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The cefazolin inoculum effect in methicillin-susceptible *Staphylococcus aureus* blood isolates: their association with dysfunctional accessory gene regulator (*agr*)

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ABSTRACT

We evaluated the clinical significance of the cefazolin inoculum effect (CIE) in methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates. In total, 146 isolates were recovered from patients with MSSA bacteremia at 9 hospitals in Korea. The CIE was observed in 16 MSSA isolates, and while type A was the only detected β-lactamase in MSSA isolates exhibiting the CIE, no strains expressing type B, C, or D β-lactamases exhibited this effect. The CIE was only observed in *agr* group III and I isolates and was significantly more common in isolates with *agr* dysfunction than in those with functional *agr* ($P < 0.001$). Even among isolates producing type A β-lactamase, the CIE was also prevalent in isolates with dysfunctional *agr* than in isolates with functional *agr* ($P = 0.025$). This study demonstrates an association between the CIE of MSSA isolates and *agr* dysfunction, in addition to those between the CIE and type A β-lactamase.

1. Introduction

Cefazolin is widely used for treating methicillin-susceptible *Staphylococcus aureus* (MSSA) infections and, due to its convenient dosing and tolerability, is recommended as an alternative agent to antistaphylococcal penicillins such as oxacillin, nafcillin, and flucloxacillin for treatment of MSSA endocarditis (Baddour et al., 2005). However, some MSSA isolates exhibit significant increases in the cefazolin MIC at high bacterial inoculum levels, which is referred to as the inoculum effect (Livorsi et al., 2012; Nannini et al., 2009; Rincón et al., 2013). The reduced efficacy of cefazolin treatment in the presence of high bacterial burdens may be due to the increased production of a certain type of staphylococcal β-lactamase (Livorsi et al., 2012; Nannini et al., 2009; Rincón et al., 2013). Four types of β-lactamases, which are encoded by the *blaZ* gene, have been identified: while the genes encoding types A, C, and D are plasmid mediated, the gene encoding type B is typically located in the chromosome (Zygmunt et al., 1992). A previous study reported cefazolin clinical failure in patients with serious MSSA infections due to high production of the type A β-lactamase (Nannini et al., 2003), and cefazolin was shown to be efficiently hydrolyzed by this enzyme (Zygmunt et al., 1992). However, not all isolates producing type A β-lactamase exhibit a significant cefazolin inoculum effect (Chapman and Steigbigel, 1983; Chong et al., 2013; Livorsi et al., 2012; Nannini et al., 2009; Rincón et al., 2013), and the precise mechanism by which some type A β-lactamase–producing MSSA strains mediate higher rates of cefazolin hydrolysis is unknown.

Density-dependent regulation of most virulence determinants in *S. aureus* is controlled by a product of the accessory gene regulator (*agr*) locus (Dunman et al., 2001). The *agr* global regulator positively regulates the expression of many murein hydrolases that are involved in autolysis (Fujimoto and Bayles, 1998). Therefore, defects in this operon might be involved in the development of an autolysis-deficient strain at high bacterial populations. Furthermore, *S. aureus* with dysfunctional

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agr exhibit enhanced adherence to polystyrene, a frequently utilized marker of biofilm production (Vuong et al., 2000). Recent data also suggest that *S. aureus* strains with dysfunctional *agr* are associated with attenuation of vancomycin bactericidal activity, as well as the vancomycin intermediate-resistant *S. aureus* phenotypes (Fowler et al., 2004; Sakoulas et al., 2003). In addition, a previous study detected a loss of vancomycin bactericidal activity against *agr* dysfunctional *S. aureus* strains at high bacterial densities (Tsuji et al., 2009). Therefore, *agr* dysfunction is known to be associated with increased mortality in patients receiving vancomycin therapy (Schweizer et al., 2011). Despite the importance of the *agr* locus for a density-dependent regulation in *S. aureus*, the relationship between *agr* dysfunction and the cefazolin inoculum effect (CIE) (significant increases in the cefazolin MIC at high bacterial inoculum levels) in MSSA isolates is yet to be investigated. Therefore, the purpose of this study was to evaluate the clinical significance of CIE in currently circulating MSSA isolates, with an emphasis on elucidating the relationship between CIE and *agr* dysfunction.

2. Methods

2.1. Patients

Patients with positive blood cultures for *S. aureus* were enrolled at 9 secondary- or tertiary-care hospitals through a prospective bacteremia surveillance study during 2 periods. The first period was between June and September 2011, and the second period spanned the entirety of 2012. Only the first episode of MSSA bacteremia for each patient was included in the analysis, and patients with polymicrobial bacteremia were excluded. In total, 151 patients were diagnosed with culture-confirmed MSSA bacteremia based on their epidemiological, antibiotic treatment, and outcome data. Among them, 5 patients were excluded because it was not possible to recover *S. aureus* on blood agar. A standardized case report form was used to collect the relevant information for each patient. Echocardiographs were performed on all patients with persistent *S. aureus* bacteremia lasting longer than 7 days. This study was approved by the Institutional Review Board of Samsung Medical Center and by each of the local review boards.

2.2. Clinical definitions

Clinically significant bacteremia was defined as at least 1 positive blood culture combined with clinical features consistent with systemic inflammatory response syndrome. Catheter-related bloodstream infection was defined as either the growth of >15 CFUs from the catheter tip in a semiquantitative culture or the growth of MSSA from a blood sample drawn from a catheter hub at least 2 h before MSSA was obtained from a peripheral vein blood sample. Infective endocarditis was defined according to the modified Duke criteria (Li et al., 2000). Neutropenia was defined as an absolute neutrophil count less than 500/mm^3. Isolates defined as "community-onset" were cultured in the outpatient setting and within 48 h of hospital admission; those classified as "hospital-onset" were obtained after 48 h of hospitalization. Community-onset infections were further categorized as either community-onset community-associated or community-onset healthcare-associated infections. Community-onset healthcare-associated infections were defined as previously described (Melzer and Welch, 2013). Mortality was defined as death from any cause within 30 days.

2.3. Species identification and antimicrobial susceptibility testing

Identification of *S. aureus* and the initial antibiotic sensitivity testing were performed at each participating center by using the Vitek2 automated system (bioMérieux, Craponne, France), and all blood culture isolates were subsequently stored at −70 °C. After recovery on blood agar, the identity of each isolate was validated with a Staphaurex Plus Kit (Murex Diagnostics Ltd., Dartford, UK). In vitro susceptibility testing was performed on all clinical MSSA isolates by using the broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). The MICs of cefazolin, oxacillin, and vancomycin were determined using a standard inoculum (SI; ~5×10^5 CFU/mL) and a high inoculum (HI; ~5×10^7 CFU/mL). Culture densities were determined at 24 h to assess whether an inoculum effect was exerted against the antibiotics. In 2 prior studies, distinct parameters were utilized to define the CIE: Nannini et al. (2009) defined the CIE as an MIC of cefazolin ≥16 μg/mL for the HI, while Livorsi et al. (2012) defined the CIE as a ≥4-fold increase in the MIC for the HI compared to that for the SI. We chose to use Nannini's definition. The *S. aureus* strain TX 0117 (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 strain (β-lactamase–negative strain) were used as controls.

2.4. β-Lactamase typing

The β-lactamase gene (*blaZ*) of all isolates was amplified by PCR, following the methodology previously described (Lee et al., 2014). Sequencing of a 355-bp fragment within the *blaZ* gene was then performed to identify putative amino acid differences at residues 128–216. Sequence analyses were performed using the NCBI BLAST network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. agr phenotyping and genotyping

The functionality of the *agr* operon was measured by δ-hemolysin production assays, as previously described (Schweizer et al., 2011). The RN4220 strain, which produces β-hemolysin, was used as an indicator in these experiments. Briefly, the presence of synergistic hemolysis within the β-hemolysin zone indicates the production of δ-hemolysin and, therefore, a functional *agr* locus. In contrast, *agr* dysfunction was defined as the complete absence of δ-hemolysin within the β-hemolysin zone, as evidenced by the lack of synergistic hemolysis. Multiplex PCR was performed, as previously described, to determine the *agr* group genotype, and appropriate control strains for *agr* groups I, II, III, and IV were included (Schweizer et al., 2011). MSSA isolates were also subjected to *spa*-genotyping, as previously described (Shopsin et al., 1999).

2.6. Statistical analyses

Discrete data are presented as frequencies and percentages, and continuous variables are presented as means ± SDs or, if the distributions were skewed, as medians and interquartile ranges. Either the χ^2 test or Fisher's exact test was used to compare categorical variables, and 2-sample *t*-test or the Mann–Whitney test was used to compare continuous variables where appropriate. To identify predictors of mortality in patients with MSSA bacteremia, a multivariate Cox proportional hazards regression model was used to control for the effects of confounding variables. Variables with $P < 0.05$ in the univariate analysis were considered candidates for multivariate analysis. Significance was defined as a $P < 0.05$. All analyses were conducted with PASW for Windows v. 18.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. The β-lactamase types and agr phenotypes and genotypes of MSSA bacteremia isolates exhibiting the CIE

In total, 146 isolates were recovered from patients with MSSA bacteremia in 2 multicenter surveillance studies. All isolates were susceptible to cefazolin at SI (MIC <8 mg/L, with the highest MIC value of 1 mg/L). The breakdown of the β-lactamase types and the *agr* phenotypes and genotypes of the isolates tested is included in Table 1. The CIE was observed in 16 MSSA isolates (11.0%), and 125 isolates