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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 antisera (designated factor 6dh [h indicates home-made]) for serotypes 6C and 6D. The factor 6b 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 3130xl 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),
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 wcN 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 further 2 isolates, which showed different colony morphologies and different antibigrams, 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.

REFERENCES


