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Original Article

The Emergence of Drug-Resistant *Streptococcus pneumoniae* and Host Risk Factors for Carriage of Drug-Resistant Genes in Northeastern Japan

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SUMMARY: Our 2-year study includes research into the occurrence, molecular characteristics, and host risk factors for the carriage of drug-resistant strains of *Streptococcus pneumoniae* as a continuation of our previous report. From September 2001 to June 2003, strains of *S. pneumoniae* were isolated from the nasopharynx of children with respiratory tract infection in Soma General Hospital. Of the total of 949 strains, 761 (81%) had a decreased susceptibility to penicillin (MIC > 0.12 μg/ml), while 818 (86%) were resistant to erythromycin (MIC > 1 μg/ml) and 789 (83%) were resistant to clarithromycin (MIC > 1 μg/ml). More than half of the strains had decreased susceptibility to meropenem. Gene analysis of 226 randomly selected strains showed that 200 strains (88.5%) had one or more altered *pbp* genes and 191 strains (84.5%) had *mef*(*A*) and/or *erm*(*B*) genes. We reviewed the patient backgrounds for previous antibiotic use, age, daycare attendance, and siblings. Previous use of oral beta-lactams has shown a strong relationship with the carriage of altered *pbp* genes (*P* value < 0.01), and previous oral macrolide use has been related to the carriage of macrolide-resistant genes (*P* value < 0.01). The controlled use of antibiotics might be an important factor in preventing the emergence of *S. pneumoniae* with antibiotic-resistant genes.

INTRODUCTION

The high occurrence of penicillin-resistant *Streptococcus pneumoniae* strains is a global issue. Resistant strains are appearing especially in Asia. Recent reports indicate that the rates of penicillin-resistant *S. pneumoniae* were 54.8% in Korea, 43.2% in Hong Kong, 38.6% in Taiwan, and 71.4% in Vietnam. Also, *S. pneumoniae* strains that are resistant to macrolides have become a considerable concern in Asia. Erythromicyn resistance occurred at rates of 92.1% in Vietnam, 86% in Taiwan, 80.6% in Korea, and 76.8% in Hong Kong (1). In Japan, penicillin-resistant strains of *S. pneumoniae* were first isolated in the late 1980s and have rapidly spread throughout the country (2). The occurrence rates of resistant strains of *S. pneumoniae* differ in Japan depending on the period and region, and range from 50 to 85% (3,4). A recent report also mentioned that the rate of macrolide-resistant strains in Japan has increased and that the occurrence of erythromycin resistance had reached 84.8% (5).

Since 2001, we began a study of the emergence of the drug-resistant *S. pneumoniae* in Soma city, located in the northeastern Japan. As we presented in our previous report, of 949 *S. pneumoniae* strains collected from the nasopharynx of children, 30% of strains were penicillin intermediately resistant *S. pneumoniae* (PISP), and 50% of strains were penicillin-resistant *S. pneumoniae* (PRSP). Our investigation of patient profiles revealed that at ages less than 3 years, attendance at a daycare center and previous antibiotic use were risk factors for carriage of penicillin-resistant strains. We also investigated the molecular mechanisms of those drug-resistant strains by polymerase chain reaction (PCR) and reported that all PISP and PRSP – and, surprisingly, 55% of penicillin-susceptible strains (PSSP) – had altered *pbp* genes (6).

In this report, as a continuation of our previous report, we discuss resistance against other antibiotics, especially macrolides, in addition to penicillin. We also report on host risk factors for carriage of *S. pneumoniae* with resistant genes.

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MATERIALS AND METHODS

S. pneumoniae: Nasopharyngeal swabs were collected from the nasopharynx of children who visited our hospital with respiratory tract infection from September 2001 to June 2003. In all cases, prior informed consent was obtained from their parents. The samples were plated on 5% sheep blood agar and then incubated for 24 h. The settlement with alpha-hemolysis was further incubated for another 24 h, and identified as *S. pneumoniae* by the Optochin test (Optochin Showa Disc; Nissui Pharmaceutical, Tokyo, Japan) and a latex coagulating reaction test (Slidex Pneumo-Kit; Biomeriux, Tokyo, Japan) was done on those samples. A total of 949 strains of *S. pneumoniae* were isolated, as we already reported.

Susceptibility testing: Antimicrobial susceptibility to seven antibiotics, such as penicillin, cefotaxim, cefditren, erythromycun, clarithromycin, clindamycin, and meropenem, was examined by a broth micro-dilution test (MICroFAST Panel Type3J-T MicroScan; Dade Behring, Tokyo, Japan) in accordance with the National Committee for Clinical Laboratory Standards (NCCLS). *S. pneumoniae* was classified on the basis of penicillin susceptibility: PSSP, <0.06 μg/ml; PISP, 0.12 - 1.0 μg/ml; and PRSP, >2.0 μg/ml.

PCR: Of the total of 949 *S. pneumoniae* strains that were isolates, 226 were chosen at random to detect antibiotic-

resistant strains by PCR. We used a commercially available primer mixture (Wakunaga Pharmaceutical, Hiroshima, Japan) presented in a previous study (7). It included oligonucleotide primers to detect the following genes: an autolysin gene named *lytA* to screen *S. pneumoniae*, three unaltered *pbp* genes named *pbp1a*, *pbp2x*, and *pbp2b*, and two macrolide-resistant genes, *mef*(*A*) and *erm*(*B*). A false positive might occur when a strain had altered sequences in the area where the primers detecting *pbp1a* anneal (7).

Patient backgrounds: Patient backgrounds, including previous use of antibiotics, age, daycare attendance, and siblings, were taken into account by a questionnaire and/or by referring to medical records. The antibiotics used in this study were as follows: oral beta-lactams: amoxicillin, cefaclor, cefditoren, cefdinir, and cefpodoxime; intravenous beta-lactams: piperacillin, cefotaxim, flomoxef, sulbactam/cefoperazon, and panipenem/betamipron; oral macrolides: clarithromycin and midekamycin; intravenous non-beta-lactams: clindamycin and fosfomycin.

Statistics: The χ^2 test was performed to compare the backgrounds of children with a resistant strain and those of children with a susceptible strain. If the *P* value was less than 0.05, the result was considered statistically significant. Antibiotic-resistant strains were determined on the basis of minimum inhibitory concentration (MIC) or gene analysis.

RESULTS

Characteristics of *S. pneumoniae*: Table 1 shows the MIC range of all 949 isolates of *S. pneumoniae* against the seven antimicrobial agents. As we presented in the previous report (6), 288 strains (30%) were PISP and 481 strains (50%) were PRSP. Twenty-two strains (2%) had intermediary resistance to erythromycin (0.5 μg/ml), and 818 (86%) had full resistance (>1 μg/ml). Correspondingly, 85 strains (9%) had intermediary resistance to clarithromycin (0.5 μg/ml) and 789 strains (83%) had full resistance to it (>1 μg/ml). Also, 544 strains (57%) had a decreased susceptibility to meropenem (>0.5 μg/ml). Cephalosporins showed moderately high levels of activity against the strains.

As we reported previously, out of the total of 226 strains tested, 98 PRSP strains had 2 or 3 altered *pbp* genes, and 69 PISP strains had at least 1 altered *pbp* gene. Of the remaining 59 PSSP, 33 strains (55.9%) had at least 1 altered *pbp* gene (6). The relationship between susceptibility to clarithromycin and the macrolide-resistant genes is exhibited in Fig. 1. Of the total of 226 strains chosen for PCR, 34 strains (15.0%) were susceptible, 21 strains (9.3%) had intermediary resistance, and 171 strains (75.7%) were resistant to clarithromycin. Gene analysis showed that 35 strains (15.9%) had no resistant genes, 91 strains (40.0%) had the *erm*(*B*) gene, 73 strains (32.5%) had the *mef*(*A*) gene, and 27 strains (11.9%) had both *erm*(*B*) and *mef*(*A*) genes. Strains having the *mef*(*A*) gene showed intermediate resistance to clarithromycin, and strains having the *erm*(*B*) gene were highly resistant to it.

Risk factors for nasopharyngeal carriage of resistant genes: The relationship between carriage of the strains with one or more *pbp* genes and each risk factor is shown in Table 2. The younger patients (2 years of age or under) that used antibiotics in the previous 3 months had a substantial relationship with the carriage of resistant strains ($P < 0.01$). Children who had attended a daycare center showed a high tendency to carry resistant strains ($P = 0.06$).

Table 3 shows the risk factors for the carriage of strains with macrolide-resistant genes. As with *pbp* genes, carriage of the strains with *mef*(*A*) and/or *erm*(*B*) genes is strongly related to younger children and to the use of antibiotics in children within 3 months ($P \leq 0.01$). Whether or not a child had one or more siblings was also related to macrolide resistance ($P \leq 0.01$).

The relationships between previous antibiotic use and resistant genes are demonstrated in detail in Figs. 2 and 3. Figure 2 shows that previous antibiotic use was significantly correlated with strains with altered *pbp* genes. Especially, strains with a point of *pbp* mutation were significantly related to the use of oral beta-lactams ($P < 0.01$). The relationships became stronger in strains with more mutations. Figure 3 shows that previous antibiotic use was related to strains with *mef*(*A*) and/or *erm*(*B*). As expected, previous macrolide use was significantly related to strains with *mef*(*A*) and/or

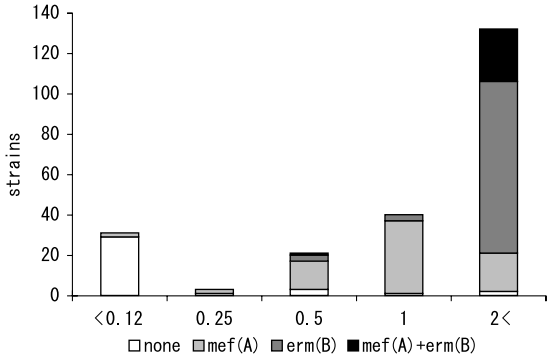

Fig. 1. Relationship between susceptibility MIC to clarithromycin and macrolide-resistant genes in *S. pneumoniae*. 34 strains (15.0%) were susceptible, 21 strains (9.3%) were intermediate resistant, and 171 strains (75.7%) were resistant to clarithromycin. Gene analysis showed that 35 strains (15.9%) had no resistant genes, 91 strains (40.0%) had *erm*(*B*) gene, 73 strains (32.5%) had *mef*(*A*) gene, and 27 strains (11.9%) had both *erm*(*B*) and *mef*(*A*) genes. Strains having *mef*(*A*) gene were ranged from MIC 0.5-2 μg/ml, strains having *erm*(*B*) gene were mostly concentrated in MIC < 2 μg/ml group.

Table 1. MICs of 7 antimicrobiotics tested against 949 isolates of *S. pneumoniae*

n = 949

Antibiotic	No. of isolates inhibited by MIC (μg/ml)										
	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
penicillin	49	131	131	56	101	160	224	97			
cefotaxim		88	80	150	288	285	58				
cefditren		111	135	170	288	245					
erythromycin			94	15	22	44	774				
clarithromycin			65	12	83	173	49	567			
clindamycin			124	20	18	479	308				
meropenem			218	187	281	32	224	7			

Table 2 . Risk factors for carriage of *S. pneumoniae* with altered *pbp* genes

n = 226

Risk factor	No (%) of patients carrying *S. pneumoniae* with altered *pbp* genes	*P*
2 years of age or under	146 (64)	<0.01
Sibling	99 (44)	0.11
Day care center exposure	61 (27)	0.06
Use of antibiotics within the last 3 months	125 (55)	<0.01

Table 3. Risk factors for carriage of macrolides resistant *S. pneumoniae* determined by gene analysis

n = 226

Risk factor	No. (%) of patients carrying *S. pneumoniae* with *mef*(*A*) and/or *erm*(*B*)	*P*
2 years of age or under	143 (63)	0.01
Sibling	133 (59)	<0.01
Day care center exposure	95 (42)	0.51
Use of antibiotics within the last 3 months	112 (50)	<0.01

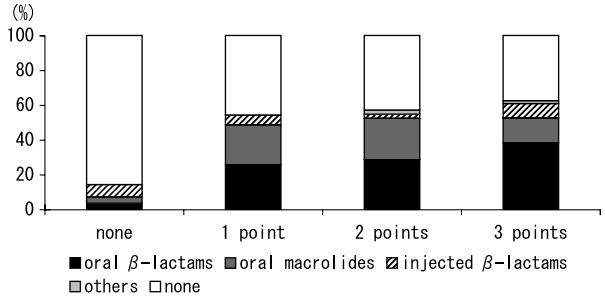

Fig. 2. Relationship between the use of antibiotics within the last 3 months and altered *pbp* genes. This figure shows that previous antibiotic was significantly correlated with strains with altered *pbp* genes ($P < 0.01$). Especially, the strains with 1 point of *pbp* mutation were significantly related to oral beta-lactam use ($P < 0.01$). These relationships were stronger in the strains with more than 2 points of mutation.

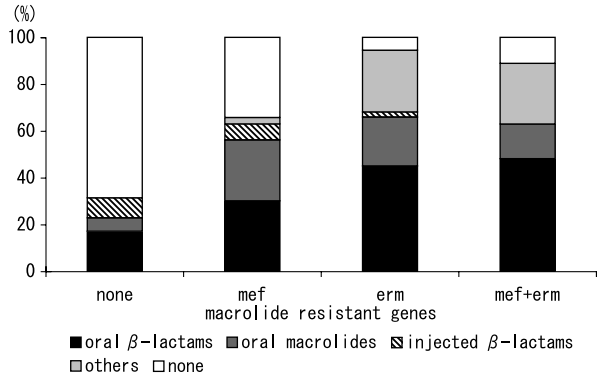

Fig. 3. Relationship between the use of antibiotics within the last 3 months and macrolide-resistant genes. Previous antibiotic use related to strains with *mef*(*A*) and/or *erm*(*B*) ($P < 0.01$). As expected, previous macrolide-use is significantly related to strains with *mef*(*A*) and/or *erm*(*B*) ($P < 0.01$). Unexpectedly oral beta-lactam use is also related to them ($P < 0.01$).

erm(*B*) ($P < 0.01$). Unexpectedly, the use of oral beta-lactams was also related to the strains ($P < 0.01$).

DISCUSSION

This report shows signs of the high incidence of antimicrobial resistance of *S. pneumoniae* in an isolated city in Japan. From our results, we realized that the emergence of drug-resistant *S. pneumoniae* might become a larger problem. The MIC ranges of all 949 isolates samples were tested with seven different antimicrobial agents in our experiments. In addition to our previous report showing a high prevalence of penicillin resistance in *S. pneumoniae*, we revealed that the rates of resistance to erythromycin and clarithromycin were much higher than those reported before in Japan (5). Another noteworthy point in our report is that more than half of the strains tested had decreased susceptibility to meropenem. Carbapenems are now considered the first choice for invasive bacterial infection in Japan. The use of meropenem should be carefully monitored for susceptibility to carbapenems.

Through gene analysis, we also exposed discouraging results about the high occurrence of *S. pneumoniae* with *mef*(*A*) and/or *erm*(*B*) genes. This relationship between susceptibility to clarithromicyn and the macrolide-resistant genes was similar to that found in a previous report (8). Also, our results corresponded with a report showing that low macrolide-resistant levels were mediated by the *mef*(*A*) gene and high resistant levels by *erm*(*B*) gene. Some previous reports maintained an association between serotypes and macrolide-resistant genes (5,9,10). They showed that serotype 19F was associated with *mef*(*A*) genes and that serotype 3 was associated with *erm*(*B*) gene, and explained that both of these strain types were globally spreading throughout Japan. One of the controversial points of our study is that we did not complete a serotyping test. We could not clarify the relationship between serotype and drug resistance. However, we consider that the macrolide-resistant strains isolated in our study might be genetically related to the serotype 19F and 3 strains.

The relationship between antibiotic use and drug-resistant *S. pneumoniae* has been well documented, including reports that the risk factors for the carriage of drug-resistant *S. pneumoniae* were recent antibiotic use, young age, daycare attendance, and/or siblings(11-18). Since drug resistance is mediated by gene mutation or the acquisition resistant genes, we tried to examine the association on a molecular level. Younger age (less than 3 years) was one of the obvious factors in carrying *pbp*, *mef*(*A*), and/or *erm*(*B*). The use of antibiotic treatment within 3 months was obviously related to the carriage of altered *pbp* genes; in particular, the relationship between previous beta-lactam use and the accretion of *pbp* genes was extremely strong. Our study shows both beta-

lactam and macrolide use was related to the carriage of *S. pneumoniae* with *mef*(*A*) or *erm*(*B*). A previous study reported that low dosage and the long-term use of antibiotics would increase the occurrence of resistant strains (19). Because our study was retrospective, we found it difficult to gather detailed information on the antibiotic usage of each patient. If we looked closer at the dosage and term of antibiotic administration, the relationship between previous antibiotic use and the carriage of resistant genes might become clearer.

After attaining our results, we considered how children might be prevented from carrying drug-resistant *S. pneumoniae*. A previous report (5) mentioned that 68% of isolated strains were included in the serotypes covered by the 23-valent pneumococcal vaccine (5). We think it is possible that vaccines will reduce the incidence of drug-resistant *S. pneumoniae* because they may reduce the chance of horizontal spreading from patient to patient. Recently, however, there have been reports on the effects of the 7-valent pneumococcal vaccine and the problems caused by drug-resistant strains whose serotypes were not included in the vaccine (20,21). Our results showed a strong relationship between antibiotic use and the carriage of drug-resistant strains, suggesting that controlled use of antibiotics might reduce the occasion to acquire resistant genes in *S. pneumoniae*. Concerning the high occurrence of resistant strains, we have to reassess a beneficial approach for the treatment of children.

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