Molecular Epidemiology of *Streptococcus pneumoniae* Serogroup 6 Isolates from Fijian Children, Including Newly Identified Serotypes 6C and 6D

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Molecular Epidemiology of *Streptococcus pneumoniae* Serogroup 6 Isolates from Fijian Children, Including Newly Identified Serotypes 6C and 6D

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Multilocus sequence typing (MLST) was applied to all unique serotype 6C and 6D isolates and a random selection of serotype 6B and 6A isolates from nasopharyngeal swabs from Fijian children enrolled in a recent vaccine trial. The results suggest that Fijian serotype 6D has arisen independently from both serotypes 6A/C and 6B.

Infection with *Streptococcus pneumoniae* is a leading cause of death in children worldwide (15, 19, 25). *S. pneumoniae* comprises 48 capsular serogroups containing more than 90 serotypes. Serogroup 6 classically consisted of serotypes 6A and 6B. The 7-valent conjugate vaccine (Prevnar; PCV7) includes the 6B antigen and confers some cross-protection against serotype 6A but not 6C (20, 22). Serotype 6C is important in carriage and invasive disease, and its prevalence has increased following widespread use of PCV7 (4, 5, 9, 10, 14, 16, 24).

The existence of serotype 6D has been postulated (8) and was created experimentally (2), but naturally occurring isolates have not been identified (2, 8, 9, 17). Recently, two serotype 6D isolates have been found in Korean children (11). Two additional isolates from Fijian children enrolled in a recent vaccine trial. The results suggest that Fijian serotype 6D has arisen independently from both serotypes 6A/C and 6B.

However, the molecular epidemiology of serotype 6D isolates is otherwise uncharacterized.

In this study, we conducted MLST of serogroup 6 isolates to determine the genetic diversity and likely evolutionary origin of serotype 6C and 6D isolates.

*S. pneumoniae* was isolated and identified as described elsewhere (18) from children aged 6 to 18 months participating in the Fiji Pneumococcal Project (FiPP) who had received 0, 1, 2, or 3 doses of PCV7 and 0 or 1 dose of 23-valent pneumococcal polysaccharide vaccine (PPV23) (23). Isolates were serotyped by a multiplex PCR-based reverse line blot (mPCR/RLB) assay and/or quellung reaction (before factor serum 6d was available), plus serogroup 6 serotype-specific PCR as previously described (11, 12).

MLST was applied to all unique serotype 6C and 6D strains from the FiPP study (*n* = 52 and 24, respectively), of which 24 and 14 isolates, respectively, were included in our previous report (without ST results) (11). For comparison, we performed MLST on a subset of randomly selected serotype 6A (*n* = 16) and 6B (*n* = 17) isolates from the FiPP study.

Fresh 18- to 24-h subcultures of *S. pneumoniae* isolates were suspended in nuclease-free water (Ambion), and genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen) per the manufacturer’s instructions. MLST was performed using primer pairs described by the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm) (aroE, recP, spi, xpt and ddl) or Enright and Spratt (6) (gdh and gki), except as described below.

PCRs were conducted with 25-μl volumes containing approximately 5 ng of genomic DNA, 1 U of AmpliTaq DNA polymerase (Applied Biosystems), 1 × PCR buffer II (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 3.0 mM MgCl₂, 250 μM each deoxynucleoside triphosphate (dNTP), 0.5 μM forward primer, and 0.5 μM reverse primer (Sigma-Aldrich). PCR cycling con-
Conclusions were drawn from the analysis of the CC founder, which contains predominantly serotype 6B (15/17) and ST4781 (Table 1). ST176 is predicted to be a CC founder (bootstrap value, 88%). Most serotype 6A isolates (10/16) belonged to STs which contain only serotype 6A in the database (i.e., ST490, ST499, and ST460).

Serotype 6B isolates had a simple population structure, comprising ST176 (15/17) and ST4781 (Table 1). ST176 is predicted to be the CC founder and contains predominantly 6B serotypes in the database. ST4781 is a new ST with a new xpt allele, xpt-325; it clusters with CC176 and is a single-locus variant (SLV) of ST639.

Serotype 6C isolates included isolates of ST4240 (14/24), ST1715, and ST4780 (Table 1). ST4240 was not assigned to a CC when the most stringent criterion was used, but when the stringency was relaxed to define a group as isolates with 5 of 7 shared alleles, it clustered with ST199, which is associated with multiple serotypes, predominantly 19A. The only other ST4240 isolate in the database is a serotype 6C isolate from Finland. These results imply evolutionary pathways somewhat different from those previously reported, mainly in developed countries, but consistent with the now recognized genetic diversity of serotype 6C (3, 4, 8, 9, 17). ST4780 is a newly identified ST in this study and is not assigned to a CC. The only other ST499 isolate in the database (serotype 6A isolate from Finland) was also not assigned to a CC. ST460 is predicted to be a CC founder (bootstrap value, 58%). Most serotype 6A isolates (10/16) belonged to STs which contain only serotype 6A in the database (i.e., ST490, ST499, and ST460).

Serotype 6D isolates comprised STs 639 (14/24), 473 (9/24), and 4240 (Table 1). ST639 is a double-locus variant (DLV) of ST176, a predicted CC founder (bootstrap value, 71%) comprising mainly serotype 6B isolates, including those in this study. Interestingly, the single Australian serotype 6D isolate (strain 8649, ST4241) is an SLV of ST176. ST473 is predicted to be the CC founder (bootstrap value, 100%) and contains mostly serotype 6A (n = 19/25) and a few serotype 6C and 6B isolates in the database. One serotype 6D isolate belonged to the ST4240 that was predominant among serotype 6C isolates.
in this study. This result was confirmed by repeating the quellung reaction and the MLST and serotype-specific PCR with new DNA extract.

Together with the small number of other serotype 6D isolates which have been analyzed to date, our results are consistent with the hypothesis that serotype 6D arose mainly from serotype 6B but had other evolutionary pathways involving serotype 6A/C and, perhaps, one or more capsule-switching events. This is also suggested by the single serotype 6D isolates among 50 ST4240 isolates, which otherwise all belonged to serotype 6C. We did not identify ST473 among our serotype 6A, 6B, or 6C isolates, which may have provided more information about the evolution of ST473 serotype 6D. An important caveat to our study is that strains previously identified as serotype 6A in the MLST database may in fact be serotype 6C; similarly, strains identified as serotype 6B may be 6D.

Naturally occurring serotype 6D isolates have so far been reported from Fiji (11; this study), South Korea (1), China, and Australia (http://spnemumiae.mlst.net) but not among isolates predominantly from Europe and the United States (2, 8, 17). It is interesting to speculate whether this predominance in Asia and Oceania is due to geographical, ethnic, or socioeconomic factors. In any case, along with the identification of four new STs and one new allele in our analysis, the importance of studying pneumococci from a range of geographical areas is highlighted.

This study is the first comprehensive characterization of the molecular epidemiology of serotype 6D isolates and of Fijian pneumococcal isolates in general. Our results suggest that serotype 6D strains may have arisen from both serotype 6A/C and 6B lineages. We thank Ping Jin, Fanrong Kong, Meng Xiao, Jacqueline Williams, Haslinawaty Kassim, and the families and staff of the Fiji Pneumococcal Project. We acknowledge the use of the pneumococcal MLST database, which is located at Imperial College London and is funded by the Wellcome Trust.

REFERENCES


7. Reference deleted.


13. Reference deleted.


