Molecular epidemiology and serogroup 6 capsular gene evolution of pneumococcal carriage in a Japanese birth cohort study

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Antibiotic resistance in Streptococcus pneumoniae is a major concern worldwide. However, it is unclear whether resistance is associated with only a few highly prevalent clones or numerous and diverse clones. We monitored 349 healthy children and obtained nasopharyngeal cultures at five time points coinciding with health check-ups (4, 7, 10, 18 and 36 months) between 2008 and 2012. A total of 497 S. pneumoniae isolates from 257 healthy children were characterized using capsular serotyping, multilocus sequence typing and antibiotic resistance genotyping (ermB, mefA/E and pbp mutations). Among these isolates, 25 serotypes and 66 sequence types (STs) were found, including 24 new STs with 11 new alleles. Although resistance was present in a variety of ST clones, most of the clones (57/66, 86.4 %) had one specific resistant or susceptible genotype. Of 233 phenotypically penicillin-non-susceptible isolates, 196 (84.1 %) belonged to only six clones, comprising ST906B, ST236 19F, ST242 23F, ST3787 6A, ST1437 23F and ST338 23A and their variants. We concluded that drug-resistant S. pneumoniae is associated with a limited number of highly prevalent clones that are capable of adapting to the community setting. Furthermore, we analysed the capsular gene evolution in serogroup 6. The strain ST2924 6D was probably the result of recombination of a 3563 bp fragment of the capsule locus acquired by an ST2924 6C strain from an ST90 6B or ST2924 6B strain. Compared with previous studies, our results showed a different recombination site (wciN and wzx) and a different cps profile (8-7-11), indicating that serogroup 6 strains have multiple sites for cps recombination as a mechanism of vaccine escape.

INTRODUCTION

Streptococcus pneumoniae is a leading cause of invasive and non-invasive bacterial infection in children worldwide. A seven-valent pneumococcal conjugate vaccine (PCV7), which includes capsular polysaccharide antigens of seven serotypes – 4, 6B, 9V, 14, 18C, 19F and 23F – has been available since 2000. A significant decrease both in colonization and in invasive diseases due to these serotypes has been observed in populations that have adopted widespread use of PCV7. However, the World Health Organization estimated that pneumococcal diseases still caused 476 000 deaths among children <5 years of age in 2008 (WHO, 2012). In Japan, PCV7 was introduced for voluntary immunization in February 2010, and has been provided as routine vaccinations since April 2013. The vaccination schedules are standardized 3+1 doses in all areas of Japan. Therefore, the impact of PCV7 in Japan is likely to be small until a larger proportion of the population is immunized.

The rate of penicillin-non-susceptible S. pneumoniae, defined as a penicillin MIC of ≥0.12 µg ml⁻¹, in Japan has remained high (76.9 % in 2010) among paediatric patients including invasive and non-invasive diseases (Tajima et al., 2013). In another report, even in healthy carriage 46.3 % of the S. pneumoniae isolates were non-susceptible to penicillin (Otsuka et al., 2013). Penicillin-non-susceptible S. pneumoniae usually harbours pbp mutations for penicillin resistance, and may have macrolide-resistant genes. In order to design
measures to reduce the resistance rate, an understanding of the distribution of resistant clones is critical.

Ninety-four different capsular serotypes of *S. pneumoniae* have been identified to date (van der Linden et al., 2013). The capsular polysaccharide of *S. pneumoniae* enables the bacterium to escape phagocytic killing and allows its survival *in vivo* (AlonsoDevelasco et al., 1995; Dhingra et al., 1977) and plays an important role during the early phase of colonization of the upper airways (Magee & Yother, 2001). In the post-PCV7 era, serotype replacement and capsular switching has led to a critical problem, i.e. a rapid increase in the incidence of invasive diseases with non-PCV7 serotypes (e.g. 19A, 15A, 23A, 35B and 6C) (Carvalho et al., 2009; Gertz et al., 2010; Kaplan et al., 2010; van Gils et al., 2010).

More recently, *S. pneumoniae* has been classified by multi-locus sequence typing (MLST) (Enright & Spratt, 1998). Each capsular serotype may include multiple sequence types (STs) with specific features, such as invasiveness and antibiotic resistance. For example, ST199 and ST320 are the major STs in the 19A serotype with multidrug resistance, whilst other STs are not associated with multidrug resistance (Ardanuy et al., 2009; Moore et al., 2008; Pillai et al., 2009; Song et al., 2009). These variations make it difficult to predict the risk of penicillin-non-susceptible *S. pneumoniae* infection based on serotype alone.

Although there are several reports on the relationship between MLST and resistant phenotypes/genotypes of *S. pneumoniae*, few studies have analysed healthy children (Lambertsen et al., 2010; Sadowsy et al., 2007; Sakai et al., 2011; Sogstad et al., 2006). Therefore, it is still unclear whether resistance is associated with only a few highly prevalent clones or with numerous and diverse clones in children, the main reservoir of *S. pneumoniae* in a population.

Among the 94 *S. pneumoniae* serotypes, 6A, 6B and 6C isolates are commonly found in healthy colonization and invasive pneumococcal disease, particularly resistant 6B strains (Otsuka et al., 2013; van der Linden et al., 2013). Genetic studies have revealed the near identity of the capsular loci of serotypes 6A and 6B, with only a single nucleotide difference in the *wciP* gene (*wciP*\(_a\) and *wciP*\(_b\*)) (Bratcher et al., 2011; Mavroidi et al., 2004). Serotype 6C is thought to have emerged by the introduction of the *wciN*\(_6\) gene, replacing *wciN*\(_a\) in the capsular locus of serotype 6A. Recently, serotype 6D was identified in Fiji (Jin et al., 2009), Korea (Bratcher et al., 2010), Japan (Chang et al., 2010) and other countries (McCullistrem & Nahm, 2012). Some of these serotype 6D isolates were resistant to penicillin.

It has been suggested that serotype 6D emerged by two proposed mechanisms: replacement and recombination. The first is replacement of the *wciP*\(_a\) gene with the *wciP*\(_b\) gene in the capsular locus of serotype 6B. The second proposed mechanism is recombination between serotype 6B and 6C *cps* loci including the *wciN* and *wciP* genes (Bratcher et al., 2011; Song et al., 2011). Therefore, serogroup 6 currently consists of four serotypes: serotype 6A (*wciN*\(_a\) and *wciP*\(_a\)), 6B (*wciN*\(_a\) and *wciP*\(_b\)), 6C (*wciN*\(_a\) and *wciP*\(_a\)) and 6D (*wciN*\(_b\) and *wciP*\(_b\)). These serotypes are highly diverse based on the observation that each serotype includes multiple STs (van der Linden et al., 2013). Bratcher et al. (2011) also showed evidence that the recombination event of serotype 6D may have occurred only once and spread worldwide, using ’*cps* profile’ analysis, which assigns a specific number based on sequencing of serogroup 6-specific genes (*wciP*, *wzy* and *wzx*) (Mavroidi et al., 2004). Their *cps* profiles were 5-1-1 and its variant. However, the evolution of serotype 6D is still controversial, because there is no direct evidence to date.

The aim of this study was to identify the prevalence of resistant clones in a birth cohort on Sado Island. We evaluated and characterized *S. pneumoniae* isolates from healthy children using MLST determination and antibiotic resistance genotyping. Furthermore, we focused on a newly discovered serotype 6D as a new clone. We proposed an explanation for the evolution of serotype 6D using sequences of capsular genes.

**METHODS**

*S. pneumoniae* isolates. The study was conducted as a part of the SADO Study (Otsuka et al., 2013). In the prospective birth cohort study, we monitored 349 children as healthy subjects, and nasopharyngeal cultures were obtained from each of the healthy subjects at five time points coinciding with health check-ups (4, 7, 10, 18 and 36 months, participation rate: 91.7–97.7 %) between 2008 and 2012. A total of 551 *S. pneumoniae* isolates were detected from 1654 samples. The serotype and antibiotic susceptibility of these isolates were determined previously (Otsuka et al., 2013). Isolates that showed negative reactions for all pooled sera were considered to be nontypable. Because of the Japanese vaccination programme, no subject in this study had been administered PCV7 by the 18-month health check-up, whilst 106/328 subjects (32.3 %) were administered PCV7 by the 36-month health check-up. Of the 106 vaccinees, 93 received one vaccine dose and 13 received two vaccine doses (Otsuka et al., 2013). The total population of Sado Island was 64120, with 2167 (3.4 %) aged <5 years, at the midpoint of the study (January 2010).

MLST. We performed MLST as described previously (Enright & Spratt, 1998). Briefly, internal fragments of each of the seven housekeeping genes *aroE*, *gdh*, *gki*, *recP*, *spn*, *spt* and *ddl* were amplified by PCR and sequenced, and their STs were determined by reference to the MLST database (http://spneumoniae.mlst.net/). New alleles and allelic profiles were submitted to the database for assignment. The relatedness of isolates and known similar strains in the database were determined by constructing a neighbour-joining tree using the online program Draw tree using our own MLST data (http://spneumoniae.mlst.net/sql/uniquetree.asp).

Resistant genes. Molecular typing for resistance was determined by PCR. Macrolide-resistant genes (*ermB* and *mejA/E*), and mutations for penicillin resistance (*pbp1a, pbp2x* and *pbp2b* mutations) in *S. pneumoniae* were detected using previously reported primers (Nagai et al., 2001; Sutcliffe et al., 1996; Tait-Kamradt et al., 1997). *S. pneumoniae* isolates were classified into resistant genotypes by the presence of *pbp1a, pbp2x* and *pbp2b* mutations: genetically penicillin-susceptible *S. pneumoniae* (*gpSPS*, no mutation), genetically penicillin-intermediate *S. pneumoniae* (*gpISP*, with one or two mutations)
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Fig. 1. Genetic tree of MLST results and resistant genes. We identified 25 serotypes and 66 STs among the 497 isolates including 24 new STs and 11 new alleles. Bar, genetic linkage distance. No subject in this study had been administered PCV7 by the age of 18 months. The number of isolates detected from PCV7 vaccinees is shown in parentheses in the (continued)
and genetically penicillin-resistant \emph{S. pneumoniae} (gPRSP, with all mutations). After all PCR experiments, the combined penicillin and macrolide-resistant genotype was assigned.

\textbf{Nucleotide sequence.} Nucleotide sequences of the \emph{cps} loci from the \textit{wchA} to the \textit{wzx} gene of serogroup 6 isolates were determined using primers reported previously (Song \textit{et al.}, 2011). \textit{cps} profiling analysis for the \textit{wciP}, \textit{wzy} and \textit{wzx} genes was performed as described previously (Mavroidi \textit{et al.}, 2004). Serotype 6A (isolates SP664, SP700, SP1095 and SP1097), 6B (SP598, SP658, SP711, SP806 and SP1107), 6C (SP391, SP412, SP622, SP829, SP1081 and SP1085) and 6D isolates (SP687 and SP1090) were used.

Informed consent was obtained from the parents/guardians of all healthy subjects prior to participation in the study. This study protocol was approved by the Ethics Committee at Sado General Hospital and was registered at the UMIN Clinical Trials Registry, Japan (trial no. UMIN000004928).

\section*{RESULTS}

\subsection*{MLST}

Of the 551 isolates obtained during the SADO Study, 497 (462 isolates obtained from non-PCV7 vaccinees and 35 isolates obtained from PCV7 vaccinees) were available for both serotyping and MLST. Among the 497 isolates recovered from the 257 subjects, 25 serotypes and 66 STs were found, including 24 new STs (ST7780–ST7789, ST7791–ST7800, ST7813, ST7814, ST7978 and ST7979) with 11 new alleles (Fig. 1).

Of the 66 STs, nine STs included isolates of two serotypes. Five of these STs had isolates that were different serotypes that belonged to the same serogroups [ST2224 (6A and 6B)], ST3594 (18B and 18C), ST3787 (6A and 6B), ST7793 (15B and 15C) and ST7797 (15B and 15C)]. Four of the nine STs included isolates that belonged to different serogroups [ST3116 (34 and non-typable), ST1437 (23F and 19F), ST338 (23A and 19F) and ST236 (19F and 6D)]. ST2924 (6B, 6C and 6D) and ST199 (15B, 15C and 19F) included isolates of three serotypes. Serotype 19F and other serogroups consisted of the STs that included two different serogroups (Fig. 1, Table 1). The serotype and ST prevalence showed no difference among samples isolated before and after PCV7 introduction (Fig. 1).

Conversely, of the 25 serotypes, ten contained one ST, five contained two STs, five contained three STs, and two contained four to six STs. The remaining serotypes (6C, 19F and 6B) had eight, nine and ten STs, respectively (Table 1). Based on the observation that serogroup 6 isolates included multiple STs, we concluded that serogroup 6 isolates are highly diverse.

\subsection*{Resistant genotypes and phenotypes}

Of the 497 isolates, 52 (10.5\%) were gPSSP, 130 (26.2\%) were gPISP \textit{pbp2x}, 104 (20.9\%) were gPISP \textit{pbp1a} + \textit{pbp2x}, 45 (9.1\%) were gPISP \textit{pbp2x} + \textit{pbp2b} and 166 (33.4\%) were gPRSP (Table 2). There was a large gap between the rates of penicillin-non-susceptible genotypes (89.5\%, 445/497) and phenotype (46.9\%, 233/497). In particular, gPISP \textit{pbp2x} isolates were almost all phenotypically penicillin susceptible.

Regarding macrolide-resistant genes, 28 (5.6\%) had both \textit{ermB} and \textit{mefA/E}, 243 (48.9\%) had \textit{ermB}, 151 (30.4\%) had \textit{mefA/E}, and 75 (15.1\%) had no macrolide-resistant genes. Combined genetic classification of the \textit{pbp} mutation and macrolide-resistant gene showed 17 resistant genotypes (Table 2). All of the gPRSP isolates had at least one of the macrolide-resistant genes.

Each ST had a specific resistant genotype(s). Of the 66 STs detected in this study, 57 STs (86.4\%) had only one specific resistant genotype (Fig. 1). For example, all 21 of the ST2922 isolates in this study had the same resistant genotype (gPISP \textit{pbp1a} + \textit{pbp2x} with \textit{ermB}). Six STs (9.1\%) had two resistant genotypes, and only three STs (4.5\%) had more than three resistant genotypes (Fig. 1). Of 233 phenotypically penicillin-non-susceptible isolates, 196 (84.1\%) belonged to only six clones, comprising ST90\textsuperscript{6B}, ST3787\textsuperscript{6A}, ST1437\textsuperscript{23F}, ST338\textsuperscript{23A}, ST236\textsuperscript{19F} and ST242\textsuperscript{23F} and their variants. Furthermore, these clones showed macrolide resistance with resistance genotype(s), except for the ST3787\textsuperscript{6A} clone. ST338\textsuperscript{23A} is the only clone that is not included in the 13-valent PCV serotypes, which includes PCV7 serotypes plus serotypes 1, 3, 5, 6A, 7F and 19A. Nine STs contained 26 isolates that showed a susceptible genotype for both penicillin and macrolide, and all of them were non-PCV7 serotypes except for 18C (three isolates).

\subsection*{Serogroup 6 capsular loci}

In order to evaluate differences in the capsular loci of serogroup 6, DNA sequence comparison was carried out for the \textit{cps} locus from the \textit{wchA} to the \textit{wzx} gene of serotype 6A, 6B, 6C and 6D isolates. The differences in the sequences in the \textit{cps} loci (Figs S1 and S2, available in JMM Online) were consistent with the results reported previously by Song \textit{et al.} (2011). The nucleotide position of nt 1 in this study corresponded to nt 5545 in a previous report by Bratcher \textit{et al.} (2011). For example, deletions in the \textit{wciN} (\textit{wciN}\textsubscript{b}) gene were found in 6C and 6D isolates corresponding to the sequences between nt 1866 and 2226 (361 bp) in ST90\textsuperscript{6B}, and nt 1866 and 2227 (362 bp) in ST2924\textsuperscript{6B} isolates. Also, there were insertions of about 300 bp between the \textit{wciN} and
wciO genes (indel sequence) between nt 2227 and 2535 (309 bp) in ST906B, and nt 2228 and 2536 (309 bp) in ST29246B isolates. ST29246C and ST29246D isolates had a partial indel sequence in the wciN gene (Fig. 2).

To clarify how the ST29246D strain emerged, the sequences of the ST906B, ST29246B, ST29246C and ST29246D isolates were compared (Fig. 2, Fig. S2). The sequences from nt 1 to nt 1872 and nt 5539 to nt 6573 of the two ST29246D isolates were identical to the sequences of the corresponding regions of the ST29246C isolates. In contrast, the sequences from nt 1936 to nt 5498 of the ST29246D isolates were identical to the corresponding sequences of the ST906B and ST29246B isolates. These results are consistent with a recombination event in which a 3563 bp fragment of the capsule locus was acquired by the ST29246C strain from the ST906B or ST29246B strain resulting in the ST29246D strain (Fig. 2). The cps profile for two ST29246D isolates was 8-7-11. These results showed a different recombination site and cps profile from a previous study (Bratcher et al., 2011).

**DISCUSSION**

Here, we have reported a genotypic surveillance of *S. pneumoniae* among healthy children and capsular gene evolution in serogroup 6 on Sado Island. We found 25 serotypes showing a total of 66 STs. Interestingly, the serotypes with multiple STs were highly prevalent serotypes, including 6B, 19F and 6C. It is possible that the more diverse serotypes were able to adapt to the community setting and spread in healthy children through their increased capacity to undergo recombination compared with other serotypes. Alternatively, the prevalent serotypes that had already spread in the community may be undergoing recombination at a rate that is not different from the other serotypes but is detected as a result of the increased prevalence of those serotypes.

*S. pneumoniae* has a variety of drug-resistant and -susceptible genotypes (17 genotypes) in this setting. Most of the STs (86.4%) had one specific resistant genotype, indicating that factors besides drug resistance play an important role in the survival and circulation of *S. pneumoniae* strains in the community. In this study, six clones and their variants accounted for 84.1% (196/233) of phenotypically penicillin-non-susceptible isolates. Furthermore, most clones showed phenotypic and genotypic macrolide resistance. We conclude that drug-resistant *S. pneumoniae* is associated with a limited number of highly prevalent clones that are capable of adapting to the community setting. We will continue

**Table 1. Relationship between capsular serotype and MLST**

Underlining indicates ST including different serotype isolates that belonged to the same serogroups. Bold type indicates ST including different serotype isolates that belonged to the different serogroups.

<table>
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<tr>
<th>Serogroup</th>
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<th>No. MLST</th>
<th>MLST</th>
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<td>3</td>
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</tr>
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<td>6A</td>
<td>42</td>
<td>3</td>
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<tr>
<td></td>
<td>6B</td>
<td>82</td>
<td>10</td>
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<td>393</td>
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<td>7</td>
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to survey and identify changes in *S. pneumoniae* clones in Japan.

Capsule switch events probably occur through genetic transformation with different serotypes of *S. pneumoniae* co-infecting the nasopharynx simultaneously in which the capsule locus is exchanged between strains (Temime et al., 2008). For example, the multidrug-resistant ST32019A strain was genetically derived from the multidrug-resistant ST32019F strain (Ardanuy et al., 2009; Moore et al., 2008; Pillai et al., 2009; Song et al., 2009).

Our data regarding serogroup 6 (Fig. 2) provide direct evidence that the ST32019A strain was genetically derived from a recombination of ST32019C and ST96 strains known as a ‘capsule switch’, and not by a point mutation in 6B strains. The recombination probably occurred on Sado Island, based on several lines of evidence: (i) ST32019A

<table>
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<tr>
<th>PCR-based genotype*</th>
<th>n (%)</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistance</th>
<th>mefA/E</th>
<th>ermB</th>
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<td>gPSSP (none)</td>
<td>52 (10.5%)</td>
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<td>0</td>
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<td>18</td>
<td>8</td>
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<td>130 (26.2%)</td>
<td>127</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>68</td>
<td>38</td>
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<td>gPISP (1a + 2x)</td>
<td>104 (20.9%)</td>
<td>73</td>
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<td>gPISP (2x + 2b)</td>
<td>45 (9.1%)</td>
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<td>gPRSP (1a + 2x + 2b)</td>
<td>166 (33.4%)</td>
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<td>130</td>
<td>34</td>
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<td>497 (100%)</td>
<td>263 (52.9%)</td>
<td>199 (40.0%)</td>
<td>34 (6.8%)</td>
<td>28 (5.6%)</td>
<td>243 (48.9%)</td>
<td>151 (30.4%)</td>
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*1a, *pbp1a*; 2x, *pbp2x*; 2b, *pbp2b*.
†One isolate was not available for the susceptibility test.

Fig. 2. Recombination of the capsular biosynthetic loci in ST906B, ST29246B, ST29246C and ST29246D. The capsule locus resides between genes *wchA* and *wzx*. The genes in the serogroup 6 loci vary in size. The position of nt 1 in this figure corresponds to nt 5545 in the previous report by Bratcher et al. (2011). The genes of the capsular biosynthetic locus of ST906B and ST29246B isolates are shown as shaded arrows and those of ST29246C isolate as open arrows. A filled box indicates a putative site of recombination. The hatched box indicates the indel sequence. The length of the nucleotide sequence and the putative recombination sites are indicated under the capsular biosynthetic locus of ST29246D isolate.
and ST906B strains have spread throughout Sado Island; (ii) one subject had the ST906B strain (SP598) before having the ST29246D strain (SP1090); (iii) there is limited movement of people (particularly children) between Sado Island and the Japanese mainland; and (iv) our study is the only report of serotype 6D in Japan. Another possible explanation is that the ST29246D strain was derived from a recombination of the ST29246C and ST29246B strains. The sequence from the wchA to the wpx gene of the ST29246B isolates was identical to that of the ST906B isolates but different from those of strains ST29246C and ST29246D. The ST29246B strain seems to have emerged by a recombination of the ST29246C and ST906B strains by exchange of the entire capsule locus. The ST29246D strain then emerged, if all recombination events occurred on Sado Island.

Bratcher et al. (2011) suggested that serotype 6D cps may have resulted from a recombination once between serotypes 6B and 6C at a location between the wciN and wciP loci and then spread worldwide, because cps profiles among their serotype 6D isolates were almost identical (5-1-1 and a single-base mutation of 5-1-1). However, our results showed a different recombination site (wciN and wpx) and a different cps profile (8-7-11), indicating that serotype 6D cps can also emerge through recombination between serotypes 6B and 6C involving wciN and wpx. These results suggest that serogroup 6 strains have multiple sites for cps recombination.

In conclusion, our results indicate that prevention of infection by drug-resistant clones could effectively reduce the prevalence of pneumococcal drug resistance. The serotypes in the 13-valent PCV include the drug-resistant clones. By contrast, our results also show that recombination in two capsular loci of serogroup 6 isolates is a mechanism of vaccine escape by S. pneumoniae.

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