First Report of Putative *Streptococcus pneumoniae* Serotype 6D among Nasopharyngeal Isolates from Fijian Children

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Background. A putative *Streptococcus pneumoniae* serotype, 6D, resulting from the introduction of *wciN* into serotype 6B has been proposed.

Methods. We studied 98 unique serogroup 6 isolates from Fijian children, two-thirds of whom had received at least 1 dose of 7-valent pneumococcal conjugate vaccine, and 51 invasive isolates from Australian children. We used a polymerase chain reaction (PCR) system that targets both *wciN* and the single-nucleotide polymorphism that differentiates serotypes 6A and 6B—*wciP584g* (6A) and *wciP584a* (6B).

Results. Two (9%) of 22 Australian isolates and 24 (38%) of 64 Fijian isolates previously identified as 6A by the Quellung reaction and *wciP584g* PCR contained *wciN* and were designated as 6C; 14 (41%) of 34 Fijian isolates previously identified as 6B by the Quellung reaction and *wciP584a* PCR contained *wciN* and were designated as the new putative serotype 6D. A significantly smaller proportion of children from whom serotype 6D was isolated (2/14 [14%]) had not received PCV-7, compared with the proportion of those from whom serotype 6B was isolated (11/20 [55%]) (*P* < .05).

Conclusion. This is the first report of naturally occurring *S. pneumoniae* serotype 6D.

*Streptococcus pneumoniae* is an important human pathogen, especially in developing countries; it accounts for >1.2 million deaths among children annually [1]. There are 48 serogroups comprising 91 serotypes, including the recently identified 6C [2, 3]. Serotypes belonging to serogroup 6 are among the most common that cause invasive disease in children [4].

Classically, serogroup 6 comprised 2 serotypes—6A and 6B [5]—that produce biochemically similar capsules, differing only in their rhamnose-ribitol linkages. The only difference in their capsular gene loci is a single-nucleotide polymorphism (SNP) in *wciP* (584a [195N] in 6B and 584g [195S] in 6A), which encodes rhamnosyl transferase [6, 7]. This SNP is the basis of polymerase chain reaction (PCR) methods developed to differentiate serotypes 6A and 6B [8, 9]. The new serotype 6C was identified in 2007 among isolates initially identified as 6A by the Quellung reaction, on the basis of differential reactions with 2 monoclonal antibodies [2].

The only difference between 6A and 6C polysaccharides is the replacement of galactose in 6A with glucose in 6C [2], which is apparently the result of homologous recombination resulting in the substitution of *wciN* of 6A (*wciN6a*) with a different gene (*wciN6c*), referred to as *wciNop*, of an unknown source [3].

It has been postulated that *wciNop* could be also introduced into the serotype 6B operon to form another new serotype, tentatively designated 6D [10]. This possibility was recently confirmed experimentally [11], but
the putative serotype 6D has not been identified among pneumococcal clinical isolates to date [11–13].

We have developed a new serotype-specific PCR assay to differentiate serotypes 6A, 6B, and 6C [9], and we used this assay in the present study to test serogroup 6 isolates from Fijian and Australian children.

**METHODS**

*S. pneumoniae strains.* A total of 179 *S. pneumoniae* serogroup 6 isolates, which had been typed by the Quellung reaction and multiplex PCR–based reverse line blot hybridization assay [14, 15] at the Centre for Infectious Diseases and Microbiology (CIDM), were used for this study. They included 121 nasopharyngeal and 7 invasive isolates collected between 2004 and 2007 from Fijian children aged 6–18 months (85 initially identified as 6A and 29 as 6B) and 51 invasive isolates collected from children aged <5 years in New South Wales in 2005 (22 initially identified as 6A and 29 as 6B).

Children enrolled in the Fijian vaccine study were randomized to receive either 0, 1, 2, or 3 doses of 7-valent pneumococcal polysaccharide vaccine (PPV-23) at 14 weeks, at 6 and 14 weeks, or at 6, 10, and 14 weeks of age. Half of each group was randomized to receive either 0, 1, 2, or 3 doses of 7-valent pneumococcal conjugate vaccine (PCV-7) at 14 weeks, at 6 and 14 weeks. ISAC was designed to test serogroup 6 isolates from different epidemiological sources.

**Serotype-specific PCR methods.** A 25-μL PCR volume contained 2 μL of template DNA, 0.125 μL of each forward and reverse primer (50 pmol/μL), 1 μL of deoxynucleoside triphosphates (2.5 mmol/L each dNTP), 2.5 μL of 10× PCR buffer (Qiagen), and 0.1 μL of Qiagen HotStar Taq polymerase (5 U/μL); molecular biology-grade H2O (Eppendorf) was added to make up a total volume of 25 μL. The PCR program was performed according to the Qiagen HotStar Taq polymerase kit instructions, as follows: 95°C for 15 min for 1 cycle; 94°C for 30 s, 62°C for 60 s, and 72°C for 60 s for 35 cycles; 72°C for 10 min for 1 cycle; and 22°C hold. Primers wciN_S1 and wciN_A2 were used to amplify wciN (359 bp), primers wciP584s and wciP-r were used to amplify wciP of serotype 6A (149 bp), and primers wciP584a and wciP-r were used to amplify wciP of serotype 6B (155 bp). PCR products were detected by electrophoresis in a 1%–2% agarose gel.

Isolates were also tested by 2 previously published PCR protocols, using primers 6C-fwd and 6C-rev, which amplify a por-

<table>
<thead>
<tr>
<th>Table 1. Oligonucleotide Primers Used in the Present Study</th>
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<tbody>
<tr>
<td>Primer</td>
</tr>
<tr>
<td>wciN_S1</td>
</tr>
<tr>
<td>wciN_A2</td>
</tr>
<tr>
<td>wciP584s</td>
</tr>
<tr>
<td>wciP584a</td>
</tr>
<tr>
<td>wciP-r</td>
</tr>
<tr>
<td>6B (6D)</td>
</tr>
<tr>
<td>5106d</td>
</tr>
<tr>
<td>3101d</td>
</tr>
<tr>
<td>6C-fwd</td>
</tr>
<tr>
<td>6C-rev</td>
</tr>
<tr>
<td>wciP584</td>
</tr>
<tr>
<td>wciP2</td>
</tr>
<tr>
<td>wciPA</td>
</tr>
<tr>
<td>wciPA2</td>
</tr>
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</table>

**NOTE.** A, antisense; fwd, forward; r and rev, reverse; S, sense.

a Nos. indicate sequence positions in corresponding GenBank sequences.

b Primers used in our previous study [9].

c Previously published primers [8].

d Previous published primers [3].

e Previously published primers [13].

f New primers designed for the present study.
Table 2. Sequence Polymorphism Sites of Serotypes 6C and 6D in Partial wchA-wciNwciO (wcANO)

<table>
<thead>
<tr>
<th>Sequence types (no. of isolates)</th>
<th>GenBank accession no.</th>
<th>wchA</th>
<th>wciN</th>
<th>wcIO</th>
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</thead>
<tbody>
<tr>
<td>wcANO-6C1 (15)</td>
<td>FJ899597</td>
<td>C b</td>
<td>A</td>
<td>A b</td>
</tr>
<tr>
<td>wcANO-6C2 (1)</td>
<td>FJ899598</td>
<td>C b</td>
<td>A T</td>
<td>A G</td>
</tr>
<tr>
<td>wcANO-EF538714 a</td>
<td>EF538714</td>
<td>T d</td>
<td>A C</td>
<td>G</td>
</tr>
<tr>
<td>wcANO-6D1 (7)</td>
<td>FJ899599</td>
<td>T b</td>
<td>A G</td>
<td>G b</td>
</tr>
<tr>
<td>wcANO-6D2 (7)</td>
<td>FJ899600</td>
<td>T b</td>
<td>A G</td>
<td>G b</td>
</tr>
<tr>
<td>wcANO-EU714777 a</td>
<td>EU714777</td>
<td>T</td>
<td>A G</td>
<td>A G</td>
</tr>
</tbody>
</table>

NOTE. Amplification was done using primer pair 5106 and 3101, and sequencing was done using primers 5106, 3101, wciN b1 and wciN b2 (see Figure 2 for details).

a Polymorphisms in Fijian serotype 6C and 6D isolates were compared with each other and with GenBank sequences EF538714 (serotype 6C cps gene cluster) and EU714777 (articulated serotype 6D/6X1 cps gene cluster). Position 1 of EF538714 was used as the alignment position.

b Four base differences, causing 4 corresponding amino acid differences between Fijian serotype 6C and 6D isolates.

c One wcAN-6C2–specific site.

d Nine base differences between EF538714 and Fijian serotype 6C; 5 were unique or specific for EF538714.

e One wcAN-6D2–specific site.

f One EU714777–specific site.

Sequencing and sequence analysis. PCR products (wciP and partial wchA-wciNwciO) of all 6D isolates and of selected 6C isolates were amplified and sequenced. Products were purified using the PCR Product Pre-Sequencing kit (USB) and were directly sequenced in both directions using the BigDye Terminator cycle sequencing kit (version 3.1) in an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Primers wciPS1 and wciPA2 were used for amplification (810 bp) of the wciP region, and the inner primer pair wciPS2 and wciPA1 were used for sequencing. For the partial wchA-wciNwciO region, primers 5106 and 3101 were used for amplification (1.8 kb), and primers 5106 and wciN b2 as well as primers 3101 and wciN b1 were used for sequencing. Sequences were compared with known sequences of serotype 6C and the artificial 6D cps loci in GenBank (accession numbers EF538714 and EU714777, respectively) [6, 11] and were analyzed with the BLASTn tool in BioManager (Sydney Bioinformatics; available at: https://www.angis.org.au/).

Six new Fijian 6C and 6D sequences were submitted to GenBank: FJ899597 (6C1), FJ899598 (6C2), FJ899599 (6D1), and FJ899600 (6D2) for partial wchA-wciNwciO, and FJ899601 (6C) and FJ899602 (6D) for near-full-length wciP (Tables 2 and 3).

RESULTS AND DISCUSSION

Fijian isolates. By means of primers wciN b1 and wciN b2, 52 (41%) of 128 serogroup 6 isolates from Fijian children were shown to contain wciN. Results of serotype 6A– and serotype 6B–specific PCRs with primers wciP584gS and wciP-r and
primers wciP584aS and wciP-r, respectively, confirmed those of conventional serotyping. Of the 85 isolates initially serotyped as 6A, 33 (39%) contained wciNp and were redesignated as 6C; of the 43 isolates initially serotyped as 6B, 19 (44%) contained wciNp and were redesignated as putative serotype 6D. Lane 3 (wciNp positive in I) amplified by wciP584aS and wciP-r (6B) and the primer pair wciP584gS and wciP-r (6A), respectively, which target wciP. Lane 1 amplified by wciP584gS and wciP-r only and was identified as serotype 6B. Lane 3 (wciNp positive in I) amplified by wciP584gS and wciP-r (as for 6A) and was designated as serotype 6C. Lane 4 (wciNp positive in I) amplified by wciP584aS and wciP-r (as for 6B) and was designated as serotype 6D.

Review of collection dates and study participant data showed that 18 isolates were duplicates (ie, were from the same specimen) and were excluded. There were 14 pairs of isolates from swab samples collected from the same participants at different times. Serotypes were the same for both members of 12 pairs and the immunization status of the children from whom they were isolated had not changed between collection of swab samples, so 1 of each pair was excluded. Two pairs of isolates from swab samples collected at different times were of different serotypes—6C and 6B were isolated at 6 and 12 months of age, respectively, from an unvaccinated child, and 6B and 6A were isolated at 12 and 17 months, respectively, from a child who had received a single dose of PCV-7 at 14 weeks of age. Both isolates of these pairs were included in the analysis. After these 30 exclusions, 98 unique serogroup 6 isolates from 96 Fijian children remained for analysis.

Of these 98 isolates, 32 were recovered from children who had not received PCV-7 (3 had received PPV-23 only at 12 months of age), and 26, 26, and 14 had received 1, 2, or 3 doses of PCV-7, respectively. The distribution of serotypes 6A, 6B, 6C, and 6D according to the immunization status of the participants is shown in Table 4. A significantly smaller proportion of the serotype 6D than 6B isolates were from children who had not received PCV-7 (P<.05). Differences in immunization status between children from whom other serotypes were isolated were not significant. All 7 invasive isolates (for 6A, 1; for 6B, 4; and for 6C, 2) were from unimmunized children.

**Australian isolates.** Serogroup 6 isolates from Australia were tested as for the Fijian isolates. With primers wciNpS1 and wciNpA2, two (4%) of 51 isolates were shown to contain wciNp. By means of primers wciP584gS and wciP-r and primers wciP584aS and wciP-r, Quellung results were confirmed (22 serotype 6A and 29 serotype 6B). The 2 containing wciNp were among those previously identified as 6A and were therefore redesignated as 6C. No serotype 6D isolates were identified.

**Is the formation of serotype 6D from 6B plausible?** Pneumococcal serotypes 6A and 6B are closely related but are distinguishable using polyclonal antisera. Apart from the one well-defined SNP in wciP, any other genetic differences have not been well defined. We have previously found several sequence polymorphisms in cpsA-cpsB [16], some of which are apparently unique to either 6A or 6B. Others have found evidence for relatively frequent switching between serotype 6A and 6B [6] and sharing of sequence types, suggesting that capsular switching occurs among serotypes 6A, 6B, and 6C [13, 17]. Serotype 6C was apparently derived from 6A by the replacement of wciN with wciNp, which replaces galactosyltransferase.

![Figure 1. Electrophoretic pattern of the polymerase chain reaction products of serotypes 6A, 6B, 6C, and 6D. Lanes 1, 2, 3, and 4 represent 4 Streptococcus pneumoniae strains that had been identified as serotypes 6A, 6B, 6A, and 6B, respectively, by Quellung methods. Lane M indicates a standard marker (100-bp DNA Ladder, catalogue number 15628-019; Invitrogen). Group I isolates were amplified using the primer pair wciNpS1 and wciNpA2, which target wciNp. Lanes 1 and 2 did not amplify. Lanes 3 and 4 amplified with wciNpS1 and wciNpA2, consistent with serotype 6C. Groups II and III isolates were amplified using the primer pair wciP584aS and wciP-r (6B) and the primer pair wciP584gS and wciP-r (6A), respectively, which target wciP. Lane 1 amplified by wciP584gS and wciP-r only and was identified as serotype 6B. Lane 3 (wciNp positive in I) amplified by wciP584gS and wciP-r (as for 6A) and was designated as serotype 6C. Lane 4 (wciNp positive in I) amplified by wciP584aS and wciP-r (as for 6B) and was designated as serotype 6D.](image-url)

Table 4. Distribution of Streptococcus pneumoniae Serogroup 6 Serotypes, According to the Immunization Histories of the Fijian Children from Whom They Were Isolated

<table>
<thead>
<tr>
<th>Serotype, no. of isolates</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A, 40</td>
<td>12 (30)a</td>
<td>12 (30)</td>
<td>8 (20)</td>
<td>8 (20)</td>
</tr>
<tr>
<td>6C, 24</td>
<td>7 (29)</td>
<td>6 (25)b</td>
<td>10 (42)a</td>
<td>1 (4)</td>
</tr>
<tr>
<td>6B, 20</td>
<td>11 (55)b,c</td>
<td>4 (20)</td>
<td>4 (20)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>6D, 14</td>
<td>2 (14)c</td>
<td>4 (29)b</td>
<td>4 (29)</td>
<td>4 (29)</td>
</tr>
<tr>
<td><strong>Total, 98</strong></td>
<td>32</td>
<td>26</td>
<td>26</td>
<td>14</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of isolates by PCV-7 dose group. PCV-7, 7-valent pneumococcal conjugate vaccine; PCV-23, 23-valent pneumococcal vaccine.

a Two of these children received a dose of PCV-23 at 12 months.

b One of these children received a dose of PCV-23 at 12 months.

c The proportion of serotype 6D isolates from children who had not received PCV-7 was significantly lower than that of serotype 6B isolates (14% vs 55%; P<.05; relative risk, 0.26 [95% confidence interval, 0.07–0.99]).
of 6A with glucosyltransferase in 6C. Another new serogroup 6 serotype would be generated if the wciN/galactosyltransferase of 6B were similarly replaced with the wciN/glucosyltransferase [11]; logically, this putative new serotype would be designated serotype 6D or genotype 6D [10]. Recently, such a strain (TIGR6X1) was produced experimentally by inserting wciN into the 6B capsule gene locus [11].

The 14 unique isolates that we have designated as serotype 6D were consistently identified as 6B by means of polyclonal antisera and 6B-specific PCR (ie, they contained the wciP584a SNP). However, 3 separate PCRs using primers wciN,S1 and wciN,A2 and the previously published primers 5106 and 3101 [3] and 6C-fwd and 6C-rev [13] amplified wciN from these isolates, producing amplicons of 359 bp, 1.8 kb, and 727 bp, respectively. We propose that these 14 putative serotype 6D isolates and are the first naturally occurring equivalents of experimental serotype 6X1 to be identified.

PCV-7 was licensed in the United States in 2000 and has been used widely in the United States and Europe since; it was introduced into the routine infant vaccination schedule in Australia in 2005 but has not been widely used in Fiji. Most of the Fijian isolates were from children enrolled in a pneumococcal vaccine trial, two-thirds of whom had received at least 1 dose of PCV-7, which contains 6B antigen and confers cross-protection against serotype 6A but not 6C [18]. Reports that the prevalence of 6C has increased in some places after the introduction of PCV-7 [12, 13, 17, 19] suggests that it may have a selective advantage, although we found similar proportions of immunized and unimmunized children among those colonized with serotypes 6A and 6C. However, 86% of children from whom serotype 6D was isolated had received at least 1 dose of PCV-7, compared with only 45% of those colonized with 6B. This suggests that serotype 6D could have a selective advantage after immunization.

To further investigate the genetic origins of serotype 6D, we sequenced capsular gene regions containing wciN and wciP from all 14 serotype 6D isolates and 16 selected serotype 6C isolates from Fiji. Two different sequence types were identified for each serotype, both of which differed slightly from GenBank sequences, with some consistent differences between 6C and 6D (summarized in Tables 2 and 3; representative sequences are shown in Figures 2 and 3). Like serotype 6C, serotype 6D has a 194-bp deletion in wciN relative to their parental se-

This figure is available in its entirety in the online version of the Journal of Infectious Diseases.

Figure 2. Sequence alignment of Streptococcus pneumoniae serotypes 6C and 6D partial wchA-wciN-wciO, amplified using primer pair 5106 and 3101 and sequenced using primers 5106, 3101, wciNS1, and wciNA2.

Figure 3. Sequence alignment of Streptococcus pneumoniae serotypes 6C and 6D partial wciP, amplified using primers wciPS1 and wciPA2 and sequenced using primers wciPS2 and wciPA1.

Acknowledgments

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References