RESEARCH LETTER

Mutational analysis of reduced telithromycin susceptibility of *Streptococcus pneumoniae* isolated clinically in Japan

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Abstract

A total of 132 *Streptococcus pneumoniae* isolates collected between 2005 and 2006 in Japan were examined for susceptibility to telithromycin (TEL) and macrolide. The overall resistance to macrolide was 80%. Among the isolates, 128 strains had low-level TEL susceptibility (minimal inhibitory concentrations [MICs] 0.03–1 $\mu g\,mL^{-1}$), suggesting that pneumococci with reduced susceptibility to TEL have appeared without prior exposure to the drug, although none of the isolates were assigned as TEL-resistant (breakpoint, $\geq 4\,\mu g\,mL^{-1}$). Eight of these isolates (MIC 0.5–1 $\mu g\,mL^{-1}$) were analyzed for macrolide resistance determinants and genetic relatedness. They all carried *mefE-mel*, which encodes the macrolide efflux genetic assembly, and three also harbored *ermB*, which encodes rRNA methylase. Allele replacement mutagenesis of the corresponding genes in the clinical isolates revealed that reduced TEL susceptibility (MIC 1 $\mu g\,mL^{-1}$) in *S. pneumoniae* may be caused by acquisition of the *mefE-mel* element only and additionally conferred by the *ermB* determinant.

Introduction

Telithromycin (TEL) is a semi-synthetic derivative of the 14-membered macrolide erythromycin (EM), and the first ketolide approved for clinical use. It has demonstrated high efficacy against *Streptococcus pneumoniae* isolates that cause community-acquired respiratory tract disease (Bozdogan *et al.*, 2003; Fogarty *et al.*, 2003). TEL and EM bind close to the peptidyl transferase region of the 50S ribosomal subunit and inhibit bacterial protein synthesis by blocking the elongation of the peptide chain through the ribosomal tunnel (Zuckerman, 2004). The primary contact site of EM and TEL is at nucleotide A2058 of 23S rRNA gene domain V, and TEL establishes additional contacts with A752 in domain II of 23S rRNA gene (Hansen *et al.*, 1999; Douthwaite *et al.*, 2000). As a result, TEL has a stronger affinity for the ribosome and can therefore overcome common macrolide resistance mechanisms including target modification directed by the methylase encoded by *ermB*, which methylates A2058, and mutations in the 23S rRNA gene and ribosomal proteins that interrupt macrolide binding (Maglio *et al.*, 2003; Farrell & Felmingham, 2004).

High-level TEL resistance in *S. pneumoniae* was experimentally generated by mutations in domain II or V of 23S rRNA gene and ribosomal proteins L4 and L22 (Leclercq & Courvalin, 2002), and is easily created from a macrolide-resistant strain by the deletion or mutation of the region upstream of *ermB* (Walsh *et al.*, 2003). In contrast, clinical TEL resistance in *S. pneumoniae* remains rare. Farrell and Felmingham initially reported that among the worldwide collection of 13 874 *S. pneumoniae* isolates isolated between 1999 and 2003, only 10 were TEL resistant (Farrell & Felmingham, 2004). The strains isolated in France, Italy, Spain, Hungary and Japan had minimal inhibitory concentrations (MICs) of 4–8 $\mu g\,mL^{-1}$. To our knowledge, the P3084055 strain (MIC 4 $\mu g\,mL^{-1}$) is currently the only TEL-resistant *S. pneumoniae* isolate in Japan (Hirakata *et al.*, 2007). Recently, the emergence of clinical isolates of *S. pneumoniae* with a very high-level TEL resistance (MIC 256 $\mu g\,mL^{-1}$) was reported (Faccone *et al.*, 2005; Wolter

et al., 2007). Sequence analysis of the strain isolated in Argentina in 2005 identified an A2058T mutation in domain V of 23S rRNA gene, a deletion located at the C-terminal portion of L22 and an S20N mutation in L4 (Faccone *et al.*, 2005). It was negative for *ermB*, *ermA* and *ermTR*, which encode rRNA methylase. Therefore, a combination of mutational changes in 23S rRNA gene and ribosomal proteins was assumed to be responsible for the high-level TEL resistance. In contrast, it was demonstrated that *ermB* with a truncated leader peptide in combination with a $_{69}GTG_{71}$-to-TPS mutation in ribosomal protein L4 was responsible for the high-level TEL resistance in the strain isolated in Canada in 2007 (Wolter *et al.*, 2007).

The objective of this study was to investigate the occurrence of TEL resistance in 132 *S. pneumoniae* isolates collected in Japan between 2005 and 2006. The results suggest that reduced-TEL-susceptibility pneumococci have certainly appeared, although none of the isolates were TEL resistant. Further analysis using isogenic *S. pneumoniae* strains demonstrated that reduced TEL susceptibility may be caused by acquisition of only the *mefE-mel* element, which encodes the macrolide efflux pump.

Materials and methods

Bacterial strains, plasmids and media

Streptococcus pneumoniae isolates collected between 2005 and 2006 in Japan and ATCC 49619 as a drug-susceptible strain were used in this study. *Escherichia coli* strain DH5α was used as a recipient in the transformation for DNA cloning. The plasmids used are shown in Table 1. Pneumococci were routinely cultured at 37 °C and 5% CO_2 in brain–heart infusion plus 0.5% yeast extract.

Antimicrobial susceptibility testing

Susceptibility to antibiotics was determined by the serial twofold dilution method using Mueller–Hinton agar plates supplemented with 5% lysed horse blood. The susceptibility or resistance of pneumococci to TEL and EM was assessed in accordance with the recommendation of the National Committee for Clinical Laboratory Standards (2007).

Detection of macrolide-resistant genes

Bacterial cells in 1 mL of overnight pneumococcal cultures were collected, suspended in 200 μL distilled water and boiled for 10 min. A portion of the lysate supernatant was subjected to PCR. Primers for *ermA*, *ermC*, *mphA*, *mphB*, *ereA* and *ereB* were described previously (Sutcliffe *et al.*, 1996). *ermB* was identified using the forward primer ermB-F (5′-TGAAAAGGTACTCAACCAAATA-3′) and the reverse primer ermB-R (5′-AGTAACGGTACTTAAATTGTTTAC-3′). *mefA/E* was detected using the primer pair mef-F1 (5′-AGTATCATTAATCACTAGTGC-3′) and mef-R1 (5′-TTCTTCTGGTACTAAAAGTGG-3′).

mefE was identified by DNA sequencing as follows: chromosomal DNA was prepared from clinical isolates as described by Blue & Mitchell (2003) and used as a temperate for PCR. The *mefE* region (+10 to +1126 to the *mefE* translational start site) was amplified using the primer pair mef-F2 (5′-CCGGAATTCTACAACAATTGG-3′) and mef-R2 (5′-CACCAAGCTTTTACACCGAT-3′). The PCR product was digested with EcoRI–HindIII and the fragment was cloned into pUC18. The resulting plasmid was subjected to DNA sequencing.

Pulse-field gel electrophoresis (PFGE) analysis

PFGE analysis was performed as described previously (Yokoyama & Uchimura, 2006) with some modifications. Briefly, the plug containing bacteria from an overnight culture was made with Seakem gold agarose (Cambrex, Rockland, ME) using a sample plug caster (Bio-Rad, Hercules, CA). The plug was treated for 18 h at 50 °C with a solution of 1 mg proteinase K mL^{-1} (Roche). After incubation, the plug was treated twice for 20 min, each with Tris-EDTA (TE) buffer containing 4 mM Pefabloc (Roche) at 50 °C, and then washed twice on ice for 20 min, each with TE buffer. The plug was digested for 18 h at 37 °C with SmaI (Roche).

Table 1. Plasmids used in this study

Plasmids	Relevant properties	References and source
pTKY856	pUC18 with 2296-bp *mefE-mel* fragment	This study
pTKY857	pTKY856 carrying *mefE-mel*-disrupted genes, Sp	This study
pTKY858	pT7Blue with 618-bp *ermB* fragment	This study
pTKY859	pTKY858 carrying *ermB*-disrupted gene, Km	This study
pTKY862	pLZ12Km2 with Sp cassette	This study
pLZ12Km2	Km cassette	Okada *et al.* (1998)
pR350	Sp cassette	Dintilhac *et al.* (1997)
pUC18	Cloning vector	Yanisch-Perron *et al.* (1985)
pT7Blue	Cloning vector	Novagen

Transformation

Synthetic competence-stimulating peptide 1 or 2 and the method of Iannelli & Pozzi (2004) were used to transform *S. pneumoniae* clinical isolates into a transformation-competent state.

Construction of *mefE-mel* and *ermB* disruption mutants

The disruption of *mefE-mel* was constructed as follows: the region encoding *mefE* and *mel* was amplified from chromosomal DNA prepared from *S. pneumoniae* strain S88 by PCR using the forward primer (5′-ACTGGATCCGCGATGGT CTT-3′) and the reverse primer (5′-CCGGAAGCTTTT TTTGCCTTAG-3′). The PCR product was digested with BamHI–HindIII and the fragment was cloned into pUC18. The resulting plasmid pTKY856 was cleaved with AccI and PstI to eliminate the inter-*mefE-mel* region. The overhanging ends were blunted with T4 polymerase and then ligated to the fragment containing the spectinomycin resistance gene (Sp), generated from pTKY862 after digestion with BamHI, followed by blunting with T4 DNA polymerase. The plasmid pTKY862 is a derivative of pLZ12Km2, with the fragment encoding Sp amplified from pR350 using the primers SpcUP and SpcDO reported previously (Martin *et al.*, 2000). The resulting plasmid pTKY857 was used to replace Δ*mefE-mel*::Sp in clinically isolated TEL-susceptible strains.

The disruption of *ermB* was constructed as follows: the *ermB* region was amplified by PCR from chromosomal DNA of *S. pneumoniae* S88 with primers ermB-F and ermB-R, and the fragment was cloned into pT7Blue. The resulting plasmid pTKY858 was cleaved with StyI and then ligated, after blunting with T4 DNA polymerase, to the fragment carrying the kanamycin resistance gene (Km), generated from pLZ12Km2 after digestion with SalI, followed by blunting with T4 DNA polymerase. The resulting plasmid pTKY859 was used to replace Δ*ermB*::Km in clinically isolated reduced TEL-susceptibility strains. To construct the Δ*mefE-mel*::Sp, Δ*ermB*::Km double mutant, the Δ*ermB*::Km mutant strains originating from each clinical isolate were transformed with pTKY857 and selected by spectinomycin resistance. The double-crossover events in all constructed mutants were assessed by Southern hybridization.

Results and discussion

A total of 132 *S. pneumoniae* isolates collected between 2005 and 2006 at one hospital in Japan were examined for susceptibility to TEL (breakpoint; resistance $\geq 4\,\mu g\,mL^{-1}$, sensitivity $\leq 1\,\mu g\,mL^{-1}$) and EM (breakpoint; resistance $\geq 1\,\mu g\,mL^{-1}$, sensitivity $\leq 0.25\,\mu g\,mL^{-1}$). A total of 106 isolates were found to be resistant to EM. A total of 128

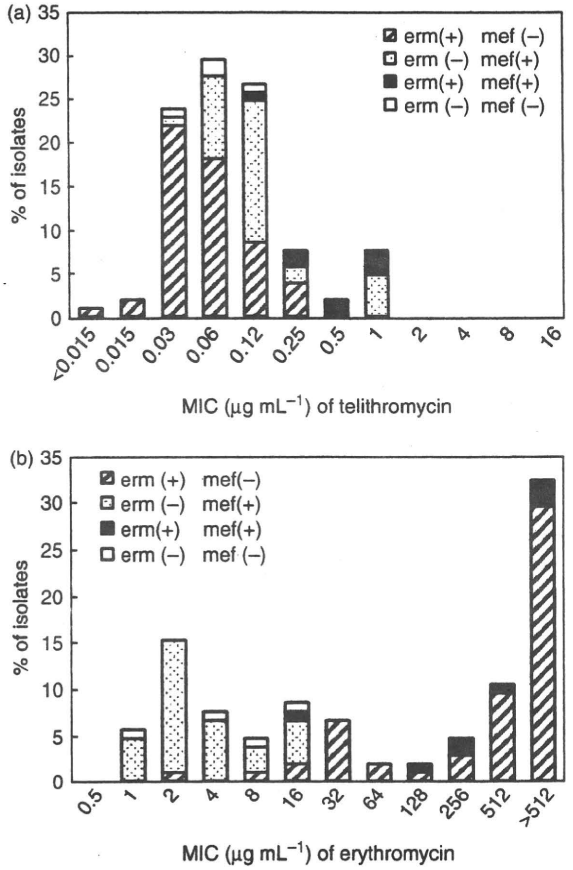

Fig. 1. MIC distribution and association with macrolide-resistant genes. MICs of telithromycin (a) and erythromycin (b) are shown. erm, *ermB*; mef, *mefA/E*.

isolates had low-level TEL susceptibility, with MICs of 0.03–1 $\mu g\,mL^{-1}$ (Fig. 1), suggesting that pneumococci with reduced TEL susceptibility have appeared without prior exposure to TEL, which has not been used in this hospital. The isolates included no TEL-resistant strains.

Distribution of macrolide-resistant determinants

To detect macrolide-resistant determinants in all isolates, PCR assays were performed for the rRNA methylase genes (*ermA*, *ermB* and *ermC*), macloride phosphotransferase genes (*mphA* and *mphB*), macrolide esterase genes (*ereA* and *ereB*) and genes encoding the macrolide efflux pump (*mefA* and *mefE*). All isolates were negative for *ermA*, *ermC*, *mphA*, *mphB*, *ereA* and *ereB* (data not shown). The distribution of *ermB* and *mef* is shown in Fig. 1. The rates of *ermB*-positive, *mef*-positive and double *ermB* and *mef*-positive isolates were 55.2%, 33.3% and 7.6%, respectively. Interestingly, all the isolates exhibiting reduced TEL susceptibility

(0.5–1 μg mL^{-1}) harbored *mef*. Two variants of *mef*, *mefA* and *mefE*, have been identified with high sequence homology (Roberts *et al.*, 1999). Because the initial PCR for detecting *mef* could not distinguish between these two variants, we performed DNA sequencing analysis to discriminate *mefA* and *mefE* in eight reduced TEL-susceptibility isolates (MIC 0.5–1 μg mL^{-1}) as described in Materials and methods. Consequently, all *mef*s in these isolates were assigned to *mefE*. It has been reported that *mefA* is the predominant efflux-associated gene found in *S. pneumoniae* in Japan (Isozumi *et al.*, 2007; Ikenaga *et al.*, 2008). In contrast, the present results demonstrated that *mefE* is also distributed with a high frequency in Japan and possibly generated the reduced-TEL-susceptibility *S. pneumoniae*.

These low-TEL-susceptibility isolates were analyzed by serotyping, multilocus sequence typing (MLST) and PFGE. Five isolates grouped to serotype 6B showed the same sequence type, which was ST2983 with MLST numbers 5-6-1-2-6-1-271 for *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*, respectively. PFGE showed that five isolates (serotype 6B) were closely related (Fig. 2). On the other hand, the sequence types of strains S43 (serotype 15A), S88 (serotype 19F) and S120 (serotype 19F) were ST361 (7-13-8-6-6-6-8), ST558 (18-12-4-44-14-77-97) and ST1464 (4-16-19-15-6-20-106), respectively. PFGE also clearly distinguished these three strains (Fig. 2). In a recent study, the most frequently occurring serogroups and serotypes of clinical pneumococcal strains isolated from children in Japan were six (32.8%), 23 (21.7%), 14 (13.2%) and 19 (12.7%) (Ikenaga *et al.*, 2008).

Decreased susceptibility to TEL in clinically isolated *S. pneumoniae* is associated with mutations in the L4 and L22 riboproteins and domains II or V of the 23S rRNA gene, and the presence of *ermB* and *mefA/E* (Faccone *et al.*, 2005; Reinert *et al.*, 2005; Al-Lahham *et al.*, 2006; Wolter *et al.*, 2007). Although a combination of these mechanisms could be responsible for TEL susceptibility in clinical isolates, the exact contribution of *mefA/E* or *ermB* to TEL susceptibility has not been revealed previously using isogenic pneumococcal strains.

Mutational analysis of reduced TEL susceptibility in *S. pneumoniae*

To ascertain the contribution of *mefE* to the reduced TEL susceptibility of *S. pneumoniae* isolated clinically in the present study, an independent insertion mutation in *mefE* was constructed by allelic replacement in five clinical isolates (MIC 0.5–1 μg mL^{-1}). *mefE* is a part of the macrolide efflux genetic assembly (mega), which includes the downstream gene *mel* (Gay & Stephens, 2001). In *S. pneumoniae*, *mefE* and *mel* are predicted to be a dual efflux pump (Ambrose *et al.*, 2005). In this study, both *mefE* and *mel* were disrupted

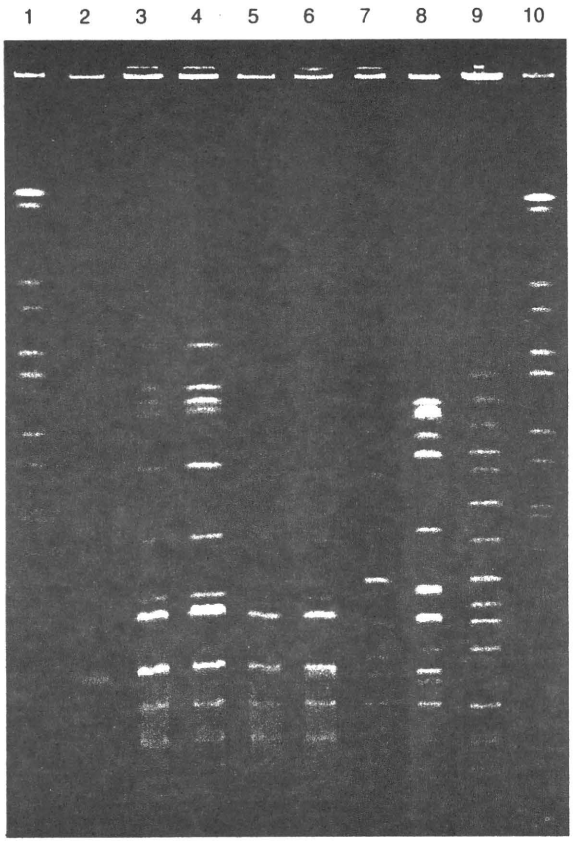

Fig. 2. PFGE profiles of SmaI restriction digests of eight TEL-reduced susceptible isolates of *Streptococcus pneumoniae*. Lanes 1 and 10, universal standard *Salmonella* serotype Braenderup H9812 (Hunter *et al.*, 2005); lane 2, strain S15; lane 3, strain S36; lane 4, strain S89; lane 5, strain S105; lane 6, strain S125; lane 7, strain S43; lane 8, strain S88; and lane 9, strain S120.

by eliminating the central 1409-bp fragment of the *mefE-mel* operon, followed by insertion of an Sp cassette. As shown in Table 2, mutations in *mefE-mel* of the serotype 6B strains S15 and S125 resulted in a significant decrease in TEL-MIC to the level of ATCC 49619 (< 0.015 μg mL^{-1}), which is used as a standard drug-susceptible strain. EM-MICs were also reduced to the level of ATCC 49619 (< 0.5 μg mL^{-1}). It is therefore concluded that *mefE-mel* is the determinant solely responsible for reduced TEL susceptibility and EM resistance in these clinical isolates.

The *mefE-mel* mutation in strain S88 (TEL-MIC 1 μg mL^{-1}), harboring both *mefE-mel* and *ermB*, resulted in a moderate reduction in TEL-MIC to 0.12 μg mL^{-1}. Independent disruption of S88 *ermB* resulted in a similar effect on TEL susceptibility (MIC 0.12 μg mL^{-1}). In contrast, disruption of both the *mefE-mel* and the *ermB* determinants further reduced TEL-MIC to the level of ATCC 49619 (MIC < 0.015 μg mL^{-1}). Similar results were obtained when

Table 2. MICs of the Δ*mefE-mel* and/or Δ*ermB* mutants derived from TEL low-susceptible isolates of *Streptococcus pneumoniae**

Strain	Serotype†	ST‡	Characteristics¶	MIC_{TEL} (μg mL^{-1})	MIC_{EM} (μg mL^{-1})
S15	6B	2983	*mefE-mel*$^+$	1	16
			Δ*mefE-mel*	< 0.015	< 0.5
S125	6B	2983	*mefE-mel*$^+$	1	16
			Δ*mefE-mel*	< 0.015	< 0.5
S43	15A	361	*mefE-mel*$^+$ *ermB*$^+$	0.5	512
			Δ*mefE-mel ermB*$^+$	0.06	246
			mefE-mel$^+$ Δ*ermB*	0.06	4
			Δ*mefE-mel* Δ*ermB*	< 0.015	< 0.5
S88	19F	558	*mefE-mel*$^+$ *ermB*$^+$	1	> 512
			Δ*mefE-mel ermB*$^+$	0.12	> 512
			mefE-mel$^+$ Δ*ermB*	0.12	4
			Δ*mefE-mel* Δ*ermB*	< 0.015	0.5
S120	19F	1464	*mefE-mel*$^+$ *ermB*$^+$	1	> 512
			Δ*mefE-mel ermB*$^+$	0.12	> 512
			mefE-mel$^+$ Δ*ermB*	0.12	4
			Δ*mefE-mel* Δ*ermB*	< 0.015	0.5

*ATCC49619 (MIC_{TEL} < 0.015 μg mL^{-1}, MIC_{EM} < 0.5 μg mL^{-1}) was used as a susceptible strain.

†Serotyping was performed by the Quellung reaction with antisera from Statens, Serum Institute (Copenhagen, Denmark).

‡Sequence type.

¶See Materials and methods for mutations in the Δ*mefE-mel* and Δ*ermB* mutants.

the mutants were constructed independently from strains S120 and S43, which carry both *mefE-mel* and *ermB* elements. Taken together, the results suggest that reduced TEL susceptibility (TEL-MIC 1 μg mL^{-1}) in *S. pneumoniae* may be caused by the acquisition of the *mefE-mel* element only and conferred additionally by the *ermB* element.

The disruption of *ermB* resulted in drastic decreases in resistance to EM; MIC declined from > 512 to 4 μg mL^{-1}. However, the *mefE-mel* mutations did not significantly affect resistance. Additional *mefE-mel* mutations in the *ermB* mutants reduced EM-MICs to the level of ATCC (MIC 0.5 μg mL^{-1}). These results suggest that *ermB* is a predominant mechanism for high resistance to EM in the pneumococcal isolates harboring both *ermB* and *mefE-mel* determinants, although the efflux assembly confers low-level resistance.

Sequence analyses of the five isolates revealed no mutations in 23S rRNA gene domains II or V. There were no mutations in the L4 ribosomal protein from any isolate, except that from strain S43, in which the S20N mutation was found (data not shown). No mutations were found in the L22 ribosomal protein from any isolate.

It has been demonstrated that the *mefE* and *mel* carried by mega may be a part of Tn*2009*, a composite element in which mega is integrated into a Tn*916*-like transposon carrying *tetM* (Franke & Clewell, 1981; Del Grosso *et al.*, 2004). The presence of *tetM* has been examined in isolates S15, S36, S89, S105 and S125, which express tetracycline resistance (MICs 16 μg mL^{-1}), using PCR with the primers TETM1 and TETM2 (Del Grosso *et al.*, 2004). This primer set produced an amplicon of approximately 2.0 kb, indicating the presence of *tetM*. The linkage between *mefE-mel* and *tetM* in these strains was investigated by Southern hybridization based on the restriction cleavage map constructed from the sequence (accession number AF376746). In these five isolates, *mefE-mel* and *tetM* were in close proximity, as shown in Tn*2009* (data not shown). Although the whole sequence of the composite transposon has not been revealed in the present study, it is speculated that transposition of a Tn*2009*-like element may be responsible for generating the reduced-TEL-susceptibility streptococci S15, S36, S89, S105 and S125. On the other hand, a recent report demonstrated that a different composite element, designated Tn*2010*, is similar to Tn*2009*, but also contains *ermB* (Del Grosso *et al.*, 2006). The presence of *tetM* in *S. pneumoniae* isolates S43, S88 and S120 was confirmed by DNA sequence analyses of PCR products of 2.0 kb amplified using the primer pair TETM1 and TETM2. Strain S43 expressed tetracycline resistance (MIC 16 μg mL^{-1}), but S88 and S120 showed a tetracycline-intermediate phenotype (MICs 4 μg mL^{-1}). In these isolates, Southern hybridization revealed a linkage between *mef-mel* and *tetM* and one between *ermB* and *tetM*, which are in Tn*2010* (data not shown).

Concluding remarks

The present study suggests that low-TEL-susceptibility pneumococci have appeared clinically in Japan without prior exposure to TEL. Mutational analysis with isogenic strains revealed that the acquisition of *mefE-mel* may reduce the susceptibility of pneumococci to TEL. It was demonstrated previously that high-level TEL resistance was easily generated from macrolide-resistant *S. pneumoniae* harboring *ermB* and *mefA* (Walsh *et al.*, 2003). It is therefore worth mentioning that the reduced TEL susceptibility clones demonstrated in the present study may have the potential to generate TEL-resistant pneumococci and spread further.

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Isolation of *Streptococcus pneumoniae* Serotypes 6C and 6D from the Nasopharyngeal Mucosa of Healthy Japanese Children

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Streptococcus pneumoniae, a primary causative agent of otitis media, pneumonia, bacteremia, and meningitis in children, results in substantial morbidity and mortality in many countries, including Japan (1–3). Of the 93 *S. pneumoniae* serotypes identified to date, serotypes 6C and 6D were recently differentiated from the classical serotypes 6A and 6B, respectively (4–6). Serotype 6C was subsequently reported to be isolated in several countries (5–9), especially as an important replacement serotype after introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) (7,9,10). The naturally occurring *S. pneumoniae* serotype 6D was isolated from the Fiji Islands, Korea, and Poland (4,11,12). In this study, 32 6C and 1 6D *S. pneumoniae* isolates were identified from the nasopharyngeal mucosa of healthy children who had not received PCV7 residing on Sado Island, Niigata Prefecture, by using serological and genetic characterization.

S. pneumoniae, *Haemophilus influenzae*, and other pathogens among children residing on Sado Island, Niigata Prefecture, are monitored as part of the Sado Island, Antimicrobials, Day-care attendance, Older siblings (SADO) Study (13). In SADO study, which was conducted in 2008, pharyngeal swabs obtained from healthy children at check-up periods of 4, 7, 10, and 18 months old (mo) were cultured. Two of the children included had received PCV7. Fifty-two percent of the children at 18 mo had been attending day nursery. All *S. pneumoniae* isolates were serotyped using the conventional Quellung reaction using commercially available pneumococcal antisera (Statens Serum Institut [SSI], Copenhagen, Denmark) and home-made factor antiserum (designated factor 6dh [h indicates home-made]) for serotypes 6C and 6D. The factor 6b antiserum used in this study could react with both serotypes 6A and 6C; the new version of the factor 6b antiserum from SSI only reacts with serotype 6A (14,15). Factor 6dh antiserum was prepared by immunization of rabbits with formaldehyde-fixed serotype 6C whole cells and subsequent absorption of the antiserum with serotype 6A whole cells. In addition to the serological examination, serotypes 6C and 6D of the isolates were confirmed by genetic characterization involving comparison of the *wciN* region of 6A, 6B, 6C, and 6D isolates using PCR with primers 5106 and 3101 (5), and DNA sequencing of the *wciP* gene. The size of the *wciN* PCR products was determined by electrophoresis with 0.8% SeaKem GTG agarose gel (Takara Bio, Otsu, Japan). The DNA sequence of the *wciP* gene was determined using BigDye v1.1 (Applied Biosystems, Foster City, Calif., USA) and 3130*xl* Genetic Analyzer (Applied Biosystems). The antibiotic susceptibility of the isolates was analyzed by the microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI M100-S18). Multi-locus sequence typing (MLST) was performed as described by Enright and Spratt (16).

A total of 337 *S. pneumoniae* isolates were obtained in this study. All isolates were initially serotyped using the Quellung reaction, and those that exhibited positive reactions with serogroup 6 antiserum were further tested using factor 6b, 6c, and 6dh antisera. Serotypes 6A and 6C exhibited positive reactions with factor 6b antiserum, whereas serotypes 6B and 6D exhibited positive reactions with factor 6c antiserum. Serotypes 6A and 6B exhibited negative reactions, and serotypes 6C and 6D exhibited positive reactions, with factor 6dh antiserum (Fig. 1). Thirty-two isolates (9.5%) exhibited positive reactions with both factor 6b and 6dh antisera, thus suggesting that they expressed the serotype 6C capsule. Furthermore, 1 isolate (0.3%) exhibited positive reactions with factor 6c and 6dh antisera, thus suggesting that it expressed serotype 6D capsule.

The *wciN* gene of the *S. pneumoniae* isolates was subsequently examined using PCR. The lengths of the PCR products for serotype 6A and 6B isolates found to be 2.0 (Fig. 2, lane 1) and 2.0/2.2 kb (Fig. 2, lanes 2 and 3), respectively. The length of each of the PCR products of the putative serotype 6C and 6D isolates was 1.8 kb (Fig. 2, lanes 4 and 5). The 2.0- and 2.2-kb *wciN* PCR products indicate the presence of capsular polysaccharide (PS) containing galactose, whereas the 1.8-kb PCR product indicates substitution of galactose by glucose (5). The DNA sequences of the *wciP* gene were determined for the isolates (4,5,11). The 138th amino acid residue in WciP for the 6A isolate is serine (AGT),

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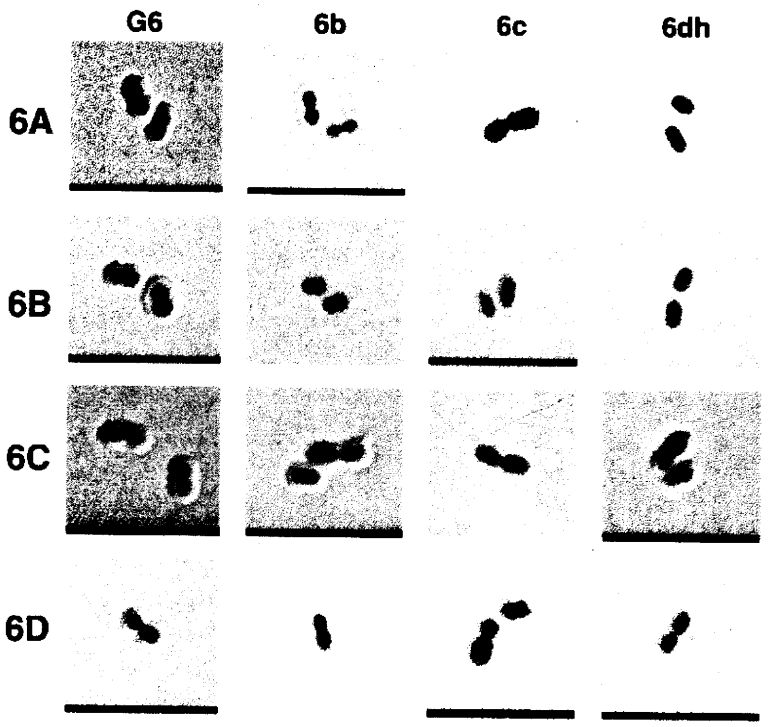

Fig. 1. Quellung reaction of *Streptococcus pneumoniae* serotypes 6A, 6B, 6C, and 6D. *S. pneumoniae* serotypes 6A (SP128) and 6B (KSP120) were isolated from cerebrospinal fluid. *S. pneumoniae* 6C (SP569) and 6D (SP687) were isolated from nasopharyngeal mucosa in this study. The antisera used are indicated on top of each column. G6, antiserum for serogroup 6; 6b, factor antiserum 6b; 6c, factor antiserum 6c; 6dh, home-made factor antiserum 6dh. Serotypes of *S. pneumoniae* are indicated on the left of the photographs. The underlined photographs illustrate positive results.

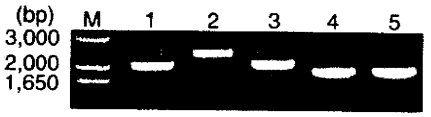

Fig. 2. PCR products of the *wciN* region of *Streptococcus pneumoniae* serogroup 6 isolates. M, 1 kb plus DNA ladder; lane 1, serotype 6A (SP128); lane 2, serotype 6B (KSP123); lane 3, serotype 6B (KSP120); lane 4, serotype 6C (SP569); lane 5, serotype 6D (SP687). The 2.0-kb or 2.2-kb fragments were obtained from serotype 6A (2.0-kb only) and 6B (2.0-kb or 2.2-kb) isolates, whereas the 1.8-kb fragments were obtained from serotype 6C and 6D isolates.

whereas that for the 6B isolate is asparagine (AAT) (17). The former amino acid is responsible for the rhamnose-(1→3)-ribitol linkage in the PS of serotype 6A, whereas the latter is responsible for the rhamnose-(1→4)-ribitol linkage in the PS of serotype 6B. The corresponding amino acids of the putative 6C and 6D isolates were serine and asparagine, respectively. The serological and genetic analyses yielded identical results in that both were consistent with the PS structure [→2)-glucose-(1→3)-glucose-(1→3)-rhamnose-(1→3)-ribitol-(5→phosphate] for 6C and [→2)-glucose-(1→3)-glucose-(1→3)-rhamnose-(1→4)-ribitol-(5→phosphate] for 6D, thus confirming the colonization of *S. pneumoniae* serotype 6C and 6D isolates in the nasopharynx of healthy Japanese children.

The 32 6C *S. pneumoniae* isolates were obtained from a total of 30 children (3 from 4-mo children, 5 from 7-mo children, 13 from 10-mo children, and 11 from 18-mo children); 2 of the isolates were obtained from the same child at 7- and 10-mo, and a furthre 2 isolates, which showed different colony morphologies and different antibiograms, were simultaneously obtained from a child at 18 mo. The *S. pneumoniae* serotype 6D was isolated from an 18-mo child. None of the children who carried the *S. pneumoniae* serotypes 6C or 6D had received PCV7. As for the children's residential area and day nursery attendance, there was no obvious association between the 30 children from whom the *S. pneumoniae* serotype 6C was isolated. The minimum inhibitory concentration (MIC) of penicillin G for the serotype 6C isolates ranged between ≤ 0.015 and 0.25 μg/ml, and that for 26 (81.3%) of the isolates being ≤ 0.06 μg/ml. All of the 6C isolates were susceptible to both cefotaxime (MIC ≤ 1 μg/ml) and meropenem (MIC ≤ 0.25 μg/ml), whereas 30 (93.8%) of them were resistant to erythromycin (MIC ≥ 1 μg/ml). The 6D isolate was susceptible to penicillin G (0.03 μg/ml), cefotaxime (0.25 μg/ml), and meropenem (≤ 0.008 μg/ml) but resistant to erythromycin (≥ 8 μg/ml). MLST analysis revealed that the frequent sequence types (STs) of the serotype 6C isolates were ST2923 (40.6%) and ST2924 (31.3%), whereas the ST of the 6D isolate was ST2924. The MLST analysis showed that the serotype 6C isolates from children on Sado Island comprised multiple clones.

The routine immunization of infants and toddlers in the United States with PCV7 has successfully reduced

the incidence of invasive pneumococcal disease (IPD) in children caused by the vaccine serotypes (18–20). Vaccination of children with PCV7 has also lowered the incidence of IPD among the elderly, a phenomenon known as the herd-immunity effect (18–20). The observed reduction in the incidence of IPD among the nonimmunized population is likely to be due to a change in the nasopharyngeal colonization of *S. pneumoniae* in immunized individuals. There has, however, been a rise in the incidence of IPD caused by non-PCV7 serotypes (known as replacement serotypes), including serotypes 19A, 6C, and others, in the United States (7,9,19, 21–24). As far as 6D is concerned, this serotype was isolated at a high rate (41%) from the nasopharyngeal mucosa of Fijian children, 86% of whom had received at least 1 dose of PCV7, thereby suggesting that serotype 6D may have a selective advantage after immunization with the vaccine (11). In addition, 5 IPD cases due to *S. pneumoniae* serotype 6D were reported in Poland (12). Because serotypes 6C and 6D were recognized after the introduction of PCV7, the surveillance data for infection with these serotypes in the United States and other countries are retrospective (12,18,19). PCV7 was released in Japan in February 2010 and widespread PCV7 vaccination is expected to lead to a similarly large reduction in pneumococcal infections, including IPD, pneumonia, and otitis media, in both the immunized and nonimmunized populations to that observed in other countries. We have initiated a population-based study to monitor the changes in IPD incidence and the serotype distribution among Japanese children, and we are also monitoring the colonized *S. pneumoniae* in the nasopharynx of healthy children. Initial results showed that *S. pneumoniae* serotype 6C was isolated from less than 2% of IPD cases without PCV7 vaccination (unpublished data) but could be isolated from the nasopharyngeal mucosa of 9.5% of the healthy children. PCV7, which includes only serotype-6B conjugate, would not affect the colonization or infection by *S. pneumoniae* serotypes 6C and/or 6D. A prospective surveillance on both colonization and infection by *S. pneumoniae* serotypes 6C, 6D, and others is therefore warranted to obtain an accurate evaluation of the effects of the 7- and 13-valent conjugate vaccines.

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Conflict of interest None to declare.

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