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Recovery of susceptibility to penicillin G in clinical isolates of *Streptococcus pneumoniae* despite increased accumulation of pbp gene alterations.

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May 4, 2008

Dear Sir:

Thank you for helpful suggestions and comments from reviewers. We agree to reform our manuscript in accordance with these comments. We decided to submit our manuscript as a short communication.

1. We agree that sequencing of the *pbp* genes will provide important information about relationship between recovery of Pen-G susceptibility and cephalosporin consumption. However, we have to do DNA sequencing of 328 plus 200 isolates to answer this question. We decided to resubmit our work without DNA sequencing data because we prefer prompt publish of the present data. We added in page 15, line 3 to read as "However, more precise analysis of *pbp* mutations may clarify the relationship between recovery of PEN-G susceptibility and reduced cephalosporin consumption."

2. We agree that antimicrobial consumption in our hospital does not have a great impact on the discussion, therefore we removed figure 3 about these data from the manuscript.

3. We shortened our manuscript to meet the regulation of the journal. Main body of our manuscript including abstract contains 2499 words, two tables and one figure. The attached file of the figure contains upper panel and lower panel. We do not mind if you put these panels side-by-side. We apologize that we could not reduce references to less than 18 although we tried to reduce them as little as possible.

We hope these changes are acceptable. Thank you.

Sincerely yours,

Yoshinobu Ohsaki, M.D.

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January 25, 2008

Dear Sir:

We believe this manuscript will be of interest to the readers of Int J Antimicrob AG for the following reasons:

1. This manuscript describes recovery of susceptibility to Penicillin G in clinical isolates of *Streptococcus pneumoniae* despite increased accumulation of *pbp* gene alterations.
2. *pbp* Gene Alterations was measured by a PCR based method.
3. We compared our results with our previous study which was published in the Int J Antimicrob AG.
4. On the other hand, the susceptibility to macrolides decreased.
5. We discussed the reason for the recovery of the susceptibility to Penicillin G might relate decreased consumption of oral cephalosporins.

All the authors have had the opportunity to review the manuscript and agree with the content. All related correspondence regarding this manuscript should be sent to Yoshinobu Ohsaki, M.D. at address in the letterhead.

This paper is not under consideration by any other journal and it has not been accepted for publication elsewhere.

Thank you in advance for your consideration of this manuscript and we hope for a favorable review.

Sincerely yours,

Yoshinobu Ohsaki, M.D.

Competing Interest Declarations

Int J Antimicrob AG

Yoshibobu Ohsaki

Declarations

The following additional information is required for submission. Please note that failure to respond to these questions/statements will mean your submission will be returned to you. If you have nothing to declare in any of these categories then this should be stated.

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There is no conflict of interests.

Please state any sources of funding for your research

There is no source of funding which needs to be stated

Please state whether Ethical Approval was given, by whom and the relevant Judgement's reference number

N/A

If you are submitting a Randomized Controlled Trial, please state the International Standard Randomised Controlled Trial Number (ISRCTN)

N/A

Recovery of Susceptibility to Penicillin G in Clinical Isolates of *Streptococcus pneumoniae* despite Increased Accumulation of *pbp* Gene Alterations

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Running title: Recovery of penicillin-susceptibility in *S. pneumoniae*.

Key words: *Streptococcus pneumoniae*, penicillin resistance, macrolide resistance, penicillin-binding protein, susceptibility.

Recovery of penicillin-susceptibility in S. pneumoniae

Abstract

Two hundred consecutive clinical isolates of *S. pneumoniae* in 2005 and 2006 were analyzed for susceptibility to antimicrobials, *pbp* alterations and macrolide resistant gene expressions (2007 analysis). The results were compared with our previous data (2003 analysis). The average minimum inhibitory concentration (MIC) of penicillin G (PEN-G) in isolates with *1a^m/2x^m/2b^m* decreased from 1.135 ± 0.503 mg/L in the 2003 analysis to 0.872 ± 0.540 mg/L in the 2007 analysis ($p=0.0046$). Isolates with *1a^m/2x^m/2b^m* increased from 30.5% to 32.3% but the difference was not statistically significant ($p=0.6979$). Isolates with a MIC of clarithromycin ≥ 1.0 mg/L increased from 65.9% to 80.0% ($p=0.0005$). Isolates with *ermB* expression increased from 46.6% to 62.6% ($p=0.0004$). We concluded that the decrease of penicillin resistance of *S. pneumoniae* did not correlate with a decrease in *pbp* mutations; on the contrary, isolates with *pbp* mutations increased. A decrease of penicillin resistance in *S. pneumoniae* with the *pbp* mutation seemed to explain our present results about the recovery of penicillin susceptibility. Our results suggested that the spread of mutated *pbp* genes among *S. pneumoniae* itself is not responsible for the acquisition of the penicillin-resistant phenotype. Use of β -lactams, especially oral cephalosporins, seems to be responsible for the acquisition of penicillin resistance.

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Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is the sixth most frequently isolated organism in human patients. It is a major cause of pneumonia, meningitis, bacteremia, otitis media, and sinusitis in humans. *S. pneumoniae* is part of the normal flora of the upper respiratory tract and is frequently carried in the oropharynx of small children. For more than 20 years, *S. pneumoniae* isolates were reliably susceptible to penicillin G (PEN-G). *S. pneumoniae* with intermediate penicillin resistance appeared in the 1970s, but they were rare. In the 1980s, highly penicillin-resistant strains began to be reported worldwide, particularly in Asian countries.

Resistant *S. pneumoniae* have penicillin-binding proteins (PBPs) of altered size and decreased affinity for penicillin (1). Resistance of *S. pneumoniae* to β -lactams becomes established via stepwise alterations in the high-molecular-weight PBPs and reduction of the binding affinity of β -lactams to the PBPs (2). Among these PBPs, the resistance of *S. pneumoniae* to penicillin and cefotaxime has been shown to be associated with mosaic mutations in the *pbp1a* (2, 3), *pbp2b* (2-4) and *pbp2x* (5, 6) genes. *S. pneumoniae* acquires exogenous low-affinity genes and causes genetic mutations that alter the PBP affinity for β -lactams (7).

We previously analyzed the *pbp* mutations in 328 clinical isolates of *S. pneumoniae* by PCR (8) and found that 84.4% of isolates with wild-type *pbp1a*, *pbp2b*, and *pbp2x* were susceptible to penicillin G, and the rest of the isolates had intermediate resistance to PEN-G. In

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contrast, all isolates that have mutations in all *pbp1a*, *pbp2b* and *pbp2x* genes had intermediate resistance or resistance to PEN-G.

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. In *S. pneumoniae*, macrolide resistance develops either by modification of the drug-binding site or by active efflux of the drug. The target modification is usually the result of dimethylation of the adenine residue at position 2058 on the 23S rRNA by a methylase enzyme such as the *ermB* gene (9). The *mefA* gene encodes a protein that has a role in the active efflux of the macrolides (10).

Antimicrobial resistant *S. pneumoniae* were isolated not only from patients with infectious disease but also from previously untreated healthy subjects. Therefore, periodic surveillance of susceptibility of clinical isolates of *S. pneumoniae* seems to be important to predict the effect of antimicrobials because *S. pneumoniae* potentially acquires mutated PBP proteins from resistant strains in the environment by intraspecific lateral gene transfer (11).

In the present study, we measured antimicrobial susceptibility, *pbp1a*, *pbp2b* and *pbp2x* alterations, and *mefA* and *ermB* expression in clinical isolates of *S. pneumoniae*. We compared these results with our previous results.

Materials and Methods

Bacteria isolation

Three-hundred twenty-eight clinical isolates of *S. pneumoniae* obtained between 1998 and 2001 were analyzed for susceptibility to antimicrobials, *pbp* alterations, and macrolide-resistance gene expressions (2003 analysis). These data were previously published elsewhere (8).

Two-hundred consecutive clinical isolates of *S. pneumoniae* collected at the Asahikawa Medical College Hospital in 2005 and 2006 were isolated and newly analyzed for susceptibility to antimicrobials, *pbp* alterations, and macrolide-resistance gene expressions using the same methods as previously reported, except that DNA extraction was skipped in the 2007 analysis. These clinical isolates were stored at -80°C. Each isolates was from a different patient. These samples included 30 sputum, 133 nasopharyngeal scrubbing, 6 ear discharge, and 31 other specimens. The patients included 143 children and 66 adults.

Susceptibility testing

The minimum inhibitory concentrations (MICs) of antibiotics for the 200 clinical isolates were determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Briefly, testing was performed in microtiter 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 a

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MacFarland 0.5 standard and further diluted in quarter-strength Ringer's solution to a final concentration of approximately 5×10^5 cfu/ml. Antibiotics were serially diluted in two-fold dilutions in microtiter 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. A standard quality control strain, *Staphylococcus aureus* ATCC 29213, was used for the susceptibility testing.

Susceptibility to penicillin G (PEN-G) was classified according to the CLSI recommendation, i.e. *S. pneumoniae* isolates for which the MIC of PEN-G was ≤ 0.06 µg/ml were classified as Pen-G^S (sensitive), those for which the MIC of PEN-G was 0.1-1.0 µg/ml as Pen-G^{Ir} (intermediate resistant) and those for which the MIC of PEN-G was ≥ 2.0 µg/ml as Pen-G^R (resistant).

The MICs of PEN-G, cefditoren pivoxil (CDTR), ceftazidime (CAZ), biapenem (BIPM), meropenem (MEPM), levofloxacin (LVFX), clarithromycin (CAM), erythromycin (EM), and telithromycin (TEL) were determined. The activity of all antibiotics was confirmed using ATCC 29213.

PCR condition

The sequences of primers were as described elsewhere (8, 12). The oligonucleotide primers used to detect three *pbp* genes were designed to amplify parts of the *pbp1a*, *pbp2x*, and

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pbp2b genes only in susceptible strains (Wakunaga Pharmaceutical, Hiroshima, Japan). These parts were positioned in blocks of highly divergent 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. Primer mixture C contained the primers to detect *mefA* and *ermB*.

PCR was done in 50 µl reaction buffer containing 1 µM of each primer, 10 µl 10X PCR buffer, 200 µM dNTP, 1U Tth DNA polymerase (Toyobo, Osaka, Japan), and pure water.

Isolates were added directly to the PCR solution. PCR conditions were, 94°C for 20 s, 57°C for 20 s, and 72°C for 15 s, for a total of 30 cycles using a thermal cycler (TP 400, Takara, Tokyo).

Following amplification, 10 µl each of the three PCR products was fractionated by 3 % agarose gel electrophoresis. The results were interpreted as follows; when all three bands of *pbp* genes were seen, the isolate was considered to have the 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 $1a^m/2x^m/2b^m$).

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. In the comparison of MIC, a *t* test was used.

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All analyses were performed using the StatView Program (Abacus Concept, Inc., Berkeley, CA).

Results

MICs of PEN-G, CDTR, CAZ, BIPM, MEPM, LVFX, CAM, EM and TEL

The MIC of PEN-G was ≤ 0.06 mg/L for 117/200 isolates of *S. pneumoniae*, 0.12 mg/L for 5 isolates, 0.25 mg/L for 16 isolates, 0.5 mg/L for 17 isolates, 1.0 mg/L for 40 isolates, 2.0 mg/L for 2 isolates and 4.0 mg/L for one isolate (Figure 1). According to the CLSI criteria, 117/200 isolates (58.5%) were classified as Pen-G^S, 78 isolates (39.0%) as Pen-G^{Ir}, and 3 isolates (1.5%) as Pen-G^F. The MIC of TEL was ≤ 0.03 mg/L for 52/200 isolates, 0.06 mg/L for 97 isolates, 0.12 mg/L for 40 isolates, 0.25 mg/L for 10 isolates and 1.0 mg/L for 1 isolate.

Correlation between Pen-G^F and pbp gene alterations

The PCR analysis was not successful in two clinical isolates of *S. pneumoniae*. The PCR product for *pbp1a* was detected in 120/198 isolates ($1a^W$), *pbp2x* in 19 isolates ($2x^W$) and *pbp2b* in 113 isolates ($2b^W$). None of these three genes was detected in 64/198 isolates ($1a^m/2x^m/2b^m$), and all of them were detected in 20 isolates ($1a^W/2x^W/2b^W$). The 3/3 Pen-G^F 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 mg/L for all 20 isolates. The MICs of PEN-G for the isolates with one or two *pbp* mutations were ≥ 0.06 mg/L for 96 isolates and 0.1-1.0 mg/L for 19 isolates. The average MIC for isolates with $1a^m/2x^m/2b^m$ was 0.872 ± 0.540 mg/L.

Correlation between susceptibility to macrolides and resistant gene expressions

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The *mefA* gene was positive in 56/198 isolates of *S. pneumoniae*, and the *ermB* gene was positive in 124 isolates (Table 2). The MIC of clarithromycin was ≥ 1.0 mg/L for 160/200 isolates (80.0%), and MIC of erythromycin was ≥ 1.0 mg/L for 162/200 isolates (81.0%). The MIC of telithromycin was ≥ 0.25 mg/L for 10 isolates and ≥ 1.0 mg/L for one isolate. Of the former 10 isolates, seven isolates were positive for both *ermB* and *mefA*, two were positive for only *ermB*, and one isolate was positive for only *mefA*. In the latter single isolate, only *ermB* was positive.

Comparison between 2003 analysis and 2007 analysis

Susceptibility to PEN-G

The average MIC of PEN-G in isolates with $1a^m/2x^m/2b^m$ significantly decreased from 1.135 ± 0.503 mg/L in the 2003 analysis to 0.872 ± 0.540 mg/L in the 2007 analysis ($p=0.0046$).

Isolates with one or two *pbp* mutations and Pen-G^S increased from 9.5% in 2003 analysis to 48.5% in the 2007 analysis (Table 1). In contrast, those with one or two *pbp* mutations and Pen-G^{I^r} decreased from 38.9% in the 2003 analysis to 9.1% in the 2007 analysis. Isolates with $1a^m/2x^m/2b^m$ and Pen-G^{I^r} decreased from 5.5% in the 2003 analysis to 1.5% in the 2007 analysis.

Isolates with $1a^w/2x^w/2b^w$ significantly decreased from 64/328 (19.5%) in the 2003 analysis to 20/198 (10.1%) in the 2007 analysis ($p=0.0046$). In contrast, those with one or two mutations increased from 164/328 (50.0%) to 114/198 (57.6%), although the difference was not

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statistically significant ($p=0.1047$), and those with $1a^m/2x^m/2b^m$ increased from 100/328 (30.5%) in the 2003 analysis to 64/198 (32.3%) in the 2007 analysis, and again the difference was not statistically significant ($p=0.6979$).

Susceptibility to CAM

Isolates with MIC of CAM ≥ 1.0 mg/L increased from 216/328 (65.9%) in the 2003 analysis to 160/200 (80.0%) in the 2007 analysis, and the difference between them was statistically significant ($p=0.0005$). Isolates with *ermB* expression significantly increased from 153/328 (46.6%) in the 2003 analysis to 124/198 (62.6%) in the 2007 analysis ($p=0.0004$). Those with *mefA* expression were 75/328 (22.9%) in the 2003 analysis and 56/198 (38.3%) in the 2007 analysis, and the difference was not statistically significant ($p=0.1768$, Table 2).

Discussion

In the present study, we found that the penicillin resistance of clinical isolates of *S. pneumoniae* decreased in the 2007 analysis in comparison to that in the 2003 analysis. Valles *et al.* (13) prospectively studied 125 cases of community acquired pneumococcal pneumonia in Spain. They found a decrease in penicillin and cephalosporin resistance, while macrolide resistance remained unchanged. Livemore *et al.* (14) observed a similar trend in the UK and the Republic of Ireland. They found that penicillin resistance had fallen significantly since 1999, whereas macrolide resistance remained unchanged. UK pharmacy sales of macrolides and oral β -lactams fell by 30% in the late 1990s. They concluded that the falling penicillin resistance in pneumococci followed reduced sales of oral β -lactams at pharmacies in the UK, but a similar fall in macrolide sales was not associated with any fall in resistance. In contrast, Hogberg *et al.* (15) analyzed antimicrobial resistance patterns in Sweden between 1997 and 2003 and reported a increase in penicillin-resistant pneumococci despite a stable prevalence and decreased antibiotic use. In our study, penicillin-resistant pneumococci significantly decreased in the 2007 analysis comparing to that in the 2003 analysis, but macrolide-resistant pneumococci significantly increased during this period of time.

We analyzed trends of *pbp* alterations and macrolide-resistant gene expressions between the 2003 and 2007 analysis. In the 2003 analysis, wild-type *pbp1a* was found in 56.4% of

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isolates of *S. pneumoniae*, wild-type *pbp2x* in 20.1%, and wild-type *pbp2b* in 64.9%. In the 2007 analysis, wild-type *pbp1a* was found in 60.6%, wild-type *pbp2x* in 9.6%, and wild-type *pbp2b* in 57.0%. The difference was statistically significant only for *pbp2x* ($p=0.0014$). Fewer isolates with $1a^w/2x^w/2b^w$ were found in the 2007 analysis than in the 2003 analysis. These results suggested that isolates of *S. pneumoniae* with the *pbp* mutation increased during this period of time, although the penicillin resistance of the isolates decreased. From these results, we concluded that the decrease in penicillin resistance of *S. pneumoniae* in the present study did not correlate with the decrease of the *pbp* mutations. The decrease of penicillin resistance in *S. pneumoniae* with a *pbp* mutation, especially in those with $1a^m/2x^m/2b^m$, seemed to explain our present results about the recovery of penicillin susceptibility.

S. pneumoniae contains six PBPs: the high molecular weight PBP1A, PBP1B, PBP2A, PBP2B, and PBP2X, and the low molecular weight PBP3. Alterations in PBP2B and PBP2X are the primary determinants of resistance conferring low-level resistance on sensitive strains, whereas alterations in PBP1A result in an increase in resistance (16, 17). Important alterations in *pbps* appear to have emerged through recombination events involving *S. pneumoniae pbp* genes and their homologous genes in human commensal streptococcal species (7, 18). Stanhope *et al.* (11) calculated the relative frequency of intraspecific lateral gene transfer of penicillin-binding proteins. Our results suggested that the spread of mutated *pbp* gene among *S. pneumoniae* itself

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is not responsible for the acquisition of a penicillin-resistant phenotype. Use of β -lactams, especially oral cephalosporins, seemed to be responsible for the acquisition of penicillin resistance because consumption of only oral cephalosporins decreased from 1999 in our hospital. However, more precise analysis of *pbp* mutations may clarify the relationship between recovery of PEN-G susceptibility and reduced cephalosporin consumption.

CAM-resistant clinical isolates of *S. pneumoniae* significantly increased in the 2007 analysis compared to the 2003 analysis. *ermB* expression significantly increased in the 2007 analysis compared to the 2003 analysis. Accumulation of these macrolide-resistant genes seemed to be responsible for the increase in macrolide-resistant *S. pneumoniae*.

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Table 1. Distribution of isolates by *pbp* genotype and susceptibility to PEN-G, and their comparison between 2003 and 2007 in percentage.

<i>pbp</i> genotype	Pen-G ^S	Pen-G ^{ir}	Pen-G ^r	Total
<i>1a^w/2x^w/2b^w</i>	20 (16.5/10.1)	0 (3.0/0.0)	0 (0.0/0.0)	20 (19.5/10.1)
One/two mutations	96 (9.5/48.5)	18 (39.9/9.1)	0 (0.6/0.0)	114 (50.0/57.6)
<i>1a^m/2x^m/2b^m</i>	1 (0.0/0.5)	60 (25.0/30.3)	3 (5.5/1.5)	64 (30.5/32.3)
Total	117 (26.0/59.1)	78 (67.9/39.4)	3 (6.1/1.5)	198 (100/100)

(2003% / 2007%)

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Table 2. Distribution of isolates according to macrolide-resistant gene expression, and their comparison between 2003 and 2007 in percentage.

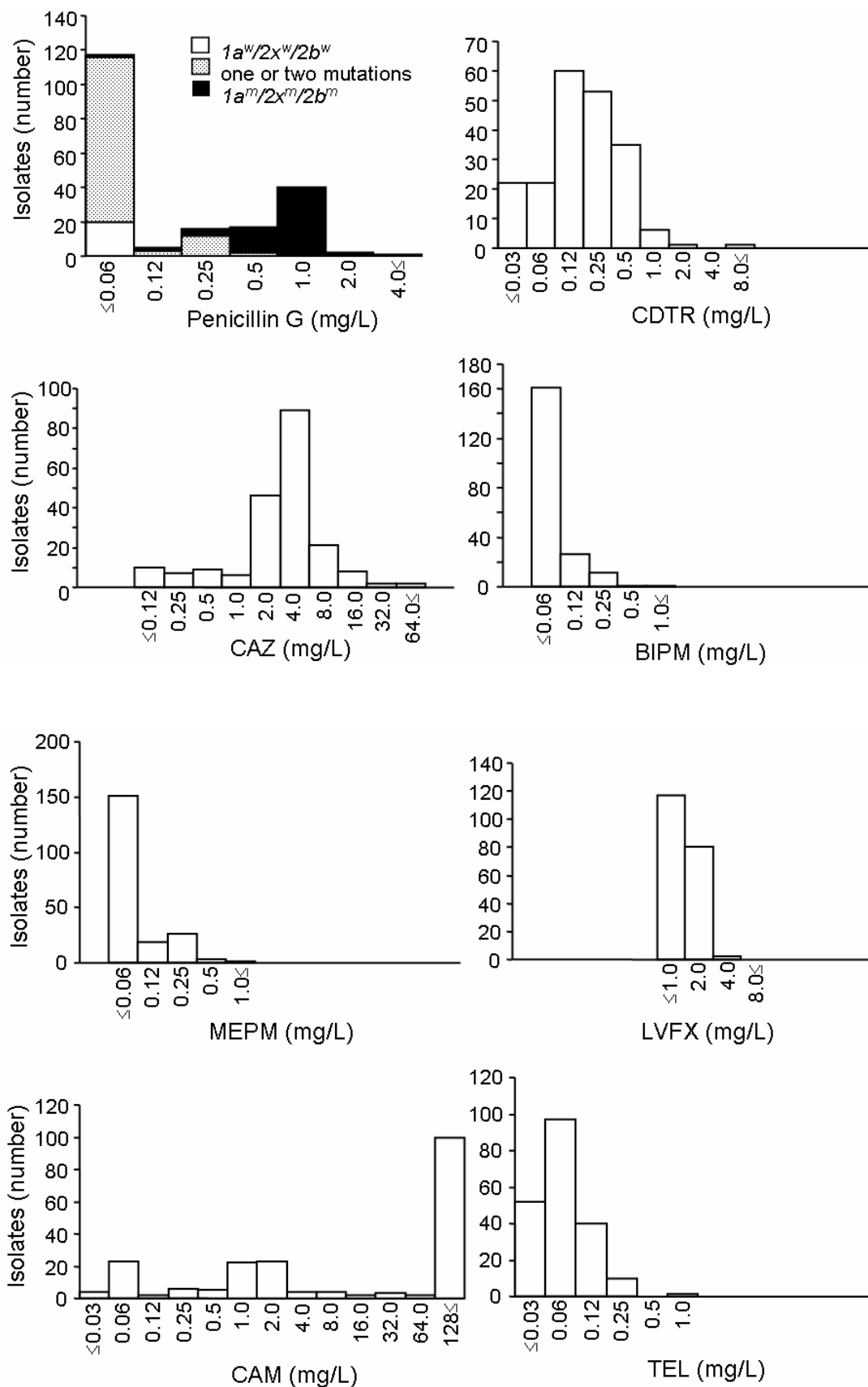
	<i>ermB</i> +	<i>ermB</i> -	Total
<i>mefA</i> +	7 (3.0/3.5)	49 (19.9/24.8)	56 (22.9/28.3)
<i>mefA</i> -	117 (43.6/59.1)	25 (33.5/12.6)	142 (77.1/71.7)
Total	124 (46.6/62.6)	74 (53.4/37.4)	198 (100/100)

(2003% / 2007%)

Figure legends

Figure 1. Distribution of MIC of various antimicrobials in clinical isolates of *S. pneumoniae*.

Figure 1. Y. Ohsaki et al.



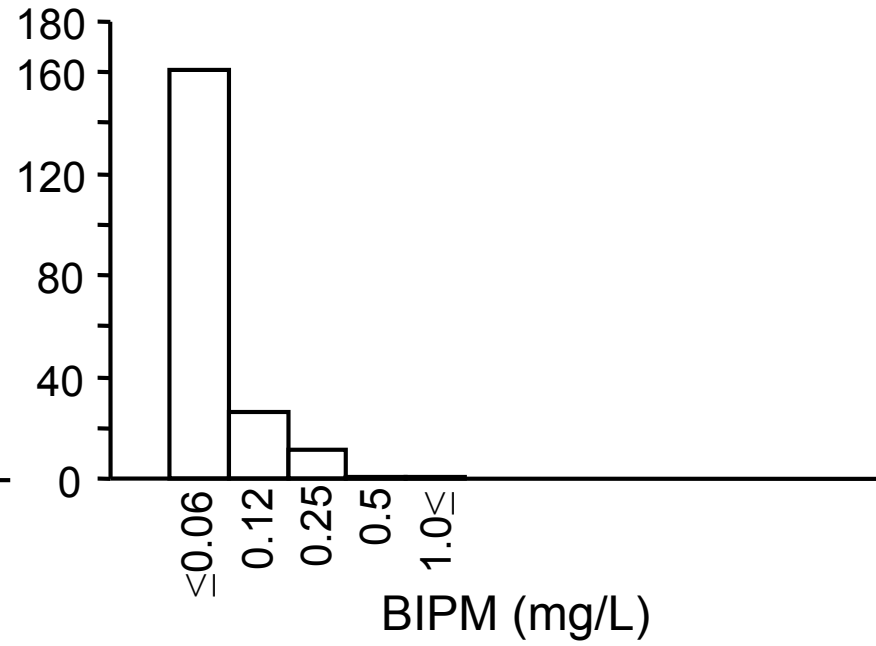
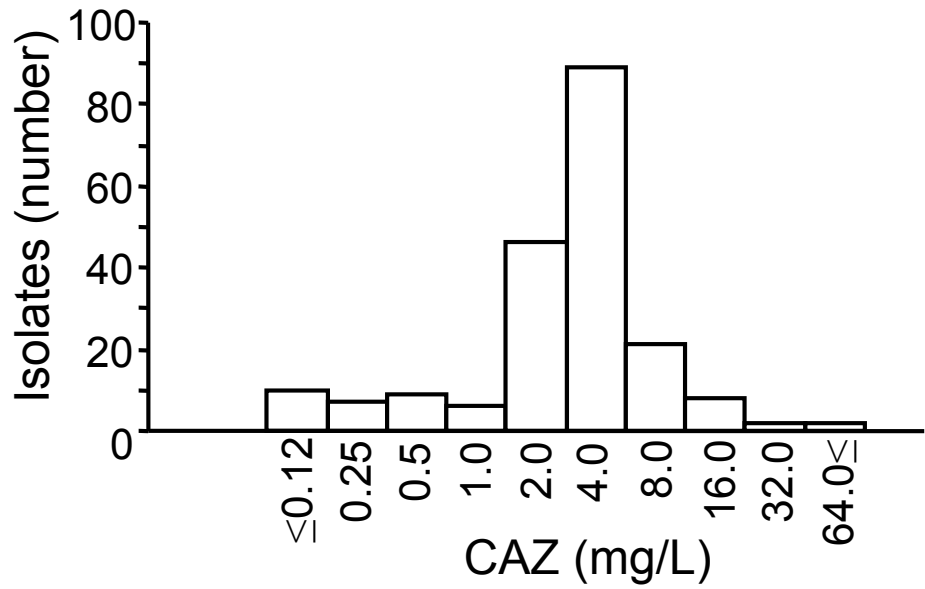
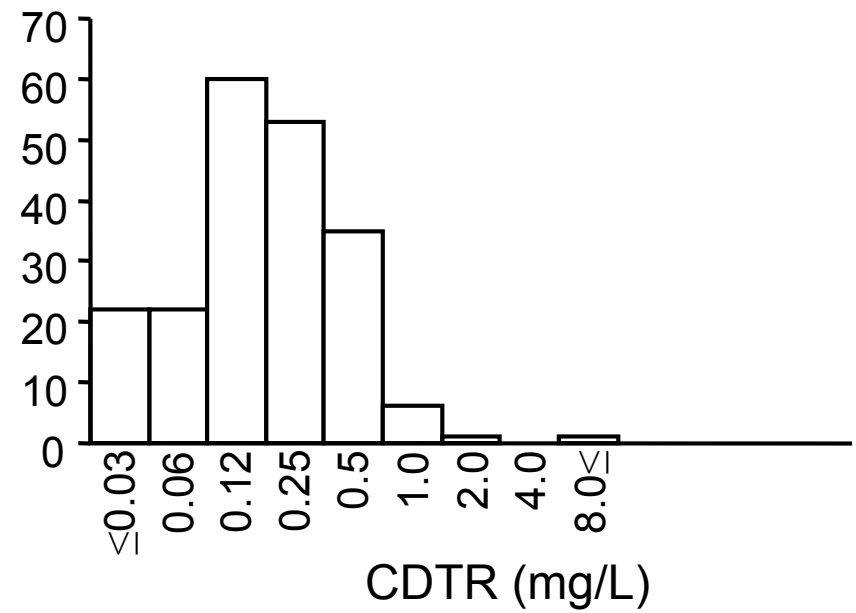
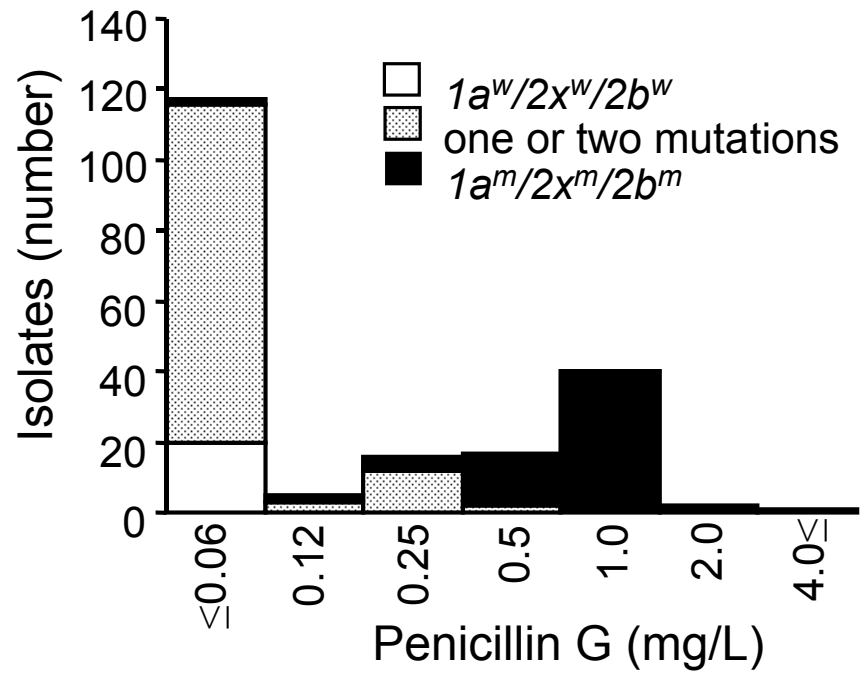
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