia [20], there is a dearth of literature regarding *agr* genotype and its functional effects in MSSA. This study was aimed to demonstrate the association between the functionality or genotypic variation of *agr* locus in MSSA and the inoculum effect of beta-lactam antibiotics and its clinical significance.

MATERIALS AND METHODS

1. Bacterial isolates and clinical information

The MSSA blood isolates were collected at a 2000-bedded tertiary care hospital in Korea during June 2014 to December 2017. Only the first bloodstream isolate from each patient was included in the study. Isolation of *S. aureus* and antimicrobial susceptibility tests were performed at the clinical microbiology laboratory of our institute using an automated system. Demographic data and clinical information about the study participants were retrospectively collected by the review of medical records. The study protocol (IRB No. H1910-030-084) was approved by the IRB at Pusan National University Hospital. and informed consents were waived.

2. Cefazolin Susceptibility tests and inoculum effects

The cefazolin minimal inhibitory concentrations (MICs) were determined by a broth microdilution method using cation-adjusted Mueller-Hinton II broth (Becton, Dickinson and Company, Sparks, MD), according to Clinical and Laboratory Standards Institute (CLSI) guidelines, except for the inoculum size of the strains [21]. MICs of high inoculum (HI, ~5 x 10^7 CFU/ml) were compared to the standard inoculum (SI, ~5 × 10^5 CFU/ml) to identify the stains with the CIE. The MIC value of each isolate was measured by two independent researchers. *S. aureus* strain TX 0117 (a high-level producer of type A β-lactamase), *S. aureus* ATCC 29213 (known to produce small amounts of type A β-lactamase), and *S. aureus* ATCC 25923 (a β-lactamase negative strain) were used as controls [1, 22]. The CIE was defined as an increase in MICs to ≥16 μg/ml at high inoculums from the susceptible range of MIC at standard inoculum [23]. The MICs of vancomycin and linezolid were measured by the broth microdilution method and E-test (bioMérieux, Marcy-l'Étoile, France). The E-test was performed according to the manufacturer's protocol. The data regarding susceptibility to agents other than cefazolin, vancomycin, and linezolid were collected through a review of medical record of microbiological data.

3. *blaZ* gene typing

Polymerase chain reactions (PCRs) were performed to amplify a 355-bp region within the *blaZ* gene by using the following primers: 5′-CAAAGATGATATAG TTGCTTATTC-3′ and

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5′-CATATGTTATTGCTTGCACCAC-3′ [3]. PCR products were analyzed by DNA sequencing, and results were analyzed using the NCBI BLAST web interface (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The β-lactamase type of each strain was classified based on the amino acid residues at positions 128 and 216 encoded by the *blaZ* gene [24].

4. *agr* functionality test and *agr/blaZ* genotyping

The functionality of the *agr* locus was measured by δ-hemolysin expression assays using *S. aureus* strain RN4220 as an indicator, and the absence of, or barely detectable, synergistic hemolysis was defined as *agr* dysfunction [25]. The genomic DNA of the isolates was extracted by the spin-column-based extraction method using a commercially available kit (Qiagen, Hilden, Germany). To determine *agr* group genotype, *agr* group specific multiplex PCR was performed using the primers that were previously described [26]. To determine *blaZ* gene genotype, PCR was performed using previously described primers and PCR products were analyzed by DNA sequencing [3].

5. Statistical analysis

R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses. Categorical variables were compared using Pearson's chi-square test or Fisher's exact test, and non-categorical variables were tested using the Mann-Whitney *U*-test or Kruskal Wallis test. All tests of significance were 2-tailed, and the results with P <0.05 were considered statistically significant.

RESULTS

A total of 197 MSSA blood isolates were collected during the study period. The mean age of patients with MSSA bacteremia was 62.3 ± 20.3 years and 67% of these patients were males. Among the MSSA bacteremia patients, 54.3% had more than one comorbidity (mean Charlson comorbidity index score: 3.4 ± 2.5). The prevalence of the community-onset bacteremia was 57.4% and bones and joints were the most common site of infection (18.3%) followed by skin and soft tissues (15.2%).

From the 197 MSSA blood isolates, two samples were excluded from the analysis due to unclear *agr* genotype. In the remaining 195 isolates, genotype I was the most common (67.5%), followed by type III (16.2%), type IV (9.1%), and type II (6.1%). Sixty-eight MSSA isolates (34.5%) showed dysfunctional *agr* gene. We did not observe significant differences between the demographic and clinical characteristics between the 4 *agr* genotypes (**Table 1**). Although we observed a trend of association between *agr* type III positive MSSA bacteremia and skin and soft tissue infection, it was not statistically significant (**Table 1**).

The proportion of dysfunctional *agr* was significantly higher in *agr* type III than other genotypes. Further, the proportion of *blaZ* genotype varied significantly according to the *agr* type; type B *blaZ* was most common in *agr* type I, whereas type A *blaZ* was most common in *agr* type III. Furthermore, more than 40% of *agr* type III MSSA exhibited CIE, whereas less than 10% of other *agr* genotype MSSA exhibited CIE (P <0.001). Significantly, resistance to erythromycin and clindamycin was most prominent in *agr* type III MSSA isolates (**Table 1**). The genotypes of *blaZ* and *agr* were significantly different between functional and dysfunctional *agr* positive MSSA (**Table 2**) isolates. However, we did not observe a statistically significant difference in the CIE positivity in these two groups (**Table 2**). The proportion of MSSA

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Table 1. Comparison of demographic, clinical, and microbiological characteristics according to the *agr* genotype of methicillin-susceptible *Staphylococcus aureus* bacteremia isolates

	agr I (n = 133)	*agr* II (n = 12)	*agr* III (n = 32)	*agr* IV (n = 18)	*P* value
Male	90 (67.7%)	10 (83.3%)	23 (71.9%)	9 (50.0%)	0.243
Old age (Age≥65)	76 (57.1%)	3 (25.0%)	16 (50%)	8 (44.4%)	0.149
Community onset	79 (59.4%)	5 (41.7%)	19 (59.4%)	9 (50.0%)	0.596
Comorbidity					
Malignancy	17 (12.8%)	1 (8.3%)	3 (9.4%)	5 (27.8%)	0.264
Diabetes Mellitus	32 (24.1%)	2 (16.7%)	5 (15.6%)	3 (16.7%)	0.663
Chronic Kidney Disease	10 (7.5%)	0 (0.0%)	3 (9.4%)	1 (5.6%)	0.740
Chronic Liver Disease	7 (5.3%)	2 (16.7%)	1 (3.1%)	2 (11.1%)	0.289
High (≥3) Charlson's Index	87 (65.4%)	5 (41.7%)	17 (53.1%)	8 (44.4%)	0.124
High (≥2) SOFA score	76 (57.1%)	6 (50%)	19 (59.4%)	8 (44.4%)	0.713
High (≥2) Pitt score	22 (16.1%)	1 (8.3%)	5 (15.6%)	2 (11.1%)	0.838
Site of Infection					
Skin, Soft tissue	18 (13.5%)	2 (16.7%)	8 (25.0%)	2 (11.1%)	0.409
Abscess, deep seated	22 (16.5%)	0 (0.0%)	7 (21.9%)	1 (5.6%)	0.196
Bone & Joint	27 (20.3%)	0 (0.0%)	8 (25%)	1 (5.6%)	0.116
Lung	16 (12%)	3 (25%)	3 (9.4%)	4 (22.2%)	0.351
Infective endocarditis	5 (3.8%)	1 (8.3%)	1 (3.1%)	0 (0.0%)	0.687
Primary bacteremia	37 (27.8%)	6 (50%)	6 (18.8%)	7 (38.9%)	0.160
agr dysfunction	45 (33.8%)	2 (16.7%)	18 (56.2%)	2 (11.1%)	0.005
blaZ type					<0.001
A	8 (6.0%)	1 (8.3%)	13 (40.6%)	5 (27.8%)	
B	53 (39.8%)	2 (16.7%)	1 (3.1%)	1 (5.6%)	
C	43 (32.3%)	4 (33.3%)	12 (37.5%)	8 (44.4%)	
D	5 (3.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
blaZ negative	24 (18.0%)	5 (41.7%)	6 (18.8%)	4 (22.2%)	
Cefazolin InE	7 (5.3%)	1 (8.3%)	14 (43.8%)	1 (5.6%)	<0.001
Ampicillin/sulbactam InE	90 (67.7%)	6 (50.0%)	24 (75.0%)	10 (55.6%)	0.313
Piperacillin/tazobactam InE	89 (66.9%)	5 (41.7%)	23 (71.9%)	7 (38.9%)	0.032
Erythromycin Resistance	4 (3.0%)	0 (0.0%)	18 (56.2%)	2 (11.1%)	<0.001
Clindamycin Resistance	3 (2.3%)	0 (0.0%)	14 (43.8%)	1 (5.6%)	<0.001
Ciprofloxacin Resistance	6 (4.5%)	0 (0.0%)	0 (0.0%)	1 (5.6%)	0.539
High dose Gentamicin Resistance	20 (15.0%)	0 (0.0%)	10 (31.2%)	3 (16.7%)	0.059
High (≥2) Vancomycin MIC (BMD)	4 (3.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.593
High (≥2) Vancomycin MIC (E-test)	5 (3.8%)	0 (0.0%)	2 (6.2%)	0 (0.0%)	0.619
High (≥4) Linezolid MIC (BMD)	4 (3.0%)	0 (0.0%)	2 (6.2%)	1 (5.6%)	0.698
High (≥3) Linezolid MIC (E-test)	2 (1.5%)	1 (8.3%)	0 (0.0%)	1 (5.6%)	0.228

SOFA, sequential organ failure assessment; InE, inoculum effect; MIC, minimum inhibitory concentration; BMD, broth microdilution.

isolates with reduced vancomycin MIC was similar in four *agr* types (**Table 1**). Similarly, the proportion of MSSA with reduced vancomycin MIC was not significantly different between the dysfunctional and functional *agr* group (**Table 2**).

Our results showed that among the four *agr* types, MSSA with the *agr* type III was associated with CIE. Isolates from *agr* III group exhibited a significantly higher CIE (40%) than the non-*agr* III group where only 5.5% isolates exhibited CIE (**Fig. 1A**). The *agr* III group also showed positive association with type A *blaZ* gene (**Fig. 1B**), and resistance to other antibiotics; erythromycin (**Fig. 1C**, 56.2% *vs.* 3.7%, P <0.001), clindamycin (43.8% *vs.* 2.5%, P <0.001), and high dose gentamicin (31.2% *vs.* 14.1%, P = 0.035).

Interestingly, all of the CIE-positive isolates had type A or type C *blaZ* genotype. Therefore, we performed a subgroup analysis of MSSA with type A and C *blaZ* genotype. In subgroup analysis of type A *blaZ* positive MSSA, our results showed that while more than 90% of *agr* III

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isolates exhibited CIE, a very low proportion of non-*agr* III isolates (10%) had CIE (**Fig. 2A**). Furthermore, CIE was also associated with erythromycin resistance (**Fig. 2B**). However, there was no significant association between the CIE and *agr* functionality (**Fig. 2C**), and the functionality of *agr* genotype was not associated with CIE in the subgroup analysis of type C *blaZ* positive MSSA as well.

Table 2. Comparison of demographic, clinical and microbiological characteristics according to the functionality of *agr* locus of methicillin-susceptible *Staphylococcus aureus* bacteremia

	Functional *agr* (n = 128)	Dysfunctional *agr* (n = 67)	*P* value
agr genotype			0.005
Type I	88 (68.8%)	45 (67.2%)	
Type II	10 (7.8%)	2 (3.0%)	
Type III	14 (10.9%)	18 (26.9%)	
Type IV	16 (12.5%)	2 (3.0%)	
blaZ type			0.034
A	14 (10.9%)	13 (19.4%)	
B	35 (27.3%)	22 (32.8%)	
C	50 (39.1%)	17 (25.4%)	
D	1 (0.8%)	4 (6.0%)	
blaZ negative	28 (21.9%)	11 (16.4%)	
Cefazolin InE	13 (10.2%)	10 (14.9%)	0.455
Ampicillin/sulbactam InE	82 (64.1%)	48 (71.6%)	0.365
Piperacillin/tazobactam InE	78 (60.9%)	46 (68.7%)	0.364
Erythromycin Resistance	13 (10.2%)	11 (16.4%)	0.301
Clindamycin Resistance	9 (7.0%)	9 (13.4%)	0.228
Ciprofloxacin Resistance	3 (2.3%)	4 (6.0%)	0.375
High dose Gentamicin Resistance	20 (15.6%)	13 (19.4%)	0.640
High (≥2) Vancomycin MIC	3 (2.3%)	1 (1.5%)	>0.999
High (≥2) Vancomycin MIC (E-test)	5 (3.9%)	2 (3.0%)	>0.999
High (≥4) Linezolid MIC	4 (3.1%)	3 (4.5%)	0.939
High (≥3) Linezolid MIC (E-test)	3 (2.3%)	1 (1.5%)	>0.999

SOFA, sequential organ failure assessment; InE, inoculum effect; MIC, minimum inhibitory concentration.

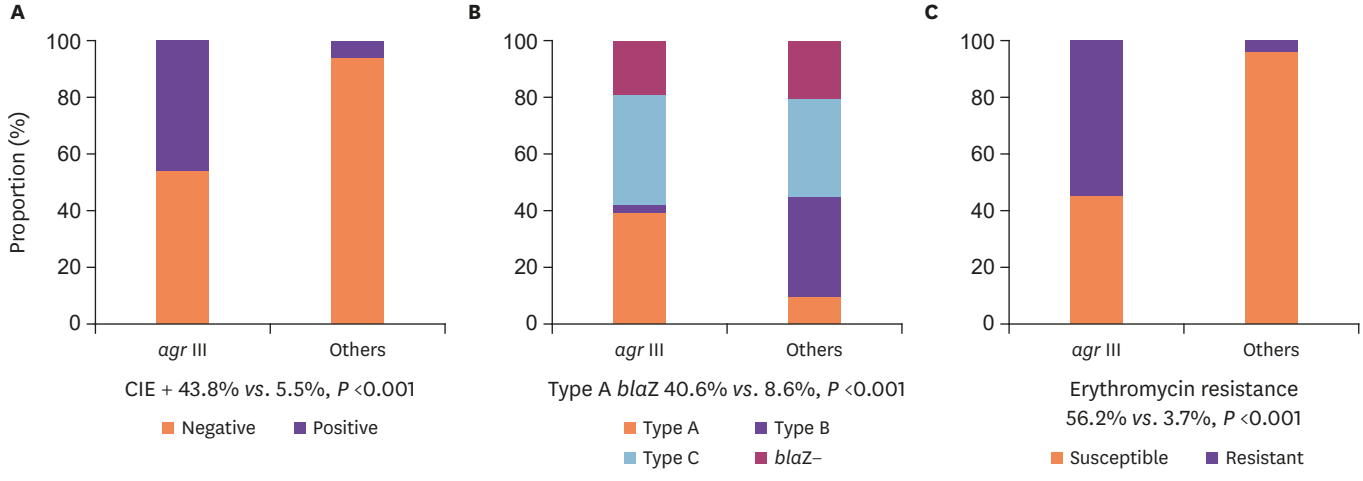

Figure 1. (A) Proportion of cefazolin inoculum effect (CIE) positivity, (B) *blaZ* genotype, (C) erythromycin resistance between *agr* type III and non *agr* type III methicillin susceptible *Staphylococcus aureus*.

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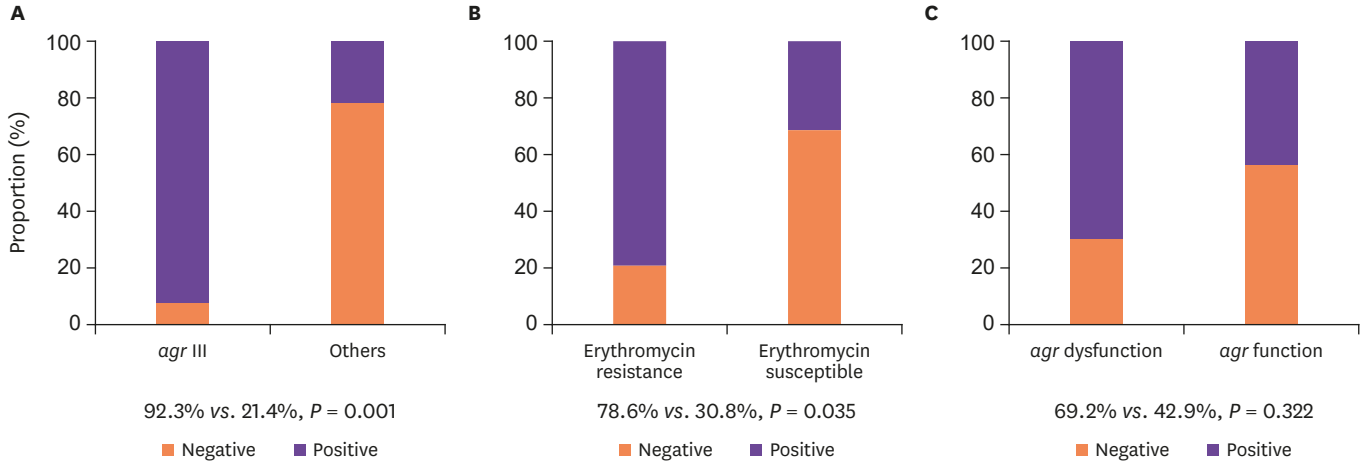

Figure 2. (A) Proportion of cefazolin inoculum effect positivity according to the *agr* genotype, (B) erythromycin resistance, (C) *agr* functionality in subgroup analysis among Methicillin susceptible *Staphylococcus aureus* with type A *blaZ* gene.

DISCUSSION

Our study demonstrated that the inoculum effect of MSSA against β-lactam antibiotics, such as cefazolin, ampicillin/sulbactam, and piperacillin/tazobactam was associated with the *agr* type III, which was also the second most prevalent *agr* genotype observed in our study. These findings are consistent with a previous study [20]. However, unlike an earlier study, which concluded that *agr* dysfunction of MSSA was independently associated with inoculum effect against cefazolin [20], our results showed that there was no association between *agr* functionality and inoculum effect against β-lactam antibiotics. Instead, we found that *agr* group III was significantly associated with CIE. Moreover, the association between the *agr* type and CIE was more apparent in the type A *blaZ* gene-positive MSSA isolates. Although type A *blaZ* genotype has been positively associated with CIE, it is not uncommon to find MSSA isolates with the type A *blaZ* gene that do not show CIE [3, 4, 27, 28]. In our study, almost all of the MSSA with *agr* type III and type A *blaZ* genotype exhibited CIE. Therefore, there is a possibility that *agr* type III can be a useful indicator to discriminate CIE positivity among type A *blaZ* positive MSSA isolates.

Our study further demonstrated that a higher proportion of *agr* type III MSSA showed clindamycin and macrolide resistance, and a majority of such isolates had type A *blaZ* genotype. Clindamycin and macrolide resistance of MSSA is associated with CIE, and the association between CIE and clindamycin and macrolide resistance was significant among MSSA with type A *blaZ* genotype [29]. Our results suggest that *agr* type III is closely related to CIE, and clindamycin and erythromycin resistance. Collectively, these results indicate that cefazolin should be used with caution in treating high-inoculum MSSA infection if the isolates exhibited resistance to clindamycin or erythromycin [23].

Earlier studies have demonstrated an association between dysfunctional *agr* locus and reduced susceptibility to vancomycin and an enhanced capacity to produce biofilms in MSSA [18, 30]. Further, many studies reported that irrespective of vancomycin use as a therapeutic agent, reduced susceptibility to vancomycin in MSSA affected treatment outcomes [17, 29, 31, 32]. Holmes NE et al. suggested that dysfunctional *agr* could be a predictor of high

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vancomycin MIC in MSSA infections [33]. However, in our study, neither dysfunctional *agr* nor *agr* genotype variation was associated with reduced vancomycin susceptibility.

The reason for the close association between the *agr* genotype with *blaZ* genotype, the inoculum effect of β-lactam antibiotics, and resistance of non- β-lactam antibiotics is not known. However, some data regarding the association between certain toxin genes and certain *agr* types is available. For instance, *agr* type III is associated with menstrual toxic shock syndrome toxins, while *agr* type IV genotype is associated with exfoliatins [34-36]. Jarraud S et al. suggested that the bacterial pathogenicity is a cumulative result of specific combinations of virulence and regulatory genes in the appropriate genetic background [35]. In light of the available scientific literature and our results, we propose that certain *agr* alleles might associate with certain antibiotic resistance genes in a particular genetic background.

Although our study has some interesting and thought-provoking outcomes, it has several limitations. Firstly, our study was conducted at a single center in the southeastern region of Korea. Earlier studies have shown a varying prevalence of CIE positive MSSA or distribution of *agr* type among clinical isolates [3, 20, 27, 37]. The inconsistent prevalence of CIE positive MSSA or distribution of *agr* types suggested that CIE positivity and its association with *agr* type could vary according to the geographic regions. Therefore, although our results form a credible reference for CIE positive MSSA and *agr* types, they lack universal relevance, and therefore, should be applied cautiously to other geographical regions. Second, we used the δ-hemolysin assay to detect MSSA isolates with *agr* dysfunction due to the convenience and lower cost than Northern blotting [38]. However, the δ-hemolysin assay may not be a sensitive marker for *agr* dysfunction and has shown an ambiguous result in an earlier report [39]. However, to minimize the shortcomings of the assay, two independent researchers analyzed the assay results. Moreover, the assays were repeated in events where discrepancies were observed in the results.

In summary, our results showed that the positivity of CIE and resistance of clindamycin could be associated with *agr* type III rather than *agr* dysfunction in MSSA bacteremia isolates. These findings were more prominent among MSSA with type A *blaZ*. Hence, we propose that Type A *blaZ* genotype with *agr* type III could be a useful indicator to genetically differentiate CIE positive MSSA isolates.

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