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Alterations in Penicillin Binding Protein Gene of Streptococcus pneumoniae and their Correlation with Susceptibility Patterns

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Running title: Alterations in *pbp* gene of *Streptococcus pneumoniae*

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Abstract

We examined penicillin binding protein (*pbp*) gene alterations among 328 clinical isolates of *S. pneumoniae*, for a correlation with their antibiotic-resistance. We determined the frequency of PEN-G resistance to clarify their susceptibility to several antibiotics, namely PEN-G, AMP, SAM, CZOP, PAPM, CLR, AZM and LVX. Oligonucleotide primers for three *pbp* genes (*pbp1a*, *pbp2x* and *pbp2b*) were used to detect mutations in *pbp*. 25.9% of the strains were classified as Pen-G^{s}, 68.0% as Pen-G^{ir} and 6.1% as Pen-G^{r}. The polymerase chain reaction (PCR) product for wild-type *pbp1a* was found in 185 isolates, that for wild-type *pbp2x* was found in 66 isolates and that for wild-type *pbp2b* was found in 213 isolates. None of these three genes was detectable in 100 isolates while all of them were detected in 64 isolates (*1a^{w}/2x^{w}/2b^{w}*). Among those 64 isolates with *1a^{w}/2x^{w}/2b^{w}*, the MIC of PEN-G was ≤0.06 μg/ml for 54 isolates and 0.12 μg/ml for 10 isolates. Among the 272 strains for which the MIC of PAPM was ≤0.03 μg/ml, there were 85 Pen-G^{s}, 184 Pen-G^{ir} and 3 Pen-G^{r} isolates. Three strains for which the MIC of LVX was ≥4.0 μg/ml included one Pen-G^{s} and two Pen-G^{ir} isolates. The MICs of CLR significantly correlated with those of AZM. The MIC of CLR was ≥1 μg/ml for 216 isolates, and the MIC of AZM was ≥1 μg/ml for 244 of them. These data suggested that PAPM is effective against

S. pneumoniae infection, although acquirement of resistance should be considered. LVX also seemed to be effective against *S. pneumoniae*.

Introduction

Streptococcus pneumoniae (*S. pneumoniae*) causes respiratory, central nervous system, dermatological, urinary, gynecological and otolaryngeal infections. Especially, *S. pneumoniae* is the commonest pathogen causative of community-acquired pneumonia and it is frequently found in adult patients with hospital-acquired pneumonia, accounting for >80% of community-acquired and 10% of nosocomial pneumonia [1, 2]. *S. pneumoniae* used be sensitive to various antibiotics, including those with a β-lactam structure such as Penicillin G (PEN-G). Penicillins was the antibiotic of first choice to treat infections caused by *S. pneumoniae*, however, the bacterium has become resistant to penicillins and cephalosporins over the past decade. Thus antibiotics should be used based on accurate information about the susceptibility of the bacteria as well as its pharmacokinetic and pharmacodynamic (PK/PD) data.

Resistant *S. pneumoniae* were identified as early as 1967 in Australia [3]. In 1999-2000 in the United States, *S. pneumoniae* for which the minimum inhibitory concentration (MIC) of PEN-G was ≥0.12 μg/ml accounted for 34.2% of 1,531 isolates from 33 medical centers, and those for which MIC was ≥2.0 μg/ml accounted for 21.5% [4]. In 1994-1995, their frequencies were 23.6% and 9.5%, respectively [5]. Another study performed in 1997 reported that the PEN-G MIC was ≤0.06 μg/ml for 65.1% of *S.*

pneumoniae isolates, 0.12-0.1 μg/ml for 22.1% and ≥2.0 μg/ml for 12.8% of the isolates [6]. The frequency of resistant strains should be considered when antibiotics with a β-lactam structure are going to be used to treat pneumococcal infections especially in geographic areas with a high prevalence of *S. pneumoniae*. The National Committee for Clinical Laboratory Standard (NCCLS) [7] recommended to classify *S. pneumoniae* isolates for which the MIC of PEN-G is ≤0.06 μg/ml as Pen-G^s (sensitive), those for which the MIC of PEN-G is 0.1-1.0 μg/ml as Pen-G^{ir} (intermediate resistant) and those for which the MIC of PEN-G is ≥2.0 μg/ml Pen-G^r (resistant). Usually, Pen-G^{ir} and Pen-G^r strains are also resistant to other penicillins and cephalosporins.

PEN-G binds to penicillin binding proteins (pbp) to inhibit the synthesis of the cell wall of bacteria. Among these pbp coding genes, mutations of *pbp1a*, *pbp2x* or *pbp2b* were reported to confer resistance to PEN-G [8-11]. Thr-371 substitution in pbp 1a was found to be associated with Pen-G^r [12] and substitution within or adjacent to the conserved amino acid motif of pbp 2x was reported [13]. Together with gene mutations in *pbp2b*, mutations in *pbp1a* and *pbp2x* were also detected by PCR [14, 15].

Macrolides are often the antibiotics of first choice for empirical treatment of community-acquired pneumonia because *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionellae* species, which cause atypical pneumonia, are susceptible to these

antibiotics. However, macrolide resistance among *S. pneumoniae* has escalated worldwide [16]. According to the NCCLS, erythromycin^{ir} (ERY^{ir}) was defined as an MIC of 0.5 μg/ml and ERY^{r} as an MIC ≥1.0 μg/ml [17]. For clarithromycin (CLR) and azithromycin (AZM), intermediate resistant is defined as an MIC of 1.0 μg/ml and resistance as an MIC of ≥2.0 μg/ml [18]. Pneumococci resistant to ERY were reported to be also resistant to AZM, CLR, and roxithromycin [19-22].

We conducted the present study to obtain data for an appropriate antibiotic therapy for patients with *S. pneumoniae* infection. We examined antibiotic-resistance of *S. pneumoniae* in relation to *pbp* gene mutations. We also tried to determine the frequency of Pen-G^{r} and to clarify the susceptibility to antibiotics of first choice in clinically isolated *S. pneumoniae*.

Materials and Methods

Bacteria isolation

Three hundred and fifty-five strains of *S. pneumoniae* were isolated from clinical samples between 1998 and 2001 at Asahikawa Medical College Hospital and stored at -80°C. The samples taken within 8 weeks from the same patient were excluded from the analyses unless they showed different susceptibility to PEN-G. Therefore, 328 strains of *S. pneumoniae* were analyzed in the present study; two to three samples were obtained from 37 patients. These samples included 87 of sputum, 62 of pharyngeal scrubbing, 19 of rear nasal discharge, 26 of ear discharge, 98 of nasal discharge and 36 of other specimens. Patients included 107 children and 221 adults.

Antibiotics

The MICs of PEN-G, ampicillin (AMP), sulbactam/ampicillin (SAM), cefozopram (CZOP), panipenem (PAPM), CLR, AZM and levofloxacin (LVX) were determined. We selected CZOP because this was one of the newest cephalosporins in our country. Each antibiotic was obtained directly from the manufacturer. Results for SAM were expressed as concentrations of AMP.

The activity of all antibiotics was confirmed using the susceptible *Staphylococcus aureus* ATCC 29213 control strain.

Susceptibility testing

The MICs of antibiotics for the 328 clinical isolates were determined according to the guidelines of the NCCLS [23]. Briefly, testing was performed in microtitre trays (Eiken Chemical, Tokyo, Japan) using cation-supplemented Mueller-Hinton broth (Eiken Chemical) containing 2.5% lysed horse blood. Test and control strains were grown at 37°C for 3 hours, the cell densities were adjusted to match a MacFarland 0.5 standard and further diluted in quarter-strength Ringer's solution to a final concentration of approximately 5 X 10^5 cfu/ml. Antibiotics were serially diluted in two-fold dilutions in microtitre trays. Inoculated trays were incubated overnight at 37°C before determining the MICs in the wells. Colonies were counted as a control procedure for the assessment of the inoculum size.

Standard quality control strains, including *Staphylococcus aureus* ATCC 29213, and *S. pneumoniae* strains, JPS-02, JPS-04, JPS-05 JPS-07, JPS-15 and JPS-48, were used for the susceptibility testing. JPS-05 is a Pen-G^s strain, JPS-04, JPS-07, JPS-15 and JPS-48 are as Pen-G^{ir} strains and JPS-02 is a Pen-G^r strain. These strains were gifts from Dr. Kimiko Ubukata of Kitazato University.

DNA extraction

The isolated strains were cultured on a 10 cm plastic dish containing culture agar for *S pneumoniae*. The bacteria were collected using a cell scraper and transferred to a 1.5

ml Eppendorf tube containing 1 ml normal saline. DNA was extracted using SepaGene kit (Sanko Junyaku, Tokyo) according to the instructions provided by the manufacturer. The DNA pellet was dissolved in 30 μl TE buffer.

PCR primers

The sequences of primers were as described elsewhere [15]. In the case of *pbp1a*, the primer sequence was modified from that in the above reference (K. Ubukata personal communication). Each primer mixture, which contains 0.1 μM of each primer and 8 mM dNTPs, is commercially available from Wakunaga Pharmaceutical (Hiroshima, Japan) as aliquots of 100 μl each. The oligonucleotide primers used to detect three *pbp* genes were designed to amplify parts of the *pbp1a*, *pbp2x* and *pbp2b* genes only in susceptible strains. These parts were positioned in blocks of highly diverged sequences identified in the mosaic *pbp* genes of penicillin non-susceptible *S. pneumoniae*. Primer mixture A contained the primers used to detect *lytA* and *pbp1a* genes. Primer mixture B contained the primers to detect *pbp2x* and *pbp2b* genes.

PCR conditions

PCR was done in 50 μl reaction buffer containing 1μM of each primer, 10 μl 10X PCR buffer, 200 μM dNTP, 1U Tth DNA polymerase (TOYOBO, Osaka, Japan), 1 μl DNA solution and pure water. PCR conditions were as follows. 94°C 20 seconds, 57°C 20

seconds, 72°C 15 seconds for 30 cycles using a thermal cycler (TP 400, Takara, Tokyo). JPS-02, JPS-04, JPS-05 JPS-07, JPS-15 and JPS-48 were used as quality control strains for the PCR analysis.

Gel electrophoresis

Following amplification, 3 μl each of the two sets of PCR product (primer set A and B) was fractionated on a GeneGel 12.5/24 acrylamide gel (Amersham Pharmacia Biotech, San Franscisco, CA) for 90 minutes using a GenePhor^{TM} apparatus (Amersham Pharmacia Biotech). Then the gel was stained by the silver staining method using a Plusone kit (Amersham Pharmacia Biotech). The results were interpreted as follows, when all three bands of *pbp* genes were seen, the isolate was considered to have wild-type *pbp* ($1a^{w}/2x^{w}/2b^{w}$), and when only two, one or none of these three bands were seen the isolate was considered to have mutated *pbps* ($1a^{m}/2x^{w}/2b^{w}$, $1a^{w}/2x^{m}/2b^{w}$, $1a^{w}/2x^{w}/2b^{m}$, $1a^{w}/2x^{m}/2b^{m}$, $1a^{m}/2x^{w}/2b^{m}$, $1a^{m}/2x^{m}/2b^{w}$ and $1a^{m}/2x^{m}/2b^{m}$). Results for the quality control strains were $1a^{m}/2x^{m}/2b^{m}$ for JPS-02, $1a^{m}/2x^{w}/2b^{w}$ for JPS-04, $1a^{w}/2x^{w}/2b^{w}$ for JPS-05, $1a^{w}/2x^{w}/2b^{m}$ for JPS-07, $1a^{m}/2x^{m}/2b^{w}$ for JPS-15 and $1a^{m}/2x^{m}/2b^{w}$ for JPS-48 (Fig. 1).

Statistical analysis

Results were compared using χ^2 test and the differences were considered

statistically significant when the value of p was less than 0.05. Correlations of MICs between two drugs were considered significant when the value of r was more than 0.8. All analyses were performed using the StatView Program (Abacus Concept, Inc., Berkeley, CA).

Results

MICs of PEN-G, AMP, SAM, CZOP, PAPM, CLR, AZM and LVX

The MIC of PEN-G was ≤0.03 μg/ml against 35/328 strains, 0.06 μg/ml for 50 strains, 0.12 μg/ml for 101, 0.25 μg/ml for 22, 0.5 μg/ml for 17, 1.0 μg/ml for 83, 2.0 μg/ml for 19 and 4.0 μg/ml for 1 isolate (Fig. 2). According to the NCCLS criteria, 85/328 (25.9%) were classified as Pen-G^s, 223/328 (68.0%) as Pen-G^{ir} and 20/328 (6.1%) as Pen-G^r. The MICs of PEN-G were almost similar to those of AMP (r=0.966, 95% CI 0.957-0.972) and SAM (r=0.964, 95% CI 0.956-0.971) (Fig. 3). MICs of PEN-G correlated with the MICs of CZOP (r=0.823, 95% CI 0.785-0. 855).

The MIC of PAPM was ≤0.03 μg/ml against 272/328 isolates, 0.06 μg/ml for 47 and 0.12 μg/ml for 9 isolates. Among the 272 isolates for which the MIC of PAPM was ≤0.03 μg/ml, there were 85 Pen-G^s isolates, 184 Pen-G^{ir} and 3 Pen-G^r isolates. Among those for which the MIC of PAPM was 0.06 μg/ml, there were 35 Pen-G^{ir} isolates and 12 Pen-G^r isolates, and among those for which the MIC of PAPM was 0.12 μg/ml, there were 4 Pen-G^{ir} isolates and 5 Pen-G^r isolates

The MIC of LVX was 0.5 μg/ml for two strains, 1.0 μg/ml for 222/328 strains, 2.0 μg/ml for 101 strains, 4.0 μg/ml for two strains and 8.0 μg/ml for one strain. These three strains for which the MIC was ≥4.0 μg/ml included one Pen-G^s and two Pen-G^{ir} isolates.

The MICs of CLR significantly correlated with those of AZM (r=0.969, 95% CI 0.962-0.975). The MIC of CLR was ≤0.03 μg/ml for 51 isolates and that of AZM was ≤0.03 μg/ml for two isolates. The MIC of CLR was ≥1.0 μg/ml for 216 isolates and that of AZM was ≥1.0 μg/ml for 244 isolates; significantly more isolates for which the MIC was ≥1.0 μg/ml were found in AZM than in CLR (p=0.0211). The MIC of CLR was ≥64 μg/ml for 114 isolates and that of AZM was ≥64 μg/ml for 137 isolates, the difference in distribution was not statistically significant (p=0.771). There were no significant correlations between MICs of PEN-G and those of CLR (r=0.214, 95% CI 0.108-0.315) and AZM (r=0.199, 95% CI 0.093-0.301).

Correlation between Pen-G^r and pbp gene alterations

The PCR product for *pbp1a* was detected in 185 isolates, *pbp2x* in 66 isolates and *pbp2b* in 213 isolates. None of these three genes were detected in 100 isolates (*1a^m/2x^m/2b^m*) and all of them were detected in 64 isolates (*1a^w/2x^w/2b^w*). 18/20 Pen-G^r isolates were a *1a^m/2x^m/2b^m* genotype (Table 1). The MICs of PEN-G for *1a^w/2x^w/2b^w* strains were ≤0.06 μg/ml for 54 isolates and 0.12 μg/ml for 10 isolates. The MICs of PEN-G for the isolates with one or two *pbp* mutations were ≥0.06 μg/ml except those were ≤0.03 μg/ml for four *1a^w/2x^m/2b^w* isolates. These four isolates were excluded from next analyses. The average MICs for isolates with *pbp* alteration were listed in table 2. The

differences among these groups were statistically significant by ANOVA ($p<0.0001$). The difference in MIC was not statistically significant between *1a*^{w}*/2x*^{m}*/2b*^{m} and *1a*^{m}*/2x*^{m}*/2b*^{w} strains.

When the cut off MIC of PEN-G between Pen-G^{ir} and Pen-G^{r} isolates was set as 2.0 μg/ml, the true positive/negative percentage of those with one or two alterations and *1a*^{m}*/2x*^{m}*/2b*^{m} was 63.9%, when the cut off level was 1.0 μg/ml, the percentage was 88.8%, and when the cut off level was 0.5 μg/ml, the percentage was 90.0%.

Correlation of susceptibility to PAPM and LVX with pbp gene alterations

Distribution of the isolates according to their *pbp* genotype and susceptibility to PAPM and LFX were shown in table 3.

Discussion

In the present study, 68.0% of clinical isolates of *S. pneumoniae* were classified as Pen-G^{ir} and 6.1% were classified as Pen-G^{r} according to the NCCLS criteria. These Pen-G^{r} *S. pneumoniae* isolates were also resistant to AMP, SAM and CZOP. On the other hand, PAPM and LVX showed uniform efficacy against Pen-G^{ir} and Pen-G^{r} isolates. However, all *S. pneumoniae* isolates for which the MIC of PAPM was ≥0.06 μg/ml were Pen-G^{ir} or Pen-G^{r} isolates. These data suggested that PAPM might prove effective against *S. pneumoniae* infections, although acquirement of resistance should be considered. Resistance to PEN-G correlated with resistance to CZOP which has a β-lactam structure, suggesting that Pen-G^{r} *S. pneumoniae* isolates might become resistant to other antibiotics with a β-lactam structure, including carbapenem. β-lactamase production is not likely to induce drug resistance of *S. pneumoniae* because susceptibility to PEN-G was similar to that to SAM in the present study.

Fluoroquinolones such as LVX, MXF, and GAT, are recommended for patients with community-acquired pneumonia which is likely to be caused by multidrug resistant *S. pneumoniae* [24]. Fluoroquinolones have a different mechanism of action compared to those with a β-lactam structure. In fact, resistance to LVX did not correlate with susceptibility to PEN-G in the present study. We found three isolates for which the MIC of

LVX was ≥4.0 μg/ml, including two Pen-G^{ir} isolates. According to the NCCLS criteria, which indicate that *S. pneumoniae* is classified as LVX intermediate resistant when the MIC of LVX is 4.0 μg/ml and resistant when the MIC is ≥8.0 μg/ml, two isolates were intermediate resistance and one was resistant [18]. The differences in mechanisms of drug resistance between β-lactam drugs and fluoroquinolones possibly explain this difference in susceptibility. In North America, resistance to LVX and failure of treatment for pneumococcal pneumonia due to acquirement of resistance to fluoroquinolones during therapy have been reported. Amino acid substitution in parC and gyrA were found in those strains [25]. However, fluoroquinolones seem to be effective against *S. pneumoniae* because resistance to fluoroquinolones has been reported to be remain low [26]. Our study confirms those findings.

Our results suggested that resistance of *S. pneumoniae* against antibiotics with a β-lactam structure is mainly due to alterations of pbp proteins. Genetic alterations in *pbp* correlated well with the susceptibility of strains to PEN-G, AMP, SAM and CZOP in the present study. Especially, alterations in the three genes, *pbp1a*, *pbp2x* and *pbp2b*, correlated with the Pen-G^{r}. The reasonable cut off level between penicillin resistance and intermediate resistance was 0.5 μg/ml from the genetic point of view; the accurate diagnostic rate was 90.0%.

According to the NCCLS criteria, more than half of *S. pneumoniae* isolates were resistant to CLR and AZM in the present study. Susceptibility to CLR correlated with that to AZM; however, more isolates were susceptible to CLR than to AZM. The MIC of PEN-G did not correlate with that of CLR or AZM. New macrolides are a good choice to treat community-acquired pneumonia because this is likely to be caused by *Mycoplasma pneumoniae* and *Chlamidia pneumoniae* as well as by *S. pneumoniae*. Physicians should be aware of MIC data as well as PK/PD data to choose antibiotics in the treatment of community acquired pneumonia.

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Table 1.

Distribution of the isolates analyzed by their *pbp* genotype and susceptibility to PEN-G

pbp genotype	Pen-G^{s}	Pen-G^{ir}	Pen-G^{r}	Total
1a^{w}/2x^{w}/2b^{w}	54	10	0	64
One or two alterations	31	131	2	164
1a^{m}/2x^{m}/2b^{m}	0	82	18	100
Total	85	223	20	328

Table 2.

Average MICs of PEN-G for isolates with *pbp* alteration

Genotype	MIC μg/ml (mean ± SD)	isolates number
1a^{w}/2x^{m}/2b^{w}	0.126 ± 0.102	n=102*
1a^{w}/2x^{m}/2b^{m}	0.325 ± 0.131	n=15
1a^{m}/2x^{w}/2b^{w}	1.0	n=2
1a^{m}/2x^{m}/2b^{w}	0.469 ± 0.503	n=41
1a^{m}/2x^{m}/2b^{m}	1.135 ± 0.503	n=100

*Four *1a^{w}/2x^{m}/2b^{w}* isolates were excluded because the MIC of PEN-G was ≤0.03 μg/ml.

Table 3.

Distribution of the isolates according to their *pbp* genotype and susceptibility to PAPM and LVX

pbp genotype	MIC fo PAPM (μg/ml)			MIC of LVX (μg/ml)					Total
	≤0.03	0.06	0.12	0.5	1.0	2.0	4.0	8.0	
1a^{w}*/2x*^{w}*/2b*^{w}	64	0	0	1	43	19	1	0	64
One or two alterations	156	6	2	0	101	62	0	1	164
1a^{m}*/2x*^{m}*/2b*^{m}	52	41	7	1	78	20	1	0	100
Total	272	47	9	2	222	101	2	1	328

Figure legends

Figure 1. Results of PCR analysis of pbp gene alterations in control strains by gel electrophoresis.

Figure 2. MICs of PEN-G, AMP, SAM, CZOP, PAPM, LVX, CLR and AZM against *S. pneumoniae* isolates. □ : isolates with a *1a*^{w}*/2x*^{w}*/2b*^{w} genotype, ■ : isolates with a *1a*^{m}*/2x*^{m}*/2b*^{m} genotype, and ▨ : isolate with other *pbp* genotypes.

Figure 3. Correlations between MICs of PEN-G and SAM, PEN-G and CZOP, PEN-G and AZM, and between those of CLR and AZM.

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Figure 3. Y. Ohsaki et al. Drug resistance of *S. pneumoniae*.

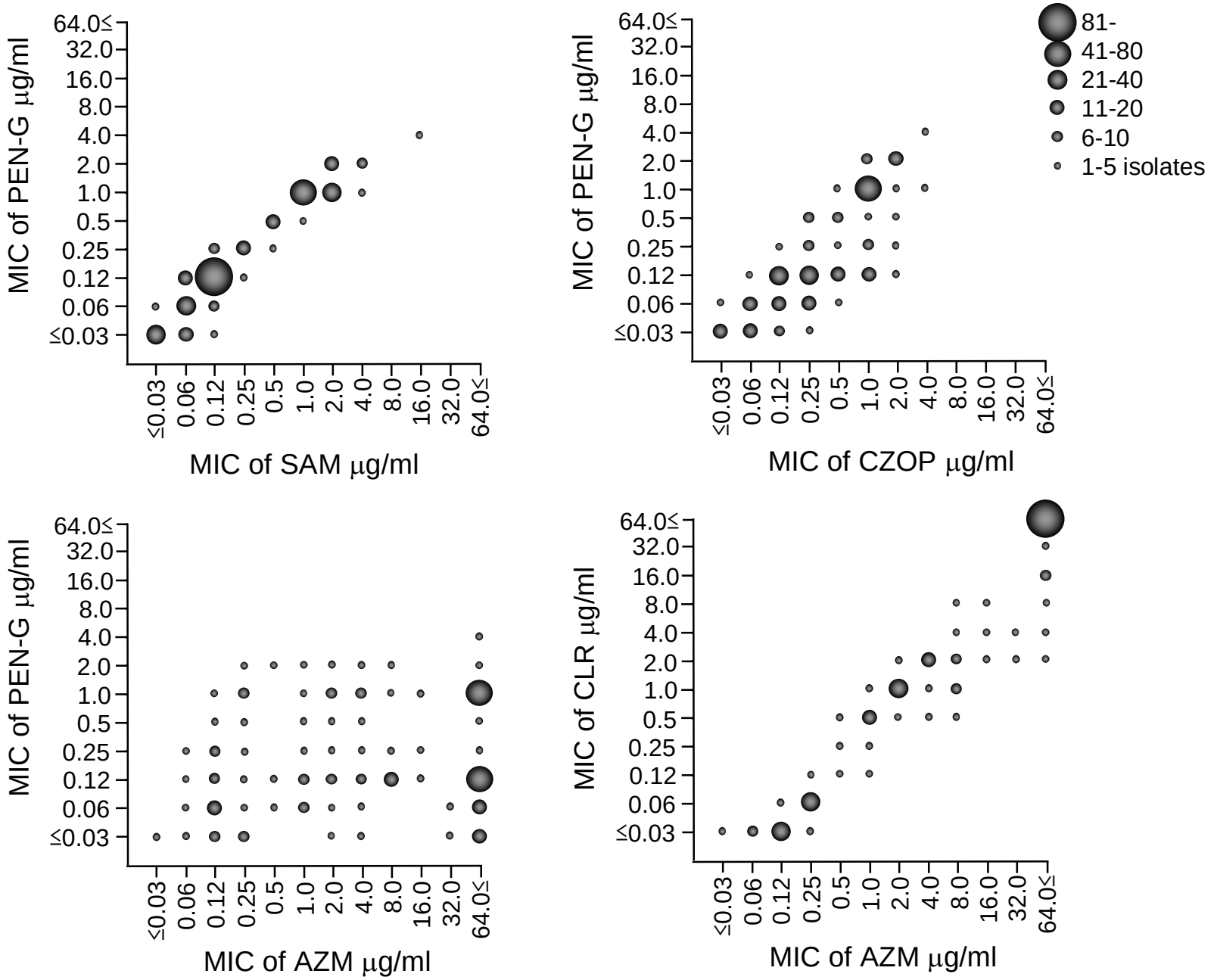

Figure 1. Y. Ohsaki et al. Drug resistance of *S. pneumoniae*.