IN VITRO ACTIVITY OF CEFOTIAM AGAINST OXACILLIN-RESISTANT Staphylococcus epidermidis STRAINS
—REEVALUATION OF BETA-LACTAM ANTIBIOTICS EFFICIENCY ON MRSE—

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We evaluated the efficacy of cefotiam (CTM) against Staphylococcus epidermidis (S. epidermidis) isolated from blood culture and central venous catheters. Of the S. epidermidis strains tested, 82.3% were methicillin (MPIPC)-resistant (MPIPC MIC ≥0.5 μg/ml) and expressed the mecA gene, and 89.2% of these MPIPC-resistant S. epidermidis (MRSE) showed less than 8.0 μg/ml CTM MIC. In vitro killing kinetics of CTM against MRSE demonstrated that strains with high CTM MIC (≥4.0 μg/ml) showed high MPIPC MIC (≥4.0 μg/ml). All strains with low CTM MIC (<2.0 μg/ml) showed MPIPC MIC lower than 2.0 μg/ml. In time-kill studies, CTM had high bactericidal activity against strains with low CTM MIC (<2.0 μg/ml), regardless of whether they were mecA positive. These results demonstrated that MRSE isolates with low CTM MIC (<2.0 μg/ml) are not easily induced CTM resistance by CTM treatment in vitro, and indicated the possibility that beta-lactams such as CTM could be an effective antibiotic agents against beta-lactam-sensitive MRSE infections.

Infections caused by coagulase-negative staphylococci are becoming clinically significant. Strains of Staphylococcus species that demonstrate resistance to multiple antimicrobial agents have limited the choice of agents available to treat both systemic and localized infections. Staphylococcus epidermidis is the most common cause of infections associated with the presence of intravascular catheters, cerebrospinal fluid shunts, prosthetic valves, orthopedic devices, pacemakers, dialysis catheters and vascular grafts). We have reported that serious S. epidermidis infections like sepsis recurred more frequently than the other staphylococcal infections3). More than 50% of S. epidermidis strains are mecA positive, showing complex antimicrobial resistance, and vancomycin (VCM) has become the first line of defense against these infections.
The Clinical and Laboratory Standards Institute (CLSI) recommends that methicillin-resistant *Staphylococcus epidermidis* (MRSE) be considered resistant to all beta-lactam antibiotics, as is methicillin-resistant *Staphylococcus aureus* (MRSA)\(^6\). However, we frequently observed beta-lactam antibiotic cefotiam (CTM)-sensitive MRSE strains with low minimal inhibitory concentrations (MIC). As the first step to investigate the possibility that CTM could be an effective antibiotic agent against MRSE, we performed the *in vitro* killing kinetics of cefotiam against MRSE by evaluating MIC, minimal bactericidal concentration (MBC).

### Materials and Methods

#### Bacterial Strains

One hundred eighty six *S. epidermidis* isolates were tested. The isolates were obtained from blood culture and central venous catheters of patients at Juntendo University Hospital, Tokyo, Japan, from 2003 to 2004. Organisms isolated from blood were cultured in Bactec system Aerobic/F\(^R\) and Anaerobic/F\(^R\) bottles (Becton Dickinson, Franklin Lakes, NJ), and organisms from central venous catheters were incubated in Trypticase soy broth (TSB, Becton Dickinson) for 7 days at 35°C. *S. epidermidis* strains were identified using conventional laboratory tests, including the Gram stain and coagulase testing, and then confirmed by the MicroScan WalkAway system pos combo panel 6.1\(^R\) (Dade Behring, Sacramento, CA).

#### *mecA* Gene Detection

PCR for *mecA* was performed as reported\(^7\). Briefly, DNA was amplified using forward and reverse primers 5’-AGTTGTAGTTGTCGGGTTTG-3’ and 5’-AGTGGAACGAAGGTATCATC-3’ in a total volume 50 µl. The DNA extract was amplified by PCR in a final volume of 50 µl, containing 13× reaction buffer, 25 µmol of each dNTP, 100 pmol of each primer, 5 unit/µl Taq DNA polymerase (TAKARA). Thermocycling parameters for a GeneAmp PCR system 9600 thermal cycler (Applied Biosystems, Foster City, CA) were as follows: 30 cycles of denaturation at 94°C for 2 min., annealing at 55°C for 1 min., extension at 72°C for 2 min. and a final extension step at 72°C for 10 min. PCR amplicons (604 bp) were visualized using a UV light box after electrophoresis on a 1.2% agarose gel stained by 0.5 mg of ethidium bromide/liter.

#### Susceptibility Testing

MIC determination and disk diffusion testing were performed as described in CLSI guidelines M100-S15\(^5\). MIC was determined by a microbroth dilution assay using the MIC-2000 system (Dynatech Laboratories, Inc., Alexandria, VA). Organisms were tested after overnight incubation in Trypticase soy agar (TSA, Becton Dickinson). Mueller-Hinton broth (MHB, Becton Dickinson) was used for susceptibility testing; for oxacillin (MPIPC) susceptibility testing, MHB with 2% NaCl was used. Tested antibiotics were MPIPC (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), ampicillin (ABPC: Meiji Seika Kaisha, Ltd., Tokyo, Japan), cefazolin (CEZ: Astellas Pharma Japan, Inc., Tokyo, Japan), cefotiam (CTM: Takeda Pharmaceutical Co., Ltd., Osaka, Japan), ceftizoxime (CZX: Astellas Pharma), cefpirome (CPR: Aventis Pharma, Ltd., Tokyo, Japan), fromoxef (FMOX: Shionogi & Co., Ltd., Osaka, Japan), imipenem (IPM: Banyu Pharmaceutical Co., Ltd.), gentamicin (GM: Schering-Plough K. K.,...
Osaka, Japan), levofloxacin (LVFX: Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan), and clindamycin (CLDM: Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Disk diffusion determinations were performed according to protocols described in CLSI guidelines M100-S15\(^6\) using Mueller-Hinton agar (Becton Dickinson) and 30\(\mu\)g CTM disks (Becton Dickinson). After overnight culture, zones were measured, alterations in spaced diameters were observed for 5 days.

The interpretation of susceptibility was based on the CLSI breakpoints\(^6\). For the antimicrobial agents out of setting in CLSI, the breakpoints were substituted by the similar agents; such as cefoxitin for CTM, cefotaxim for CPR, and moxalactam for FMOX, and assumed less than 8\(\mu\)g/ml to be sensitive for the grouping of bacterial strain to mention later, we used the Japanese Society of Antimicrobial Chemotherapy (JSAC) breakpoints for sepsis\(^8\) together with the CLSI breakpoints.

**Minimal Bactericidal Concentration of CTM**

The minimal bactericidal concentration (MBC) of CTM were determined in accordance with published NCCLS methods\(^9\). MBC were determined by subculturing in 10\(\mu\)l of TSA broth from each well with no visible growth after incubation for 24 hours. The MBC was interpreted as the lowest CTM concentration at which no growth occurs on TSA\(^10,11\).

### Results

**Susceptibility Pattern of MPIPC-Resistant S. epidermidis**

The 82.3% of 186 S. epidermidis tested were MRSE (MPIPC MIC \(\geq 0.5\)\(\mu\)g/ml). We investigated the antibiotic susceptibility pattern of these 153 isolates. As shown in Table 1, CTM exhibited the highest antibacterial activity among the drugs tested (89.2% sensitive). High susceptibility was also observed with beta-lactam antibiotics such as CEZ (66.7%), CPR (67.9%), FMOX (58.2%), and IPM (56.9%).

**Association of MPIPC and CTM Susceptibility and mecA Gene Expression in S. epidermidis**

Microdilution MICs of MPIPC and CTM together with mecA positivity of 186 S. epidermidis are shown in Fig. 1. CTM showed potent activity against both MPIPC-susceptible and MPIPC-resistant S. epidermidis. One hundred percent of the MPIPC-susceptible S. epidermidis (MSSE; MPIPC \(\leq 0.25\)\(\mu\)g/ml) were sensitive to CTM with the MICs of less than 1\(\mu\)g/ml. MRSE with MPIPC MICs ranging from 0.5 to 2.0\(\mu\)g/ml showed CTM sensitivity with MICs ranging from 1.0 to 2.0\(\mu\)g/ml. MRSE with high MPIPC MICs (\(\geq 4\)\(\mu\)g/ml) showed widely ranging CTM MICs (from 1.0 to \(\geq 128.0\)\(\mu\)g/ml).

We then classified the 186 S. epidermidis isolates into 5 groups: A, strains with MPIPC MIC

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>ABPC</th>
<th>CEZ</th>
<th>CTM</th>
<th>CPR</th>
<th>CZX</th>
<th>FMOX</th>
<th>IPM</th>
<th>GM</th>
<th>LVFX</th>
<th>CLDM</th>
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<tr>
<td>Breakpoint ((\mu)g/ml)</td>
<td>(\leq 0.25)</td>
<td>(\leq 8)</td>
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<td>(\leq 4)</td>
<td>(\leq 4)</td>
<td>(\leq 1)</td>
<td>(\leq 0.5)</td>
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<td>Susceptibility (%)</td>
<td>1.7</td>
<td>66.7</td>
<td>89.2</td>
<td>67.9</td>
<td>23.5</td>
<td>58.2</td>
<td>56.9</td>
<td>18.3</td>
<td>28.1</td>
<td>3.3</td>
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Table 1. The antibiotic susceptibility of 153 MPIPC-resistant S. epidermidis isolates.
Fig. 1. MPIPC and CTM susceptibility and mecA gene expression in 186 S. epidermidis isolates.

<table>
<thead>
<tr>
<th>CTM (MIC: µg/ml)</th>
<th>0.063</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
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<td>MPIPC (MIC: µg/ml)</td>
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Underlined numbers represent numbers of mecA positive strains. The solid line is the breakpoint defined by CLSI. The dashed line of MPIPC is the breakpoint recommended by NCCLS prior to 1998\(^{13}\), and the dashed line of CTM is the breakpoint defined by JSAC\(^{14}\).

Group A, strains with MPIPC MIC ≤0.25 µg/ml and CTM MIC ≤2.0 µg/ml; B, strains with MPIPC MIC 0.5~2.0 µg/ml and CTM MIC ≤2.0 µg/ml; C, strains with MPIPC MIC ≥4.0 µg/ml and CTM MIC ≤2.0 µg/ml; D, strains with MPIPC MIC ≥4.0 µg/ml and CTM MIC 4.0~8.0 µg/ml; E, strains with MPIPC MIC ≥64 µg/ml and CTM MIC ≥16.0 µg/ml.

≤0.25 µg/ml and CTM MIC ≤2.0 µg/ml; B, strains with MPIPC MIC 0.5~2.0 µg/ml and CTM MIC ≤2.0 µg/ml; C, strains with MPIPC MIC ≥4.0 µg/ml and CTM MIC ≤2.0 µg/ml; D, strains with MPIPC MIC ≥4.0 µg/ml and CTM MIC 4.0~8.0 µg/ml; and E, strains with MPIPC MIC ≥64 µg/ml and CTM MIC ≥16.0 µg/ml.

All group A isolates (17.7% of the total) were mecA negative, while isolates of groups B, C, D, and E (19.4%, 11.3%, 41.9%, and 9.7% of the total number, respectively) were all mecA positive.

Association between Susceptibility and Bactericidal Activities of CTM in S. epidermidis

MBCs of all tested S. epidermidis isolates were less than four-fold greater than the MIC (Table 2), indicating that CTM is highly bactericidal to S. epidermidis. In particular, more than 50% of CTM-susceptible strains (groups A, B, and C) showed equal MIC and MBC. 38.9% of the group E strains (highly resistant to both MPIPC and CTM) and 9.1% of group A strains (MPIPC- and CTM-susceptible) showed MBCs four times greater than MICs. These results indicate that the higher MICs of MPIPC may predict poor bactericidal activity of CTM against S. epidermidis.
Table 2. Bactericidal activities of CTM against *S. epidermidis* strains (n=186).

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of strains</th>
<th>1 × MIC (%)</th>
<th>2 × MIC (%)</th>
<th>4 × MIC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>33</td>
<td>20 (60.3)</td>
<td>10 (30.3)</td>
<td>3 (9.1)</td>
</tr>
<tr>
<td>B</td>
<td>36</td>
<td>18 (50.0)</td>
<td>14 (38.9)</td>
<td>4 (11.1)</td>
</tr>
<tr>
<td>C</td>
<td>21</td>
<td>14 (66.7)</td>
<td>2 (9.5)</td>
<td>5 (23.8)</td>
</tr>
<tr>
<td>D</td>
<td>78</td>
<td>20 (25.6)</td>
<td>38 (48.7)</td>
<td>20 (25.6)</td>
</tr>
<tr>
<td>E</td>
<td>18</td>
<td>6 (33.3)</td>
<td>5 (28.8)</td>
<td>7 (38.9)</td>
</tr>
</tbody>
</table>

a) groups A–E: for definition, see Fig. 2.

Fig. 2. Inhibition zones of CTM disk diffusion tests and time dependent changes (3 strains).

Representative CTM disk diffusion test results. Isolates of groups D and E showed small colonies inside the inhibition zones at day 5 (arrows).

Disk Diffusion Determinations for CTM

Isolates evaluated for CTM time-kill kinetics were subjected to CTM disk diffusion testing. Groups A and B had large inhibition zones (more than 30 mm diameters) that were unchanged until day 5 of incubation. The inhibition zones of group C isolates were less than 30 mm with no diameter change observed during the 5 day incubation. Isolates belonging to groups D and E, however, showed less than 25 mm inhibition zones in the susceptible range at 24 hours of incubation, in which small colonies appeared on day 5 (Fig. 2).

The observed inhibition zones and time-dependent changes in the 186 *S. epidermidis* isolates were compared. As shown in Fig. 3, all isolates belonging to group A showed more than a 30 mm diameter inhibition zone with no change in diameters. The mean diameters of group B and C isolates were 7
Fig. 3. Inhibition zones of CTM disk diffusion tests and time dependent changes (186 strains).

Graphs show the mean (circles) and maximum and minimum inhibition zone diameters (squares).

to 10 mm less than that of group A, and the decrease in diameters was less than 3 mm after 5 days of incubation. The mean diameter of group D at 24 hours was 25.3 mm (ranging from 15 to 30 mm), and small colonies appeared inside the inhibition zone at day 4. Isolates belonging to group E showed smaller inhibition zones in the resistant range (≤12 mm).

Discussion

We performed bacteriological in vitro study on the efficacy of CTM against S. epidermidis isolated from blood culture and central venous catheters.

In CLSI guidelines, sensitive/resistant interpretation on beta-lactam antibiotics against Staphylococcus is determined by the results of MPIPC susceptibility. The MPIPC-resistant strains are interpreted as resistant against all beta-lactam antibiotics because of their mecA positivity. Whereas most of MRSA strains show the obvious resistance against beta-lactam antibiotics, MRSE stains with low MICs against beta-lactam antibiotics are frequently observed. Such a variety of sensitivity against beta-lactams between MRSA and MRSE has been reported because of the difference of their mecA transcription regulators. It is also considered that the more frequent exposure to antibiotic reagents is another reason of lower beta-lactams MIC of MRSE compared to MRSA. The MRSA strains isolated from inpatients, being frequently exposed to antimicrobial agents, more easily acquire multidrug-resistance than the MRSA from outpatients, which are often observed their low beta-lactams MIC, so called border-line MRSA. We have previously reported that the S. epidermidis strains isolated from blood culture and central venous catheters were not nosocomial epidemic-strains but carry-on-strains of individual patients. These observations provide an argument that few opportunities of exposure to antibiotics in S. epidermidis might be one of the reason of lower beta-lactams MIC of MRSE.
We observed the same level of CTM bactericidal activities against *S. epidermidis* of less than 2 μg/ml CTM MIC as of CTM sensitive *S. epidermidis*. MRSE with more than 4 μg/ml CTM MIC, however, exhibited the low susceptibility against CTM. Low susceptibility isolates contain subpopulations that can grow at more than MIC concentration of antimicrobial agents. As many reports on VCM\(^{16,17}\), hetero-resistance were also observed against CTM in this study. The small colonies appeared inside the inhibition zone in groups D and E of our disk diffusion assay exhibited the subpopulations which decreased the sensitivity against CTM, indicating that no bactericidal efficacy of CTM can be expected against these isolates. These findings further need to be confirmed by the population analysis.

These results indicate the possibility of efficacy of CTM against MRSE with less than 2.0 μg/ml CTM MIC, which agrees with the CTM MIC breakpoint for sepsis by the JSAC. Our bacteriological investigation could valid this CTM MIC breakpoint.

Reevaluation of beta-lactam antibiotics efficacy on MRSE might be useful to choose more profitable and safety antimicrobial agents to patients\(^{19,20}\).

The clinical utility of beta-lactam antibiotics requires further bacteriological testing and clinical trials.

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