

Temporal Trends and Molecular Epidemiology of Recently Described Serotype 6C of *Streptococcus pneumoniae*[∇]

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Received 14 October 2008/Returned for modification 22 November 2008/Accepted 1 December 2008

We studied the epidemiology of the recently described serotype 6C of *Streptococcus pneumoniae* among a collection of carriage isolates recovered between 1996 and 2007 in Portugal. Of 4,064 isolates, 106 (2.6%) were of serotype 6C, 17.9% of which were multidrug resistant. The strains were genetically diverse.

The polysaccharide capsule of *Streptococcus pneumoniae* is considered to be the most important virulence factor in this species. It is antigenically diverse, and its identification by serology (Quellung reaction) has been used for decades as a primary criterion for the classification of pneumococci (18). Up to now, 91 serotypes have been described, with serotype 6C being the most recent one (14). The latter is indistinguishable from serotype 6A by the Quellung reaction, although it contains a galactose molecule instead of a glucose in the capsular repeating oligosaccharide unit due to the presence of a different glycosyl transferase (*wciN*) gene (14). Serotypes 6A and 6C, together with serotype 6B, constitute serogroup 6, which has related capsular structures (1, 10). The putative serotype 6D that would result from the introduction of *wciN*_{6C} into the serotype 6B operon has been proposed, although it remains unidentified (7).

The 7-valent pneumococcal conjugate vaccine (PCV7, targeting serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) was introduced in the United States in 2000 and became commercially available in several European countries during 2001. PCV7 confers protection, within serogroup 6, against serotype 6B. The most recent data available suggest that it provides cross-protection against disease caused by serotype 6A but not that caused by serotype 6C (12).

Due to its recent identification, data on the epidemiology of serotype 6C are still very scarce (13). A recent study from South Africa found that serotype 6C strains had a higher propensity to cause meningitis than did serotype 6A or 6B strains (5). A study from the CDC in the United States reported that in 2006, the rate of invasive disease caused by serotype 6C was significantly higher than that in 1999 but lower for serotype 6A (3). Another very recent publication from the United States also documented the increasing prevalence of this serotype after 2001 (8).

In this study, we describe the epidemiology of serotype 6C

strains colonizing healthy Portuguese preschool children in studies conducted between 1996 and 2007.

We screened 4,064 *S. pneumoniae* strains isolated from a total of 6,559 nasopharyngeal samples obtained from children attending day care centers in the Lisbon and Oeiras areas of Portugal, in studies conducted in nine different years between 1996 and 2007: 1996 to 1999, 2001 to 2003, and 2006 to 2007. All samples were collected between January and March of each year. Identification of pneumococci was done as previously described (17). The serotypes of drug-resistant isolates ($n = 1,659$) were reported previously (9, 11, 16, 17).

Antimicrobial susceptibility testing was performed using the Kirby-Bauer technique according to Clinical and Laboratory Standards Institute recommendations and definitions (4) for chloramphenicol, erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim. MICs of penicillin and ceftriaxone were determined by use of an Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations and were interpreted according to CLSI guidelines (4). Multi-drug resistance was defined as resistance to three or more antibiotics tested.

To identify strains of serogroup 6, all isolates lacking serotype assignment were screened by PCR for the presence of a specific region of the *wzy* gene as described previously (2). Specifically, two primer pairs were used, 6Bwzy-f (5'-CGA CGT AAC AAA GAA CTA GGT GCT GAA AC-3') and 6Bwzy-r (5'-AAG TAT ATA ACC CTG TAA AAC TCT GAC-3'), generating a product of 200 bp for all serogroup 6 strains, and *cpsA*-f (5'-GGT GTT CTC TAT CCT TGT CAG CTC TGT GTC GCT C-3') and *cpsA*-r (5'-GTG TGA ATG GTC GAA TCA ACT CTA TAA ATG CC-3'), generating a product of 657 bp. The highly conserved *cpsA* gene exists in all but two capsular loci and was used as an internal control (2). To assign serotypes 6A, 6B, and 6C (and the putative serotype 6D), all serogroup 6 isolates were (i) typed by the Quellung reaction (18) and (ii) screened by PCR for a region of *wciN* using previously described primers (5'-TACCATGCAGGGT GGAATGT-3' and 5'-CCATCCTTCGAGTATTGC-3') that result in product sizes of 2.0 kb for serotypes 6A and 6B and 1.8 kb for serotype 6C (13). The PCRs were done using a 10- μ l volume containing 1 \times GoTaq Flexi buffer (Promega, Madison, WI), 2.5 mM of MgCl₂, 0.08 mM of deoxynucleoside triphos-

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[∇] Published ahead of print on 10 December 2008.

TABLE 1. Distribution of serogroup 6 pneumococci over time^a

Yr	Total no. of Pn isolates	No. of isolates (%): of:			
		SG 6	Serotype 6A	Serotype 6B	Serotype 6C
1996	277	77 (27.8)	33 (11.9)	40 (14.4)	4 (1.4)
1997	353	84 (23.8)	26 (7.4)	46 (13.0)	12 (3.4)
1998	465	66 (14.2)	5 (1.1)	60 (12.9)	1 (0.2)
1999	596	137 (23.0)	45 (7.6)	68 (11.4)	24 (4.0)
2001	466	135 (29.0)	57 (12.2)	75 (16.1)	3 (0.6)
2002	559	97 (17.4)	30 (5.4)	61 (10.9)	6 (1.1)
2003	559	94 (16.8)	41 (7.3)	36 (6.4)	17 (3.0)
2006	392	64 (16.3)	46 (11.7)	2 (0.5)	16 (4.1)
2007	397	55 (13.8)	31 (7.8)	1 (0.3)	23 (5.8)
Total	4,064	809 (19.9)	314 (7.3)	389 (9.6)	106 (2.6)

^a Pn, pneumococcus; SG, serogroup.

phages, 1.0 μM of each primer, and 0.1 U μl^{-1} of GoTaq Flexi DNA polymerase (Promega, Madison, WI). DNA was isolated from freshly grown bacterial cultures, picked with a sterile tip, and briefly immersed into the PCR mix. Thermocycling was performed using a My Cycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) under the following conditions: 94°C for 4 min; 35 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s (for *wzy*) or 2 min (for *wciN*); and a final extension step at 72°C for 5 min. PCR analysis was done by electrophoresis on 1% or 2% Seakem LE agarose gels in $1\times$ Tris-acetate-EDTA buffer. Gels were stained in a 0.1- $\mu\text{g ml}^{-1}$ ethidium bromide solution.

Preparation of chromosomal DNA, restriction with SmaI endonuclease, pulsed-field gel electrophoresis (PFGE), and analysis of patterns with Bionumerics software (version 5.10; Applied Maths, Gent, Belgium) were done as previously described (15, 17). Multilocus sequence typing was performed, as previously described (6), on selected strains of each PFGE cluster, choosing at least one representative of each year within each group.

Out of 4,064 pneumococcal strains recovered from colonization samples in studies conducted between 1996 and 2007, 809 (19.9%) were of serogroup 6: 7.3% were of serotype 6A, 9.6% were of serotype 6B, and 2.6% were of serotype 6C. Temporal fluctuations were observed for all serotypes (Table 1). Serotype 6B was dominant among serogroup 6 isolates from 1996 until 2002, when it started to decline, being almost absent by 2006. For serotype 6A, a sharp decrease was observed in 1998, and no significant fluctuations were observed with the availability of PCV7 (since June 2001). The prevalence of serotype 6C ranged from 0.2% to 5.8%, reaching the highest value in 2007. There were no strains of the hypothetical serotype 6D.

Of the 106 serotype 6C strains identified, 35.8% were resistant to at least one of the antibiotics tested. The proportions of penicillin-resistant isolates according to parenteral meningeal and nonmeningeal criteria were 30.2% and 0%, respectively, as 29.2% of the 106 isolates had an MIC of 0.12 $\mu\text{g ml}^{-1}$, and a single isolate (1%) had an MIC of 0.25 $\mu\text{g ml}^{-1}$ (4). Resistance levels were 21.7% for erythromycin, 18.9% for clindamycin, 18.9% for tetracycline, and 1.9% for sulfamethoxazole-tri-

TABLE 2. Characteristics of serotype 6C strains^a

PFGE group (no. of isolates)	ST (no. of isolates) ^b	CC	Resistance pattern (no. of isolates)	No. of DCCs
6C-1 (52)	ST395 (5) ST1714 (1) ST1692 (1) ST3711 (1)	395	Susceptible	12
6C-2 (18)	ST1150 (4)	138	P (11) Susceptible (7)	3
6C-3 (1)	ST1150 (1)	138	P	1
6C-4 (4)	ST3673 (2)	138	Ery	2
6C-5 (1)	ST2789 (1)	Singleton	Susceptible	1
6C-6 (2)	ST3671 (1)	Singleton	Susceptible	1
6C-7 (6)	ST1150 (1) ST2689 (2)	138	P (3) Susceptible (3)	3
6C-8 (2)	ST2185 (1)	3034	Ery, Clin, Tet, SXT	1
6C-9 (3)	ST395 (2)	395	Susceptible	1
6C-10 (11)	ST3396 (1)	386	P, Ery, Clin, Tet (8) Ery, Clin, Tet (3)	1
6C-11 (6)	ST3396 (1)	386	P, Ery, Clin, Tet	1

^a P, penicillin G MIC of 0.12 $\mu\text{g ml}^{-1}$, with the exception of a single isolate that had an MIC of 0.25 $\mu\text{g ml}^{-1}$; Ery, erythromycin; Clin, clindamycin; Tet, tetracycline; SXT, sulfamethoxazole-trimethoprim; No. of DCCs, number of day care centers where the PFGE group was found.

^b Number of representative strains tested.

methoprim. Multidrug resistance was detected in 17.9% of the isolates and was observed in 2006 and 2007 only.

PFGE fingerprinting clustered the 106 serotype 6C strains into 11 groups (Table 2). By multilocus sequence typing, the 27 strains representing all PFGE groups yielded 11 sequence types (STs) that, using eBURST and the complete database of *S. pneumoniae* (available at <http://spneumoniae.mlst.net/>), fell into four clonal complexes (CCs) based on a minimum similarity of five identical loci (numbered by ST of the predicted founder as CC138, CC395, CC386, and CC3034) and two singletons (ST3671 and ST2789) (Table 2). Three novel STs were found in this study (STs 3671, 3673, and 3711).

The dominant group 6C-1 (STs 395, 1692, 1714, and 3711) included 52 strains susceptible to all antibiotics that were recovered from several day care centers and were detected in all but one sampling period (Fig. 1 and Table 2). The next two most frequently found groups, 6C-2 (ST1150, with 18 isolates) and 6C-10 (ST3396, with 11 isolates), contained strains that were resistant to penicillin (according to meningeal criteria). In addition, strains of group 6C-10 were also resistant to erythromycin, clindamycin, and tetracycline (Table 2). While group 6C-2 was found in 1997 and between 2001 and 2003, group 6C-10 was identified in 2007 only (Fig. 1). The remaining eight groups were sporadic, representing 0.9 to 5.7% of the serotype 6C collection.

Our study shows that the recently described serotype 6C is frequently carried by healthy young children in Portugal. Over-

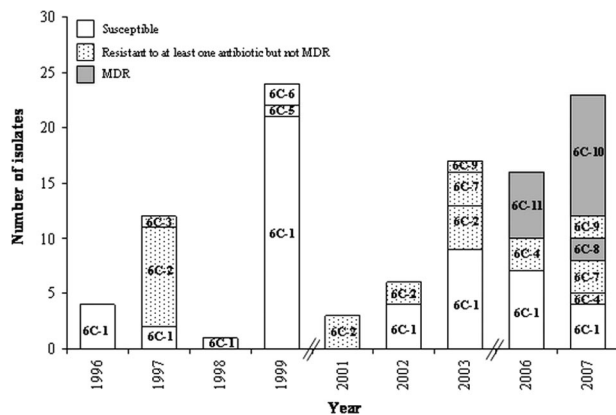


FIG. 1. Distribution of serotype 6C PFGE groups through the nine study periods. MDR, multidrug resistant.

all, of all isolates that would have been conventionally typed by the Quellung reaction as belonging to serotype 6A, close to one-quarter (25.2%) were identified as belonging to serotype 6C; this value ranged between 5.0% and 42.6% depending on the sampling period.

Serotype 6C strains circulating in Portugal are genetically diverse. Similar observations were recently reported in a study from the United States (8), and, of interest, there were no common STs among serotype 6C isolates from both studies. This observation implies that if the introduction of *wciN*_{6C} into the capsular operon of serotype 6A strains occurred only once, as suggested previously by Park et al., it must have occurred a sufficiently long time ago to yield such lineage diversity (13). In our collection, one fully antibiotic-susceptible lineage (6C-1, with STs 395, 1692, 1714, and 3711) persisted over one decade and accounted for close to half of all serotype 6C isolates, suggesting that it is well adapted even when considering the natural selection driven by antibiotic pressure. Still, strains of serotype 6C circulating in Portugal are often drug resistant, and in particular, multidrug resistance has been detected in 2006 and 2007. These data are in contrast with recent observations from South Africa, where most invasive strains of this serotype were fully susceptible or resistant to macrolides only, but are comparable to resistance rates reported in a recent study from the United States (8).

In summary, serotype 6C has been circulating in Portugal since at least 1996. It is genetically diverse and often antibiotic resistant. Continued surveillance of this serotype is important since it is not targeted by PCV7, and it is not known if PCV13 (which targets not only serotype 6B but also serotype 6A) will confer cross-protection to it. Additional data from other countries and estimates of its invasive disease potential will help us understand the epidemiology of this serotype.

This work was supported by projects PREVIS (LSHM-CT-2003-503413) and GRACE (LSHM-CT-2005-518226) from the European Commission and PTDC/SAU-ESA/65048/2006 from the Fundação para a Ciência e Tecnologia (FCT), Portugal. S.N. was supported by grant SFRH/BD/40706/2007 from the FCT.

We acknowledge the use of the pneumococcal MLST database, which is located at the Imperial College of London and is funded by the Wellcome Trust.

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