Abstract

Purpose. In Japan, the 7-valent pneumococcal vaccine (PCV7) was introduced in 2010 and, in 2013, the PCV7 was replaced with the 13-valent pneumococcal vaccine (PCV13). This study was conducted to investigate serotypes, antimicrobial resistance and prevalence of pilus islets in pneumococcal isolates from inpatients in a Japanese tertiary hospital.

Methodology. From April 2011 to February 2016, 151 isolates [95 (18 children, 77 adults) and 56 (19 children, 37 adults) in the PCV7 and PCV13 periods, respectively] were collected. All isolates were serotyped using genetic methods and were tested for susceptibility to 18 antimicrobials. Unaltered penicillin-binding protein (PBP) genes, macrolide resistance genes and pilus islets were identified by PCR.

Results. Between the two periods, the prevalence of non-PCV13 serotypes was shown to increase from 50.0 to 78.9 % in children, and serotype 3 increased from 14.3 to 24.3 % in adults. Six of seven isolates from invasive diseases were assigned to non-PCV13 serotypes. Overall, multidrug resistance (MDR) was detected in 46.4 % of isolates, which included the dominant non-PCV13 serotypes 6E, 15A and 23A (prevalence ≥ 75.0 %). gPRSP (three altered genes pbp1a, pbp2b and pbp2x) and macrolide resistance genes [erm(B) and/or mef(A/E)] were detected in 35.8 and 93.4 % of all isolates, respectively. Pilus islets [PI-1 (clade I, II and III) and/or PI-2] were found in 22.5 % (34/151) of isolates belonging to six different serotypes (19F, 23F, 19A, 6E, 15B and 35B) and 88.2 % (30/34) of these exhibited MDR.

Conclusion. This study revealed the spread of MDR in several non-PCV13 serotypes and in isolates with pilus islets.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) remains the leading cause of invasive pneumococcal disease (IPD) such as bacteremia and meningitis, and non-IPD including otitis media, mainly in children and the elderly. The capsular polysaccharides (CPSs) of pneumococci are the principal virulence factors utilized for effective vaccines [1]. The pneumococci have been classified into nearly 100 different serotypes [2]. In 2000, a 7-valent pneumococcal conjugate vaccine (PCV7) for serotypes 4, 6B, 9V, 14, 18C, 19F and 23F was introduced for children in the USA. Since then, PCVs, including PCV10 (PCV7 plus serotypes 1, 5 and 7F) and PCV13 (PCV7 serotypes plus 1, 3, 5, 6A, 7F and 19A), have been implemented in childhood immunization schedules in more than 120 countries and PCV13 has been approved for use in adults in more than 100 countries [3].

In the USA, after the replacement of PCV7 by PCV13 in 2010, a decline in the rate of IPD and non-IPD with PCV serotypes was observed through direct and indirect effects of vaccination among all age groups [4]. However, after
widespread implementation of the PCVs, multidrug resistance (MDR) among non-PCV13 serotypes, especially serotypes 15B, 35B and 23A, increased in the USA [5]. The spread of non-PCV13 serotypes such as 15A and 35B with penicillin-nonsusceptible \textit{S. pneumoniae} (PNSP; 37.8\%) was reported in France [6]. Increases of non-vaccine serotypes and resistance to both penicillin and erythromycin have also been observed in a pneumococcal carriage study among children in Portugal [7]. A more recent study suggested that the rate of non-vaccine serotype carriage was 90.9\% in infants aged 2 months who had not been vaccinated with PCV13 [8].

Another bacterial virulence factor, a pneumococcal pilus, was first identified in 2006 [9]. To date, two types of pil encoded by the pilus islets (PI-1 and PI-2) of pneumococcus have been described and proposed as vaccine candidates [10–12], of which PI-1 is classified into three clades [13]. PI-1 was identified in 14.4\% of IPD isolates and 18\% of carrier isolates in Spain [14], and the rate of PI-2 among IPD isolates was shown to increase from 3.6\% in 1999 to 21\% in 2006 in the USA [15]. The presence of PI-1 and/or PI-2 is mainly associated with penicillin-nonsusceptible isolates of vaccine serotypes [16]; however, information regarding the prevalence of pilus islets among non-vaccine serotype pneumococci is limited.

In Japan, the first pneumococcal conjugate vaccine, PCV7, was introduced as a voluntary vaccination in children aged <5 years in February 2010. In April 2013, PCV7 was approved for the national immunization programme and was replaced by PCV13 in November 2013. Our previous cross-sectional study on non-IPD in outpatients across all age groups demonstrated an increase of non-PCV13 serotypes from 39.7\% in 2011 to 72.9\% in the PCV13 period (P<0.001) [17].

The aim of the present study was to evaluate the prevalence of serotypes, genotypes and antimicrobial susceptibility among clinical isolates of pneumococci causing IPD and non-IPD in a university hospital. The prevalence of pilus islets and their relatedness to other bacteriological characteristics were of particular interest in this study.

**METHODS**

**Pneumococcal strains**

A total of 151 clinical isolates (37 from children, 114 from adults) of \textit{S. pneumoniae} from patients with IPDs (seven isolates from blood or cerebrospinal fluid) and non-IPDs (144 isolates from nasal discharges, sputum or other nonsterile sites) were collected at the Sapporo Medical University Hospital in Hokkaido, Japan. Children and adults were grouped by age (<16 years of age and ≥16 years of age) based on previously published studies [6, 18, 19]. Among the isolates, 95 (18 children, 77 adults) and 56 (19 children, 37 adults) were collected in the PCV7 (April 2011 – October 2013) and PCV13 (November 2013 – February 2016) periods, respectively. All isolates were stored by Microbank (Pro-lab Diagnostics, Richmond Hill, Canada) at −80°C until analysed.

**Serotyping and antimicrobial susceptibility testing**

For serotyping and antimicrobial susceptibility testing, isolates were cultivated overnight at 37°C in 5\% CO\textsubscript{2} on trypticase soy agar with 5\% sheep blood agar (Nippon Becton Dickinson). All isolates were serotyped by sequential multiplex PCR methods targeting the \textit{cps} locus using primers previously designed and recommended by the Centers for Disease Control and Prevention (CDC) [20, 21] for pneumococcal serotype deduction of 70 serotypes. After the PCRs, additional genetic subclassing for serogroups 6 and 15 was performed as reported previously [22, 23]. All PCRs were performed in a 25 µl PCR mixture containing 2.5 µM 10× Ex Taq buffer, 200 µM dNTP, 0.5–2.0 µM of each primer, 0.25 U Ex Taq DNA polymerase (Takara Bio, Japan) and 2 µl DNA template extracted from isolates. The PCR products were visualized by electrophoresis on 2% agarose gel.

Antimicrobial susceptibilities for all isolates were assessed using the broth microdilution method using the Dry Plate Eiken HW04 (Eiken Chemical, Tokyo, Japan), measuring MIC within a limited concentration range. The antimicrobial agents examined were penicillins (penicillin, PEN; ampicillin, AMP; ampicillin-sulbactam, SAM), macrolides (erythromycin, ERY; clarithromycin, CLR; azithromycin, AZM), clindamycin (CLI), cephalosporins (cefepime, FEP; cefuroxime, CXM; ceftriaxone, CRO), carbapenems (imipenem, IPM; meropenem, MEM), quinolones (levofloxacin, LVX; moxifloxacin, MXF; gatifloxacin, GAT), vancomycin (VAN), tetracycline (TET) and trimethoprim-sulfamethoxazole (SXT). The reference strain \textit{S. pneumoniae} ATCC 49619 was used for quality control. The Clinical and Laboratory Standards Institute (CLSI) breakpoints (susceptible, S; intermediate, I; or resistant, R) were employed [24]. For interpretation of PEN, FEP, CRO and CXM breakpoints, the CLSI criteria for meningitis cases or oral administration were used. Based on the CLSI criteria for meningitis cases, PEN was considered S at MIC ≤0.06 µg ml\textsuperscript{-1} and R at MIC ≥0.12 µg ml\textsuperscript{-1}, and FEP, CRO and CXM were considered S at MIC 0.5 µg ml\textsuperscript{-1}, I at MIC 1 µg ml\textsuperscript{-1} and R at MIC ≥2 µg ml\textsuperscript{-1}. For oral administration, PEN was considered S at MIC ≤0.06 µg ml\textsuperscript{-1}, I at MIC ≥0.12–1 µg ml\textsuperscript{-1} and R at MIC ≥2 µg ml\textsuperscript{-1}, and FEP, CRO and CXM were considered S at MIC ≤1 µg ml\textsuperscript{-1}, I at MIC 2 µg ml\textsuperscript{-1} and R at MIC 4 µg ml\textsuperscript{-1}. Because breakpoints for AMP and SAM are not provided by the CLSI, the EUCAST breakpoint for \textit{S. pneumoniae} [25] was used to interpret susceptibility to the two antimicrobial agents. Non-susceptibility to PEN (MIC ≥0.12 µg ml\textsuperscript{-1}) combined with resistance to two or more non-β-lactam antimicrobial classes was defined as MDR.

**Detection of pilus islets and resistance genes and assignment of PBP genotypes**

All isolates were examined for the presence of the two pilus islets (PI-1 and PI-2) by PCR and all PI-1 positive isolates were classified into three clades (I, II or III) by a subsequent PCR using previously described primers [16]. Multiplex PCRs were used to identify macrolide resistance genes \textit{erm} (B) and \textit{mef}(A/E) [26, 27], three unaltered PBP genes.
\((\text{pbp1a}, \text{pbp2x})\) and pneumococcal virulence factor gene \(\text{lytA}\) encoding autolysin \cite{26,28}. Based on the previously published scheme \cite{28}, the genotype of penicillin resistance (PBP genotypes) was represented as gPRSP (three \(\text{pbp}\) gene alterations), gPISP (one or two \(\text{pbp}\) gene alterations) and gPSSP (non-altered \(\text{pbp}\) genes). gPISP was expressed with the altered PBP gene(s) in parentheses, for example, gPISP \((\text{pbp2b})\).

**Statistical analysis**

Statistical analysis was carried out using SPSS 19.0 (IBM, Armonk, NY) with the level of significance set at \(P<0.05\). A two-tailed chi-square test or Fisher’s accurate probability methods (for small group sizes) was used to assess correlations between antimicrobial susceptibility and pilus genes among different serotypes.

**RESULTS**

**Serotype distribution**

The distribution of pneumococcal serotypes among isolates from children and adults is shown in Table 1. In children, no PCV7 serotypes were detected during the PCV13 period, while prevalent serotypes (accounting for 57.9\%) were 15A, 23A, 19A and 6E. The proportion of the non-PCV13 serotypes in children increased from 50.0\% in the PCV7 period to 78.9\% in the PCV13 period; however, this difference was not significant \((P=0.091)\). In contrast, in adults, serotype 3 (a PCV13 serotype) was the most prevalent and showed an increasing trend (from 14.3\% in the PCV7 period to 24.3\% in the PCV13 period), resulting in an increased rate of PCV13 serotypes from the PCV7 to the PCV13 period (from 48.1 to 59.5\%). Throughout this study, seven (4.6\%) isolates with six different serotypes were collected from adults with IPD. Among them, six isolates belonged to non-PCV13 serotypes (Table 2).

**Antimicrobial susceptibility**

Of the 151 isolates, 70 (46.4\%) were PNSP (MIC \(\geq 0.12 \mu g/ml\)) \cite{40.4\%; PEN-intermediate \(\text{S. pneumoniae}\), 6.0\%; PEN-resistant \(\text{S. pneumoniae}\), 75.0\% non-susceptibility rates were found against macrolides (ERY, CLR and AZM; \(\geq 94.1\%\)), TET (88.1\%) and CLI (64.9\%). Non-PCV13 serotypes 6E, 15A and 23A were fully resistant to ERY and TET. Overall, MDR was identified in 46.4\% of the isolates with the highest MDR rate in serotypes 19F and 23A.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Serotype} & \text{PCV7 period} & \text{PCV13 period} \\
\hline
& \text{Children} & \text{Adults} & \text{Total} & \text{Children} & \text{Adults} & \text{Total} \\
\hline
\text{PCV7} & \text{Children} & \text{Adults} & \text{Total} & \text{Children} & \text{Adults} & \text{Total} \\
\hline
\text{6B} & 1 (5.6) & 2 (2.6) & 3 (3.2) & 0 & 0 & 0 \\
\text{9V} & 0 & 1 (1.3) & 1 (1.1) & 0 & 0 & 0 \\
\text{14} & 0 & 3 (3.9) & 3 (3.2) & 0 & 0 & 0 \\
\text{19F} & 4 (22.2) & 5 (6.5) & 9 (9.5) & 0 & 4 (10.8) & 4 (7.1) \\
\text{23F} & 0 & 6 (7.8) & 6 (6.3) & 0 & 4 (10.8) & 4 (7.1) \\
\hline
\text{Additional PCV13} & & & & & & \\
\text{6A} & 1 (5.6) & 4 (5.2) & 5 (5.3) & 1 (5.3) & 1 (2.7) & 2 (3.6) \\
\text{3} & 0 & 11 (14.3) & 11 (11.6) & 1 (5.3) & 9 (24.3) & 10 (17.9) \\
\text{19A} & 3 (16.7) & 5 (6.5) & 8 (8.4) & 2 (10.5) & 4 (10.8) & 6 (10.7) \\
\hline
\text{Non-PCVs} & & & & & & \\
\text{6C} & 2 (11.1) & 4 (5.2) & 6 (6.3) & 1 (5.3) & 0 & 1 (1.8) \\
\text{6E} & 0 & 7 (9.1) & 7 (7.4) & 2 (10.5) & 3 (8.1) & 5 (9.9) \\
\text{7B/7C/40} & 0 & 0 & 0 & 0 & 1 (2.7) & 1 (2.7) \\
\text{10A} & 0 & 4 (5.2) & 4 (4.2) & 0 & 1 (2.7) & 1 (1.8) \\
\text{11A/11D} & 1 (5.6) & 1 (1.3) & 2 (2.1) & 1 (5.3) & 3 (8.1) & 4 (7.1) \\
\text{15A} & 0 & 5 (6.5) & 5 (5.3) & 4 (21.1) & 1 (2.7) & 5 (8.9) \\
\text{15B} & 0 & 2 (2.6) & 2 (2.1) & 0 & 1 (2.7) & 1 (1.8) \\
\text{15C} & 1 (5.6) & 3 (3.9) & 4 (4.2) & 1 (5.3) & 1 (2.7) & 2 (3.6) \\
\text{22F/22A} & 1 (5.6) & 2 (2.6) & 3 (3.2) & 1 (5.3) & 0 & 1 (1.8) \\
\text{23A} & 2 (11.1) & 8 (10.4) & 10 (10.5) & 3 (15.8) & 1 (2.7) & 4 (7.1) \\
\text{33A/33F/37} & 0 & 0 & 0 & 1 (5.3) & 2 (5.4) & 3 (5.4) \\
\text{35B} & 2 (11.1) & 4 (5.2) & 6 (6.3) & 1 (5.3) & 2 (5.4) & 3 (5.4) \\
\hline
\text{Total} & 18 & 77 & 95 & 19 & 37 & 56 \\
\end{array}
\]
Prevalence of altered pbp genes and macrolide resistance genes

Among PBP gene genotypes in all isolates, gPISP (pbp2x; 37.1 %) and gPRSP (35.8 %) were the most prevalent, while only 5.3 % were gPSSP (Table S1, available in the online Supplementary Material). The detection rates of gPRSP were significantly higher in serotypes 19F (92.9 %, P<0.001), 23F (90.0 %, P<0.001) and 6E (75.0 %, P=0.009) than in other serotypes, and a high rate was also seen in serotypes 19A (50 %, P=0.256) and 15A (80 %, P=0.168) (Fig. 1). Most of the serotype 3 (90 %) and 23A (92.9 %) isolates exhibited the gPISP (pbp2x) and gPISP (pbp2x+2b) genotypes, respectively (P<0.001). Among all isolates, 74.2, 29.1 and 9.9 % harboured erna(b), mef(A/E) and both these genes, respectively (Table S1).

Prevalence of the pilus islets PI-1 and PI-2

Of the 151 isolates, 34 (22.5 %) possessed at least one pilus islet, and the pilus islet-positive isolates belonged to six serotypes (19F, 23F, 19A, 6E, 15B and 35B) (Table 4). The prevalence of pilus islets (solely PI-1, solely PI-2 and both these islets) was 13.3, 1.3 and 7.9 %, respectively. None of the seven IPD isolates had pilus islets. Isolates carrying solely PI-1 clade I, -clade II and -clade III were found in serotypes 19A, 6E and 23F/35B, respectively. Solely PI-2 was positive in serotype 19F and 15B isolates (one isolate each) that were PNSP. Two PIs (PI-1 clade I and PI-2) were found in only serotype 19F and 19A isolates, all of which were PNSP. In addition, all the 15B and 35B PNSP isolates had pilus islets and exhibited non-susceptibility to CXM and SXT. The pilus islet rate in PNSP was significantly higher in serotype 35B than in other piliated PNSP isolates (P=0.012). Overall, 88.2 % (30 isolates) of 34 isolates with PI-1 and/or PI-2 exhibited MDR.

**DISCUSSION**

A global increase in the prevalence of non-vaccine serotypes and their antimicrobial resistance has been noted following the worldwide implementation of PCVs. After the implementation of PCV13, increased MDR caused by the non-PCV13 serotypes 35B, 15B and 23A was observed in 42 medical centres in the USA in 2012–2013 [5]. In studies on pneumococcal carriage in children from Asian countries, serotype replacement due to vaccine introduction has also been observed. In Hong Kong, a predominance of non-PCV13 serotypes such as serogroup 15 and serotypes 23A and 6C was found, with non-susceptible rates for PEN and ERY being 7.3 and 79.3 %, respectively [8]. In a study from Korea, non-PCV13 serotypes were detected in 88.3 % of isolates containing the most prevalent serotypes 23A, 15B and 15C, and proportions of non-susceptibility to PEN, ERY and MDR were 86.0, 90.5 and 81.5 %, respectively [29]. These studies illustrate the high prevalence rates of MDR associated with non-PCV13 serotypes.
Table 3. Antimicrobial susceptibility of the 151 pneumococcal isolates among individual serotypes

<table>
<thead>
<tr>
<th>Serotype*</th>
<th>No. of isolates</th>
<th>Percentage of intermediate resistance (I), resistance (R), and MDR in each serotype†‡§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEN I/R</td>
<td>AMP R</td>
</tr>
</tbody>
</table>
| 6B        | 3               | 66.7/0                                 | 33.3                                  | 33.3    | 0/100  | 0/100   | 0/66.7   | 0/0    | 33.3/0  | 0/0    | 33.3/0  | 0/100   | 33.3/66.7 | 66.7 /
| 9V        | 1               | 0/0                                    | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/0      | 0/0    | 0/0     | 0/0    | 0/0     | 0/0     | 0/0          |
| 14        | 3               | 33.0/0                                 | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 33.0/0       |
| 19F       | 13              | 69.2/23.1                              | 61.5                                  | 61.5    | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 76.9/7.7    |
| 23F       | 10              | 80.0/100                               | 60/60                                 | 0/100   | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 80.0/200    |
| 6A        | 7               | 28.6/28.6                              | 28.6                                  | 28.6    | 0/71.4 | 0/71.4   | 0/71.4    | 0/0    | 0/0     | 0/0    | 0/0     | 0/0     | 0           |
| 3         | 21              | 0/0                                    | 0/0                                   | 0/0     | 0/0    | 0/0     | 0/0      | 0/0    | 0/0     | 0/0    | 0/0     | 0/0     | 0           |
| 19A       | 14              | 429/7.1                                | 14.3                                  | 14.3    | 0/85.7 | 0/85.7   | 0/85.7    | 0/0    | 0/0     | 0/0    | 0/0     | 0/0     | 429/7.1     |
| 6C        | 7               | 0/0                                    | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 85.7/0      |
| 6E        | 12              | 75.0/0                                 | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 16.7/83.3   |
| 7B/7C/40  | 1               | 0/0                                    | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 0/0          |
| 10A       | 5               | 0/0                                    | 0/0                                   | 0/0     | 0/60.0 | 0/60.0   | 0/60.0    | 0/0    | 0/0     | 0/0    | 0/0     | 0/60.0  | 0/0           |
| 11A/11D   | 6               | 0/0                                    | 0/0                                   | 0/0     | 0/83.3 | 0/83.3   | 0/83.3    | 0/0    | 0/0     | 0/0    | 0/0     | 0/83.3  | 100/0        |
| 15A       | 10              | 70.0/20.0                              | 80/80                                 | 80/80   | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 80/20.0      |
| 15B       | 3               | 33.3/0                                 | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 33.3/0       |
| 15C       | 6               | 16.7/0                                 | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 16.7/0       |
| 22F/22A   | 4               | 0/0                                    | 0/0                                   | 0/0     | 0/75.0 | 0/75.0   | 0/75.0    | 0/0    | 0/0     | 0/0    | 0/0     | 0/75.0  | 25.0/0        |
| 23A       | 14              | 78.6/7.1                               | 0/7.1                                 | 0/7.1   | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 78.6/7.1     |
| 35F/33A/37| 2               | 0/0                                    | 0/0                                   | 0/0     | 0/100  | 0/100   | 0/100    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 0/0          |
| 35B       | 9                | 33.3/0                                 | 33.3                                  | 33.3    | 0/88.9 | 0/88.9   | 0/88.9    | 0/0    | 0/0     | 0/0    | 0/0     | 0/100   | 22.2/77.8    |
| Total     | 151             | 40.4/6.0                               | 19.9                                  | 19.9    | 0/79.3 | 7.6/86.8 | 2.0/92.1  | 2.0/62.9| 0.7/0.7 | 26.5/12.6| 0.7/2.0 | 15.2/0  | 9.3/0       | 1.3/86.8 | 37.7/16.6 | 46.4 |

*PCV7- and PCV13-serotypes are shown in bold and bold with underline, respectively.
†All isolates were susceptible to LVX, MXF, GAT and VCM.
‡The CLSI does not provide MIC breakpoints for AMP and SAM. Therefore, the EUCAST breakpoints of AMP (S<0.5µg ml⁻¹, R>2µg ml⁻¹) were employed. SAM susceptibility was inferred from the MIC of AMP. Only resistance rates were shown for AMP and SAM.
§Multidrug resistance (MDR) was defined as non-susceptibility to PEN (MIC ≥0.12µg ml⁻¹) combined with resistance to two or more antibiotic classes.

Kawaguchiya et al., Journal of Medical Microbiology 2017;66:643–650
In Japan, studies on patients with IPD after the introduction of PCV7 in 2010–2013 indicated an increase in non-PCV13 serotypes such as 15A and 35B with IPD in children [30] and adults [3]. Our previous study in the community also revealed an increase of non-PCV13 serotypes causing non-IPD in both children and adults (from 39.7 % in 2011 to 72.9 % in the PCV13 period; \( P < 0.001 \)) and MDR was prevalent in non-PCV13 serotypes (15A, 23A, 6C and 35B) among children with non-IPD in 2014 [31]. Similarly, in our present study on hospital-associated \( S. \ pneumoniae \) isolates, the rate of non-PCV13 serotypes in children was 78.9 % after the implementation of PCV13. Similar rates in children have been documented in recent studies on meningitis in France [6] and pneumococcal carriage in Hong Kong [8]. The MDR rate in the present study was 46.4 %, and prevalent non-PCV13 serotypes 15A, 23A and 6E showed the highest MDR rates within each serotype (75–90 %). Other non-PCV13 serotypes 15B and 35B also contained MDR isolates (33.3 % each). The findings of the present study are therefore in agreement with those of reports from Japan and other countries where, notably, the high prevalence of serotype 6E was observed. Taken together, serotype replacement and the emergence of MDR have been occurring in the PCV13 period globally, and it remains an important concern after the widespread use of the polysaccharide vaccines (PSVs).

In adults, serotype 3 is the most prevalent, and its prevalence has increased in the present study period. Vaccination for adults in Japan [two pneumococcal vaccines, a 23-valent pneumococcal polysaccharide vaccine (PPSV23) and PCV13] has been available for use in adults 65 years or older for routine and voluntary vaccinations, respectively, since 2014. Although PPSV23 was first introduced as a voluntary vaccination in Japan in 1988, the immunization rate among the elderly in the PPSV23 voluntary period was low (less than 18 % in 2012) [3]. Further, although PPSV23 has been approved as a routine vaccination for adults since October 2014, the vaccine is administered at specific ages at five-year intervals (aged 65, 70, 75, 80, 85, 90, 95 and ≥100 year of age) at present. In the present study, PPSV23 and PCV13 serotypes were highly prevalent among adults in the PCV13 period (53.6 and 70.3 %, respectively), mostly due to the increase of serotype 3, which is included in both PCV13 and PPSV23. Similar persistence of serotype 3 among adults with IPD has been observed in 16.4 % in 2010–2011 to 18.5 % in 2012–2013 in Japan [3]. The high prevalence of serotype 3 among adults is therefore still thought to persist and to be associated with a low rate of vaccinations with the PPSV23 and/or PCV13 for adults. In addition, six of seven IPD isolates from adults belonged to non-PCV13 serotypes. Among them, non-vaccine serotype 7B/7C/40 was first detected in an IPD case in the present study; although this
serotype was detected in non-IPD isolates in a study from the USA [32].

In the present study, the pilus islet was found in vaccine serotypes 19F, 23F and 19A as well as in non-PCV13 serotypes 6E, 15B and 35B. Most of the isolates were PNSP (n=30/34, 88.2%), and all the piliated PNSP isolates were MDR. The presence of pilus islets has been described previously in PCV types such as 6B, 9V, 14, 19A and 19F [15, 33–35]. In fact, only serotypes 19F and 19A had both PI-1 and PI-2, and most of the isolates with PCV13 serotypes harboured pili genes [35]. The MDR strains of serotypes 19F and 19A were furthermore strongly associated with the presence of pilus islets [33]. In our study, the presence of both pilus islets was also only observed in serotypes 19F and 19A and most serotype 19F isolates had both pilus islets (n=12/13, 92.3%). More recently, the emergence of pilus islets in non-PCV13 serotypes 15B/C, 35B, 11A and 19B was described [11]. In the present study, pilus genes among non-PCV13 serotypes 6E, 15B and 35B belonged to different pilus islet groups and/or clades. Notably, pilus islets were first detected in serotype 6E in the present study. Our findings indicated a correlation between serotypes, the presence of pilus islets including clade types, as well as antimicrobial resistance, especially β-lactam resistance. Of note, there was an association between several non-PCV13 serotypes and the presence of PIs. These findings may provide a basis for pilus islets as potential antigens for future protein vaccines.

In conclusion, our results reveal the prevalence of non-PCV13 serotypes with MDR, such as 15A, 23A and 6E, in hospital S. pneumoniae isolates in the PCV13 era. A correlation between pilus islets and non-PCV13 serotypes was furthermore demonstrated. Ongoing surveillance on serotypes and antimicrobial resistance with virulence proteins including pilus islets is therefore required for designing vaccines independent of serotype.

### Funding information
This study was supported in part by a Grant-in-Aid for Scientific Research (NO. 16K09101) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

### Conflicts of interest
The authors declare that there are no conflicts of interest.

### Ethical statement
In this study, no human participants were involved directly. Hence, human ethics clearance was not required. We used S. pneumoniae isolates routinely cultured from clinical specimens in the hospital.

### References

---

### Table 4. Pilus islet 1 (PI-1) and/or pilus islet 2 (PI-2) clades detected in each serotype of PSSP and PNSP

<table>
<thead>
<tr>
<th>Serotype*</th>
<th>Total</th>
<th>No. of isolates</th>
<th>PSSP/PNSP†</th>
<th>Pilus islet (%)</th>
<th>Pilus islets (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV7/13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19F</td>
<td>13</td>
<td>PSSP</td>
<td>1</td>
<td>1 (100)</td>
<td>PI-1 clade I+ PI-2 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNSP</td>
<td>12</td>
<td>11 (91.7)</td>
<td>PI-1 clade I+ PI-2 (10), PI-2 (1)</td>
</tr>
<tr>
<td>23F</td>
<td>10</td>
<td>PSSP</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNSP</td>
<td>9</td>
<td>3 (33.3)</td>
<td>PI-1 clade III (3)</td>
</tr>
<tr>
<td>19A</td>
<td>14</td>
<td>PSSP</td>
<td>7</td>
<td>1 (14.3)</td>
<td>PI-1 clade I (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNSP</td>
<td>7</td>
<td>5 (83.3)†</td>
<td>PI-1 clade I (4), PI-1 clade I + PI-2 (1)</td>
</tr>
<tr>
<td>non-PCVs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6E</td>
<td>12</td>
<td>PSSP</td>
<td>3</td>
<td>2 (66.6)</td>
<td>PI-1 clade II (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNSP</td>
<td>9</td>
<td>7 (77.8)</td>
<td>PI-1 clade II (7)</td>
</tr>
<tr>
<td>15B</td>
<td>3</td>
<td>PSSP</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNSP</td>
<td>1</td>
<td>1 (100)</td>
<td>PI-2 (1)</td>
</tr>
<tr>
<td>35B</td>
<td>9</td>
<td>PSSP</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNSP</td>
<td>3</td>
<td>3 (100)§</td>
<td>PI-1 clade III (3)</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>PSSP</td>
<td>20</td>
<td>4 (20.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PNSP</td>
<td>41</td>
<td>30 (73.2)</td>
<td></td>
</tr>
</tbody>
</table>

*The pilus islet was found in serotypes 19F, 23F, 19A, 6E, 15B and 35B of all isolates. Other serotypes, 6B, 9V, 14, 6A, 3, 10A, 11A/11D, 22F/22A, 33A/33F/37, 6C, 7B/7C/40, 15A, 15C and 23A have no pilus islet (date not shown).
†PSSP, penicillin-susceptible pneumococci; PNSP, penicillin-nonsusceptible pneumococci.
‡P=0.103, between PSSP and PNSP.
§P=0.012, between PSSP and PNSP.
||P=0.001, between PSSP and PNSP.


