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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, 17) until recently, when we identified 14 naturally occurring serotype 6D isolates from nasopharyngeal swabs from Fijian children (11). Recently, two serotype 6D isolates have been found in Korean children (1).

Initially, serotype 6C was postulated to have arisen from a single, possibly recent, evolutionary event in which serotype 6A *wciN* was replaced by serotype C *wciN* (21). Subsequent analyses, predominantly by multilocus sequence typing (MLST), have shown that 6C is genetically diverse, believed to be a consequence of multiple separate conversion events or a single event occurring sufficiently early in pneumococcal evolution (3, 4, 8, 9, 17).

Serotype 6C is usually associated with clonal complexes (CCs) containing predominantly serotype 6A, and less commonly, 6B or non-serogroup-6 serotypes (3, 4, 9, 17). To date, MLST has been conducted on two serotype 6D isolates from Korea (ST282) (1), two from China (ST982 and ST4190), and one from Australia (ST4241) (<http://spneumoniae.mlst.net>).

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- μ l volumes containing approximately 5 ng of genomic DNA, 1 U of AmpliTaq DNA polymerase (Applied Biosystems), 1 \times PCR buffer II (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 3.0 mM MgCl₂, 250 μ M each deoxynucleoside triphosphate (dNTP), 0.5 μ M forward primer, and 0.5 μ M reverse primer (Sigma-Aldrich). PCR cycling con-

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TABLE 1. Distribution of STs and eBURST analysis for 109 serotype 6 isolates from Fijian children

Serotype	ST	No. of isolates	eBURST analysis ^a		
			Serotype(s) shared by ST in database (no. of isolates)	Predicted CC founder ST (bootstrap value [%]) ^c	No. of ST (total no. of isolates) in CC in database ^c
6A	490	7	6A (16)	490 (96)	50 (90)
	4778 ^b	3	N/A	N/A	N/A
	4779 ^b	3	N/A	N/A	N/A
	499	2	6A (1)	N/A	N/A
	460	1	6A (14)	460 (88)	64 (94)
6B	176	15	6B (22), 6A (1)	176 (71)	374 (714)
	4781 ^b	2	N/A	176 (71)	374 (714)
6C	4240	49	6C (1)	N/A	N/A
	1715	2	6B (1)	1715 (23)	4 (7)
	4780 ^b	1	N/A	N/A	N/A
6D	639	14	6B (1)	176 (71)	374 (714)
	473	9	6A (19), 6B (3), 6C (3)	473 (100)	83 (124)
	4240	1	6C (1)	N/A	N/A

^a eBURST analyses based on a minimum of six identical loci to define a group or clonal complex (CC).

^b New STs identified in this study; ST4781 contains a new *xpt* allele, *xpt*-325.

^c N/A, STs not assigned to any CC by eBURST.

ditions were a 5-min hold at 94°C, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. Some isolates which produced no or small amounts of PCR product from *spi* (seven 6C and three 6D isolates) and/or *recP* (three 6C and one 6A isolate) were successfully amplified with primers described by Enright and Spratt (6) at an annealing temperature of 52°C in 4.5 mM MgCl₂.

Amplicons were sequenced in both directions using capillary separation on the ABI 3730xl DNA analyzer with ABI BigDye Terminator labeling (version 3.1) (Australian Genome Research Facility) using the same primers as for amplification. Contiguous sequences were formed and edited using Sequencher 4.9 (Gene Codes Corporation).

Allelic profiles and sequence types (STs) were obtained and compared to those of other isolates in the MLST database (<http://spneumoniae.mlst.net>). Relationships between STs were explored using eBURST version 3 software (Imperial College, London), which is available at the MLST website. For this study, a group was defined as two or more isolates which shared alleles at six of seven loci. Using this stringent definition, a group also defined a clonal complex (CC). Bootstrap analyses of the CC founder are also presented where appropriate.

Generally, Fijian serogroup 6 isolates were highly clonal, with the predominant clone in each serotype representing >44% of the isolates. This is not surprising, given that all strains were isolated from children of similar ages over a relatively short period of time in a small, geographically isolated area.

Serotype 6A isolates included STs 490 (7/16), 4778, 4779, 499, and 460 (Table 1). ST490 is predicted to be the CC founder (bootstrap value, 96%) and contains predominantly serotype 6A isolates in the MLST database. ST4778 and ST4779 are newly identified in this study and were not assigned to a CC. The only other ST499 isolate in the database (sero-

type 6A isolate from Finland) was also not assigned to a CC. ST460 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 ($n = 49/52$), 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 Australia (26). ST1715 was predicted by eBURST to be a CC founder, but with a low bootstrap value (23%). The only other ST1715 isolate in the database is a serotype 6B 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.

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://spneumoniae.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.

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