Effects of Calcium Concentrations on Antimicrobial Susceptibility Testing Against *Pseudomonas aeruginosa*

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Abstract: Accurate determination of the antimicrobial susceptibilities of clinical isolates is important in establishing adequate treatment with antibiotics, particularly for infections caused by multiple drug-resistant strains of *Pseudomonas aeruginosa*. However, current laboratory antimicrobial susceptibility testing is affected by various conditions, including cation concentrations. In this study, we analyzed the relevance of the resistance determinants, metallo-β-lactamase (MBL) and aminoglycoside-modifying enzyme production, and examined the effects of calcium concentration on the *in vitro* activity of antibiotics. The broth microdilution method using Mueller-Hinton broth supplemented with various concentrations of calcium (5.2, 25, and 50 mg/L) was used to evaluate 58 clinical strains of *P. aeruginosa*. We observed increases in the minimum inhibitory concentrations (MICs) of aminoglycosides, tetracyclines, quinolones, and some β-lactam antibiotics with higher concentrations of calcium in the media. Strains that did not produce MBLs would have been affected by calcium concentration. Strains that did not produce aminoglycoside-modifying enzymes tended to lower the MICs of aminoglycosides more substantially than strains that produced aminoglycoside-modifying enzymes. In conclusion, the calcium concentration of the media may affect the results of antimicrobial susceptibilities of *P. aeruginosa* in relation to each resistance gene.

Keywords: Multiple-drug-resistant *Pseudomonas aeruginosa*, Cation, Metallo-β-lactamases, Aminoglycoside-modifying enzymes.

1. INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic pathogen that exhibits high intrinsic resistance to many antimicrobial agents. *P. aeruginosa* is commonly identified as the causative agent of life-threatening nosocomial infections in immunocompromised patients. The increasing incidence of multiple drug-resistant strains of *P. aeruginosa* (MDRP) recovered from hospitals worldwide is of great concern [1]. MDRP poses serious health risks owing to the lack of drugs effective against infections caused by MDRP strains. The major determinants of antimicrobial resistance in MDRP strains are the production of metallo-β-lactamases (MBLs) and aminoglycoside-modifying enzymes. MBLs including IMP, VIM, SPM, and GIM have been reported in *P. aeruginosa* worldwide [2-4]. Hence, it is critically important to obtain accurate information on the antimicrobial susceptibilities of clinical strains to ensure the appropriate use of antibiotics, particularly in the case of MDRP infections [5].

The minimum inhibitory concentrations (MICs) of aminoglycosides and tetracyclines increased when *P. aeruginosa* growth media was supplemented with cations [6, 7]. To date, there have been no reports of similar increases in MICs for other antibiotics, and to the best of our knowledge, the relationship between resistance determinants and calcium concentrations has yet to be reported.

We previously reported an increase in the MICs of oxacillin (MPIPC) with the addition of calcium to the growth media of *Staphylococcus aureus*. The Clinical and Laboratory Standards Institute (CLSI) recommends the use of cation-adjusted Mueller-Hinton broth (MHB) containing 20 to 25 mg of Ca²⁺ per liter and 10 to 12.5 mg of Mg²⁺ per liter for broth microdilution susceptibility tests [8]. These concentrations differ markedly from those in the human body (e.g. 42.5–51 mg/L for Ca²⁺), and as such we evaluated Ca²⁺ concentrations both within and beyond the breakpoints proposed by the CLSI.

In this study, we analyzed the relevance of the resistance determinants, MBLs and aminoglycoside-modifying enzymes, and examined the effects of
calcium concentration on the in vitro activity of a range of antibiotics (including β-lactam antibiotics, fluoroquinolones, aminoglycosides, and tetracyclines) against *P. aeruginosa*, using the standard broth microdilution method, and Mueller-Hinton broth (MHB) supplemented with Ca$^{2+}$ at 5.2, 25, and 50 mg/L.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

Fifty-eight clinical isolates of *P. aeruginosa* were obtained from the diagnostic microbiology laboratories of Tokyo Medical University Hospital and Todachuo General Hospital, Japan, between January 2007 and February 2012. Strains were isolated from blood, urine, stool, and sputum samples of patients. All isolates were identified using standard methods for *P. aeruginosa*. *P. aeruginosa* strain ATCC 27853 was included as a control.

2.2. Screening of MBL Producers

All clinical isolates were screened for MBL production using disks impregnated with an MBL inhibitor. Two Kirby–Bauer (KB) disks containing 30 μg of ceftazidime and a disk containing 3 mg of sodium mercaptoacetic acid (SMA) (Eiken Chemical Co. Ltd., Tokyo, Japan) were used in the screening test (9).

2.3. Detection of Resistant Genes

PCR analyses for the detection of MBL genes were carried out for all strains for which the screening test using SMA disks gave positive results. The *bla*IM, *aac(6')-lae* and *aac(6')-Ib* genes were detected by PCR using previously reported primers (10–12). The *bla*IM-specific primers IMP-F and IMP-R used in this study were designed to amplify *bla*IM-1, *bla*IM-2, *bla*IM-3, *bla*IM-4, *bla*IM-5, *bla*IM-6, *bla*IM-7, *bla*IM-8, *bla*IM-9, *bla*IM-10, and *bla*IM-11.

2.4. Culture Media

The basal concentration of Ca$^{2+}$ and Mg$^{2+}$ in commercial MHB (Eiken Chemical Co. Ltd.) are 5.2 mg/L and 4.2 mg/L, respectively. Sterile MHB was supplemented with Mg$^{2+}$ and Ca$^{2+}$ using sterile stock solutions of reagent-grade MgCl$_2$ ·6H$_2$O and CaCl$_2$ ·2H$_2$O, to final calcium concentrations of 25 mg/L and 50 mg/L, and a magnesium concentration of 12.5 mg/L. Atomic absorption spectrometry was used to quantify calcium and magnesium concentrations in the supplemented media.

2.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was carried out in 96-well microtiter plates (Dry Plate Eiken DP-35; Eiken Chemical Co. Ltd.) using a twofold standard broth microdilution method with MHB. MHB was inoculated with *P. aeruginosa* at concentrations of $10^4$ to $10^5$ CFU/mL. All other conditions were as per the methods recommended by the CLSI. The 96-well plates were used with the following concentration of each antibiotic: piperacillin (PIPC), 1–64; tazobactam/piperacillin (TAZ/PIPC), 4/1–4/64; sulbactam/cefoperazone (S/C), 8/8–32/32; ceftazidime (CAZ), 0.5–16; cefepime (CFPM), 0.5–16; imipenem (IPM), 0.25–8; meropenem (MEPM), 0.25–8; doripenem (DRPM), 0.25–8; gentamicin (GM), 0.25–8; tobramycin (TOB), 0.25–8; amikacin (AMK), 1–32; aztreonam (AZT), 0.5–16; minocycline (MINO), 0.25–8; fosfomycin (FOM), 32–128; ciprofloxacin (CPFX), 0.06–2; levofloxacin (LVFX), 1–4; sulfamethoxazole/trimethoprim (ST), 19/1–38/2; colistin (CL), 2–8.

We also modified microtiter plates (originals from Eiken Chemical Co. Ltd.) to measure wider ranges of MICs. These plates consisted of the following: PIPC, 1–128; TAZ/PIPC, 4/1–4/64; CAZ, 1–64; IPM, 1–64; MEPM, 1–128; DRPM, 1–64; GM, 0.5–64; TOB, 0.5–64; AMK, 0.5–64; AZT, 1–128; CPFX, 0.25–32; LVFX, 0.25–32. Breakpoints of each antibiotic were in accordance with CLSI breakpoints M100-S22.

In this study, MDRP strains were defined as being resistant to three major drug classes (i.e., antipseudomonal carbapenems, fluoroquinolones, and aminoglycosides). *P. aeruginosa* strains resistant to two major drug classes named above were defined as 2-drug resistant strains of *P. aeruginosa* (2DRP).

3. RESULTS

3.1. Resistance Patterns of Tested Strains

Of the 58 strains tested, 28 (48.3%) were characterized as MDRP and 30 (51.7%) as 2DRP. The 2DRP group consisted of 25 strains sensitive to AMK, three to CPFX, and two to IPM.

3.2. Analysis of MBL and Aminoglycoside Resistance

Of the 58 strains tested, 23 (39.7%) produced MBLs, as determined by the preliminary screening of isolates using SMA-impregnated disks. PCR analyses identified *bla*IM genes for each of these 23 strains. The
aac(6')-ib gene was detected for 24 strains (41.4%), whilst the aac(6')-lae was notably absent for all strains.

3.3. Effects of Ca\(^{2+}\) Concentration on Antimicrobial Susceptibilities

The distribution of susceptible, intermediate, and resistant strains varied considerably for media containing 25 and 50 mg/L Ca\(^{2+}\) for the aminoglycosides (AMK, GM, TOB) and carbapenems (MEPM, DRPM) (Figure 1). PIPC, TAZ/PIPC, CPFX, and LVFX were difficult to evaluate because many strains exceeded the range of the MICs of DP-35. As shown in Figure 2, the resistance rate to IPM and AMK increased with an increase in calcium concentration from 25 to 50 mg/L using DP-35, whilst the resistance rate remained unaltered for CPFX. An increase in Ca\(^{2+}\) concentration from 25 to 50 mg/L resulted in an increase in MICs of IPM and AMK, and a change in susceptibility from sensitive to resistant. When Ca\(^{2+}\) concentrations increased from 25 to 50 mg/L, five strains previously classified as 2DRP were confirmed as MDRP.

Table 1 compares the MIC 50 and MIC 90 at Ca\(^{2+}\) concentrations of 25 and 50mg/mL using modified microtiter plates with expanded MICs. Six (50%) of the antibiotics exhibited a change in MIC 50 at different calcium concentrations, whilst all antibiotics were unaffected by a change in calcium concentration at MIC 90. Figure 3 summarizes the results of the broth microdilution method using modified microtiter plates to clarify the range of the MICs of DP-35. Qualitative

IPM, imipenem; MEPM, meropenem; DRPM, doripenem; AMK, amikacin; GM, gentamicin; TOB, tobramycin

Figure 1: Effects of calcium concentration using DP-35.

Comparison of the effects of calcium concentration on the in vitro activity of antibiotics against P. aeruginosa using the standard broth microdilution method, MHB, and calcium concentrations of 5.2, 25, and 50 mg/L. When Ca\(^{2+}\) concentrations were increased from 25 to 50 mg/L, the MICs of aminoglycosides and carbapenems increased, with a concomitant shift in the position of the ogive observed.
interpretation of susceptibility based on dilution tests with IPM, MEPM, DRPM, AMK, GM, TOB, CPFX, LVFX, PIPC, and TAZ/PIPC were markedly affected by cation supplementation.

With regard to the determinants of multidrug resistance, the 23 MBL-producing strains were highly resistant to IPM, whilst the non-MBL-producing strains were sensitive (Figure 4). Table 2 lists the number of strains resistant to carbapenems in MBL-producing P. aeruginosa, and to aminoglycosides in aminoglycoside-modifying enzyme producers. The 23 MBL-producing strains had no appreciable effect on the determination of resistance. The seven strains with increased resistance to IPM (MICs ≥ 8) were non-MBL-producing P. aeruginosa. Of the 24 strains of aminoglycoside-modifying enzyme producers tested, 17 (70.8%) were highly resistant to aminoglycoside. Of the 34 strains lacking the aminoglycoside resistance gene, only a single strain was considered highly resistant to aminoglycosides.

4. DISCUSSION

The CLSI recommends calcium and magnesium be used at concentrations of 20–25 mg/L and 10–12.5 mg/L, respectively, for dilution susceptibility testing. Since the 1980’s, dilution susceptibility testing has been performed in Japan using the standard broth microdilution method as specified by the CLSI. To date, the CLSI has not standardized cation concentrations for the agar used in the agar disk method. In addition, cation concentrations differ according to the type of antibiotic tested. The calcium concentration for daptomycin is set at 50 mg/L for the broth microdilution method [13, 14]; similar to the calcium concentration in the human body. Daptomycin MICs, as determined by E-tests, have been shown to differ when Mueller-hinton agar is supplemented with different calcium concentrations, and as such this method is not recommended. P. aeruginosa is an opportunistic pathogen with a high intrinsic resistance to many antimicrobial agents.

It has been reported elsewhere that the MICs of aminoglycosides and tetracyclines increased upon addition of cations to the test media of P. aeruginosa [6, 7]. In the current study, we showed that the addition of calcium elicited the same effect for aminoglycosides, tetracyclines, carbapenems, quinolones, and some β-lactams. IPM MICs increased in the presence of 50 mg Ca²⁺/L for seven P. aeruginosa strains, and these strains were subsequently determined to be IPM-resistant.

Strains that produce IMP-type MBLs and aac(6’)-account for 66.8% of all P. aeruginosa strains in Japan [15]. In the current study, we found this number to be higher. Of the 58 clinical isolates investigated,
IPM, imipenem; MEPM, meropenem; DRPM, doripenem; AMK, amikacin; GM, gentamicin; TOB, tobramycin

Figure 3: Effects of calcium concentration using modified microtiter plates with expanded MICs.

The effects of calcium concentration on the \textit{in vitro} activity of antibiotics against \textit{P. aeruginosa} using the standard broth microdilution method, MHB, and calcium concentrations of 25 and 50 mg/L.
Table 2: Number of Strains Resistant to Carbapenems in MBL-Producing *P. aeruginosa* and to Aminoglycosides in Aminoglycoside-Modifying Enzyme Producers

<table>
<thead>
<tr>
<th></th>
<th>IPM</th>
<th>AMK</th>
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<tr>
<td></td>
<td>MBLs (+)</td>
<td>aac6 (+)</td>
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<tr>
<td></td>
<td>n = 23</td>
<td>n = 24</td>
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<td></td>
<td>Ca 25</td>
<td>Ca 50</td>
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<td><strong>S</strong></td>
<td>0 (0)</td>
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<tr>
<td><strong>R</strong></td>
<td>23 (100)</td>
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Abbreviation: IPM: imipenem; AMK: amikacin; MBLs: metallo-β-lactamases; S: susceptible; R: resistant.

29 strains were not MDRP and they produced neither MBL nor aac(6’), whilst 25 strains were identified as 2DRP. MBL-producing strains were unaffected by changes in calcium concentration because of their high-level resistance at the MICs of carbapenems, whilst non-MBL-producing strains would have been affected by changes in calcium concentration (Table 2). The production of MBLs may have little consequence in the determination of resistance, but we did observe an increase in MICs at a calcium concentration of 50 mg/L.

Figure 4: Comparison of IPM MIC for MBL-producing *P. aeruginosa* and non-MBL-producing *P. aeruginosa* strains at a calcium concentration of 25 mg/L.

The MBL-producing strains tended to exhibit high MIC levels, whilst the non-MBL-producing strains had low MIC levels.

A small number of strains identified as aminoglycoside-modifying enzyme producers remained susceptible to aminoglycosides (Table 2). In addition, the MICs increased with the addition of cations for these strains, although an accurate detection of resistance was not possible. Thirty-four strains lacked the gene for aminoglycoside-modifying enzymes and as such, with the exception of a single strain, were susceptible to aminoglycosides. Strains not producing aminoglycoside-modifying enzymes had lower MICs for aminoglycosides compared with enzyme producing strains, and this difference is believed to be attributed to the overexpression of efflux pumps.

Quinolone MICs were high as a measure of resistance, and increased with a change in calcium concentration from 25 to 50 mg/L. They have little relationship to determining resistance.

In the current study, we demonstrated an increase in MICs with the addition of cations, not only for aminoglycosides and tetracyclines but also for carbapenems, quinolones, and some β-lactams for a number of *P. aeruginosa* isolates. Differences in the effects of calcium concentration among resistance genes was also observed. Our results suggest the accurate detection of carbapenem MICs for the non-MBL-producing *P. aeruginosa* strains would have failed if MHB medium supplemented with 25 mg/L calcium had been used. Furthermore, we suggest that the accurate detection of MICs of aminoglycosides would have failed for strains producing aac(6’).

REFERENCES


