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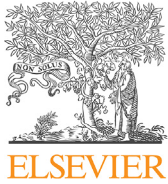

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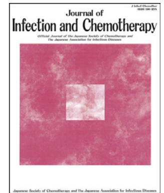

Original Article

Inoculum effect of high concentrations of methicillin-susceptible *Staphylococcus aureus* on the efficacy of cefazolin and other beta-lactams

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ABSTRACT

The existence of a cefazolin inoculum effect (InE) of methicillin-susceptible *Staphylococcus aureus* (MSSA), which is speculated to be a reason for cefazolin treatment failure in MSSA infections, is controversial. In Japan, although cefazolin is one of the therapeutic choices for patients with MSSA infection, there are few reports of this effect. Additionally, the association between InE and *blaZ* type in beta-lactams other than cefazolin has not been well documented. In this study, we confirmed an MSSA InE in several beta-lactams, including cefazolin, and its relationship with *blaZ*, using 52 MSSA isolates from blood cultures. Three isolates (5.8%) that possessed type A *blaZ* showed a pronounced cefazolin InE. Five isolates (9.6%) showed pronounced InE with sulbactam/ampicillin; four isolates had type C *blaZ* and one had type A *blaZ*. However, we confirmed InE in MSSA isolates with *blaZ* not only type A and C but also B and D. For cefotaxime, ceftriaxone, imipenem, and meropenem, regardless of the presence of *blaZ*, we did not observe a significant increase in MICs at a high inoculum of MSSA. Hence, our results suggest that the above four beta-lactams are good alternatives to cefazolin if InE leads to treatment failure in a patient.

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1. Introduction

Inoculum effect (InE) is a well-known phenomenon that affects minimum inhibitory concentrations (MICs) of antimicrobial agents, especially beta-lactams. This effect is most pronounced when a high concentration of bacteria is used for antimicrobial susceptibility testing (AST) [1,2]. Previous reports [3] have demonstrated an in vitro InE on cefazolin (CEZ), one of the major beta-lactams used against methicillin-susceptible *Staphylococcus aureus* (MSSA) infections. Specifically, Nannini et al. showed an MSSA InE on CEZ in an endocarditis patient [4]. The degree of InE differs by MSSA strain, and some studies have demonstrated a pronounced InE when an MIC of CEZ at high inoculum was four-fold high or more compared to that at standard inoculum, changing the interpretation from susceptible to resistant [5–10].

CEZ is the antibiotic of choice against MSSA infections in Japan. However, there have been a few reports of sporadic cases with treatment failure [11]. One possible reason for this failure is the emergence of CEZ InE, namely, the attenuation of antimicrobial activity as a result of a pronounced InE in MSSA infections. The presence of *blaZ*, which encodes a beta-lactamase, and specific types of this gene, have not yet been associated with this phenomenon. However, there have been few reports of clinically confirmed CEZ InE in Japan [12]. Furthermore, an association between MIC increase and the presence of *blaZ* in isolates has not been established. Therefore, we investigated the occurrence of InE and examined its association with the presence and type of *blaZ* in clinical isolates of MSSA.

2. Material and methods

2.1. Isolates and antimicrobial susceptibility testing

Fifty-two MSSA isolates from patients with bacteremia in Sapporo Medical University Hospital from January 2012 to December

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2014 were included in this study. The isolates tested did not include isolates which were repeatedly identified from a single patient. Each isolate was grown on sheep blood agar (Nissui Pharmaceutical, Japan) and incubated at 35 °C for 24 h. The MICs of oxacillin and cefoxitin were determined using a Microscan WalkAway plus System (Beckman Coulter, Japan) and MicroScan Pos Combo 3.1J (Beckman Coulter, Japan). We confirmed MSSA infections according to Clinical and Laboratory Standards Institutes (CLSI) M100-S22 [13].

Nine antimicrobial agents were used in antimicrobial susceptibility testing: CEZ, penicillin (PCG), ampicillin (ABPC), cefotaxime (CTX), ceftriaxone (CTRX), cefepime (CFPM), imipenem (IPM), meropenem (MEPM), and sulbactam/ampicillin (SBT/ABPC). MICs were measured using the broth microdilution method with Eiken dry plates (Eiken Chemical, Japan). For the standard inoculum (SI), 25 μL of a 1.0 McFarland colony suspension in sterilized saline was added to 12 mL of Mueller-Hinton broth (MHB) for a final concentration of 2–8 × 10^5 CFU/mL (Eiken Chemical, Japan). For the high inoculum (HI) [5], 2 mL of a 1.0 McFarland colony suspension was added to 10 mL of MHB for a final concentration of 2–8 × 10^7 CFU/mL without centrifugation. One hundred microliters of each mixture was inoculated into wells of the dry plates. After 18 h of incubation at 35 °C, two clinical laboratory technicians determined the MICs according to CLSI criteria [13]. This research was approved by the Institutional Review Board at Sapporo Medical University Hospital (http://web.sapmed.ac.jp/byoin/chiken/record.html) (number 272-156).

2.2. Definitions

The InE were divided into three categories [5,8]: (i) non InE, (ii) InE, and (iii) pronounced InE. (i) MICs at HI of MSSA were within two-fold of those at SI, limiting quality control [14]. (ii) MICs at HI were four-fold or more than those at SI, but the results at HI were still interpreted as "susceptible" according to CLSI criteria [13]. (iii) MICs at HI were four-fold or more than those at SI, changing the interpretation of MICs from "susceptible" to "intermediate" or "resistant."

To make clear the association between pronounced InE/InE and clinical outcomes in patients with beta-lactams, we reviewed clinical database of all MSSA cases. Among them, we selected bacteremia patients caused by MSSA alone and extracted those who were administered any of nine beta-lactams for more than 75% of the antimicrobial course [11]. Treatment failure was defined as re-detection of MSSA from blood culture within 12 weeks after antimicrobial therapy.

2.3. Typing of blaZ

At first, a 0.5 McFarland bacterial suspension was subjected to a temperature of 95 °C for 5 min to extract DNA. A 421-bp region of the structural gene of *blaZ* including Ambler position 128 and 216 was amplified by PCR as described by Kaase et al. [15] using the following primers: forward, 5′-CAAAGATGATATAGTTGCTTATTCTCC-3′), and reverse, 5′-TGCTTGACCACTTTTATCAGC-3′. *S. aureus* ATCC 25923 and 29213 were used as negative and positive controls, respectively. *blaZ* was typed according to Ambler positions 128 and 216; in type A *blaZ*, threonine is found at position 128 and serine at position 216; in type B, lysine is at position 128 and asparagine at position 216; in type C, threonine is at position 128 and asparagine at position 216; and in type D, alanine is at position 128 and serine at position 216 [16,17]. After direct sequencing of the PCR products, we confirmed the two codons using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Statistical analysis

Comparisons of the degree of MIC increase at HI against that at SI between *blaZ*-negatives and -positive MSSA isolates were performed by non-parametric Mann–Whitney *U* testing. When calculating a significant difference, we regarded "≤, ≥" as "=". SPSS software version 24 (IBM SPSS, Chicago, IL, USA) was used for the analysis. With P values < 0.05, differences were considered significant.

3. Results

First, a confirmation of the presence and genotype of *blaZ* in 52 isolates was displayed in Table 1. Thirty-eight isolates were positive for *blaZ* (73.1%), which were more than negative. The classification of *blaZ* type showed similar frequency in type A, B and C (31.2%, 34.2% and 34.2%). Type D of *blaZ* was detected in only 1 isolate (2.6%). A comparison of the MICs between SI and HI in MSSA isolates with or without *blaZ* is shown in Table 2. For CEZ, difference in the MIC_{50} at HI compared to that at SI was not confirmed in the *blaZ*-negative group, but the MIC_{50} at HI was 4-fold higher than that at SI in the *blaZ*-positive group, and maximum one had 32-fold. For PCG and ABPC, the marked MIC_{90} increase at HI was observed in the *blaZ*-negative group, while many isolates in the *blaZ*-positive group exhibited the MIC of beyond the measurement range. For CFPM, the MIC_{50} at HI compared to that at SI was 2-fold in the *blaZ*-negative group and *blaZ*-positive group, and the significant difference was not detected. For CTX, CTRX, IPM, and MEPM, the MIC_{50} at SI and HI exhibited the same value in both the *blaZ*-positive and -negative groups; that is, neither *blaZ* gene possession nor bacterial count affected antimicrobial activity in vitro. For SBT/ABPC, no isolates showed differences in MICs at SI and HI in the *blaZ*-negative group, whereas we observed a significant increase in MICs associated with the presence of *blaZ*.

Twenty-one (55.3%) of 38 isolates carrying *blaZ* exhibited a significant increase (four-fold or more) in MICs of CEZ at HI compared to that at SI (Table 3). Among them, 18 isolates (47.4%) were categorized as CEZ InE. The most common type of *blaZ* in these isolates was type C (55.6%), followed by type B (22.2%) and then type A (16.7%). Three (7.9%) of 38 isolates with *blaZ* were categorized as exhibiting pronounced CEZ InE; all isolates possessed type A *blaZ*. Of 38 isolates, 1 isolate (2.6%) carrying type C *blaZ* showed pronounced PCG InE. For PCG and ABPC, some isolates without *blaZ* indicated pronounced InE and InE, although there was only one case which fitted the definition of pronounced InE. Five (13.2%) isolates with *blaZ* indicated the MIC increase (four-fold or more) of CFPM, which were interpreted as CFPM InE, but pronounced CFPM InE was not observed (Table 3). Sixteen (42.1%) of 38 isolates expressing *blaZ* showed MICs of SBT/ABPC at HI that were four-fold or higher than that at SI (Table 3). Of the 38 isolates with *blaZ*, 11 (28.9%) demonstrated SBT/ABPC InE; the most prevalent type of *blaZ* in these isolates was type C (45.5%), followed by type A and then type C (both

Table 1
Presence or absence of *blaZ* in methicillin-susceptible *Staphylococcus aureus* isolates.

blaZ gene type	Number of isolates (%)
Negative	14 (26.9)
Positive	38 (73.1)
A	12 (31.6)
B	13 (34.2)
C	12 (31.6)
D	1 (2.6)

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