

Type distribution of serogroup 6 *Streptococcus pneumoniae* and molecular epidemiology of newly identified serotypes 6C and 6D in China^{☆,☆☆,★}

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Abstract

The recently determined serotypes 6C and 6D *Streptococcus pneumoniae*, as well as subtypes 6B-I and 6B-II, were not reported in China. Among the 171 invasive isolates, 19 were identified as serogroup 6. There were equal distribution (42.1%) of 6B-I and 6B-II, 15.8% of 6A and lack of 6C and 6D. Among 1662 noninvasive isolates, 210 were identified as serogroup 6. The rates of types 6A, 6B-I, 6B-II, 6C, and 6D were 42.4%, 21.0%, 29.1%, 4.8%, and 2.9%, respectively. Subtype 6B-II was more resistant to antibiotics than others. The main sequence types (STs) of serotype 6C and 6D isolates were ST2912 and ST982, respectively. These results suggested that all recognized types of serogroup 6 can be found in China and that subtype 6B-II was more drug resistant. The epidemic STs of serotype 6C and 6D did not show genetic association with the STs spreading in other countries.

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Streptococcus pneumoniae remains the leading cause of community-acquired respiratory infections and is one of the most important human pathogens causing severe invasive infections (World Health Organization, 2007). So far, more than 90 pneumococcal serotypes classified into 46 groups have been identified. Serogroup 6 is one of the most common causes of invasive pneumococcal disease (IPD) in humans. Serogroup 6 traditionally comprised 2 serotypes, 6A and 6B. However, a new serotype, previously identified as serotype 6A by quellung reaction, was identified as a distinct serotype

by multibead assay with monoclonal antibodies (mAbs) and was designated serotype 6C (Park et al., 2007a). In the 6C polysaccharide, glucose replaces the galactose present in the 6A polysaccharide. Homologous recombination resulted in the substitution of *wciN* in the capsule biosynthesis operon of serotype 6A strains (*wciN6A*) with a different gene (*wciNβ*, also referred to as *wciN6C*) in serotype 6C strains (Park et al., 2007b). Accordingly, it was demonstrated that *wciNβ* can be experimentally introduced into the serotype 6B operon to form another new serotype, 6D (named as 6X1 in the original report) (Bratcher et al., 2009). However, 6D capsules were not identified among pneumococcal clinical isolates (Campos et al., 2009; Hermans et al., 2008) until 2 recent reports on Fijian (Jin et al., 2009a) and Korean isolates (Bratcher et al., 2010). Another recent report showed that 2 subtypes of 6B, labeled as 6B-I and 6B-II, can be distinguished by polymerase chain reaction (PCR) (Jin et al., 2009b). However, so far, it was not clear whether differentiation of these subtypes is of significance in epidemiology surveys of pneumococci.

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In view of the demonstration of several new types and subtypes of group 6 *S. pneumoniae*, which was one of the commonest serogroups in China (Yao and Yang, 2008), we have reexamined isolates previously characterized as serogroup 6 in several strain collections from children in the hinterland of China to determine the serotypes or subtypes using traditional capsule quellung test, PCR, and multibead assay. Our study shows that the distribution of the serotypes or subtypes changed over time.

1. Materials and methods

1.1. Pneumococcal isolates

A total of 1883 *S. pneumoniae* isolates were included in the study. Details of these *S. pneumoniae* collections have been published before (Li et al. 1999; Xue et al., 2010; Yao et al., 2007; Yao et al., 2009; Yu J et al., 2008; Yu SJ et al., 2008). All isolates were collected in the hinterland of China. The IPD isolates from children aged less than 14 years amounted to a total of 171, including 100 (58.5%) isolates from blood, 48 (28.1%) from cerebrospinal fluid, 22 (12.9%) from pleural fluid, and 1 (0.6%) from joint cavity fluid. The IPD isolates were recovered at 9 children's hospitals and at 2 pediatric departments of academic hospitals located in Beijing ($n = 7$), Tianjin ($n = 10$), Shenyang ($n = 32$), Shanghai ($n = 33$), Shenzhen ($n = 28$), Nanjing ($n = 11$), Suzhou ($n = 22$), Wenzhou ($n = 10$), Chongqing ($n = 10$), Wulumuqi ($n = 4$), and Hefei ($n = 4$) during the period from 2006 to 2008. The non-IPD isolates from children aged less than 5 years consisted of 1662 isolates collected from 6 children's hospitals located in Beijing ($n = 893$, 1997–2007), Shanghai ($n = 284$, 2001–2002 and 2005–2006), Guangzhou ($n = 327$, 2000–2002 and 2005–2006), Xi'an ($n = 64$, 2001), Chongqing ($n = 45$, 2005), and Shenzhen ($n = 49$, 2008). Non-IPD isolates were cultured from nasopharyngeal specimens except those from Beijing in 2006–2007 and from Shenzhen in 2008, which were cultured from hypopharyngeal aspirate specimens. All *S. pneumoniae* isolates had been identified based on typical colony morphology, α -hemolysis, morphology in Gram-stained smears, and the identity was confirmed by optochin (BBLTM; Becton, Dickinson, Sparks, MD) sensitivity and bile solubility tests in a central laboratory (Beijing Pediatric Research Institute, Beijing, China).

1.2. Serological typing

All isolates were serogrouped by capsule quellung test with the Pneumotest kit (Statens Serum Institut, Copenhagen, Denmark). The serogroup 6 isolates were further examined using type-specific antisera against serotypes 6A and 6B (Statens Serum Institut). Typing was done with phase contrast microscopy according to the procedure published before (Sørensen, 1993).

1.3. Multibead assay

All serotype 6A isolates identified by quellung test were sent to the Department of Pathology, University of Alabama at Birmingham, AL, to determine serotype 6C by multibead assay. This assay was performed as described previously (Yu J et al., 2005, 2008; Yu SJ et al., 2008). Two sets were included in this assay. Set 1 contained beads coated with capsular polysaccharides of serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9N, 9V, 11A β , 14, 18C, 19A, 19F, and 23F. Set 2 contained beads coated with polysaccharides of serotypes 2, 6A, 8, 10A, 11A α , 12F, 15B, 17F, 20, 22F, and 33F. Each set of bead mixture was incubated with a bacterial lysate and an appropriate mixture of mAbs. After incubation, the reaction mixture was washed with phosphate buffered saline containing 0.1% Tween 20 and mixed with fluorescein-conjugated goat anti-mouse immunoglobulin. After 30 min of incubation, the latex beads were washed and analyzed for their fluorescence with a flow cytometer (FACSArray; Becton Dickinson).

1.4. PCR for 6A, 6B-I, 6B-II, 6C, and 6D

Chromosomal DNA was isolated from overnight cultures of *S. pneumoniae* isolates grown on 5% trypticase soy agar (OXCID, English) using the Simax™ Genome DNA Kit (SBS Genentech), according to the manufacturer's instructions. The 6C isolates were differentiated from 6A using PCR with the primers 5106 and 3101 described previously (Carvalho et al., 2009; Jin et al., 2009b), which generated a 1.8-kbp-size amplicon for 6C and a 2.0-kbp amplicon for 6A. The pair of primers was also used to differentiate the 6D *cap* locus from that of 6B, which generated a 1.8-kbp-size amplicon for 6D and a 2.0- or a 2.3-kbp-size amplicon for subtypes 6B-I and 6B-II, respectively. The primers 5101 and 3101 used to differentiate 6B-I and 6B-II more definitively generated a 956-bp-size amplicon for 6B-I and a 1.2-kbp-size amplicon for 6B-II (Jin et al., 2009b). The PCR procedures were as previously described (Carvalho et al., 2009; Jin et al., 2009b).

1.5. Antimicrobial susceptibility

For all serogroup 6 isolates, MICs were determined against penicillin, amoxicillin–clavulanic acid, ceftriaxone, cefuroxime, erythromycin, imipenem, levofloxacin, and vancomycin using E-test strips (AB BIODISK, Solna, Sweden). The penicillin interpretive breakpoints for nonmeningitis isolates were modified by the Clinical and Laboratory Standards Institute (CLSI) in 2008 (CLSI, 2009). Use of the modified breakpoints for susceptible, intermediate, and resistance rates to compare the penicillin susceptibilities in this study blurred the differences between the serotypes. Therefore, the 2006 interpretive breakpoints (CLSI, 2006) were used in the present study for the analysis of the penicillin susceptibilities. S.

pneumoniae ATCC49619 was used as a quality control strain in the susceptibility tests. The serotyping and antimicrobial resistance data were analyzed using the WHONET version 5.3 software, as recommended by the WHO (<http://www.who.int/drugresistance/whonetsoftware/en/>).

1.6. Molecular epidemiology of serotype 6C and 6D strains

For serotype 6C and 6D isolates, molecular characterization was done by genotyping of the penicillin and erythromycin resistance genes, by pulsed-field gel electrophoresis (PFGE) typing, and by multilocus sequence typing (MLST). The procedures can be found in the website of the Pneumococcal Molecular Epidemiology Network (PMEN) (<http://www.sph.emory.edu/PMEN/>). Briefly, chromosomal DNA extraction was done as mentioned above. Restriction fragment length polymorphism (RFLP) patterns of the PCR products were analyzed after digestion with restriction enzymes *HinfI* or *HaeIII* and *RsaI* for *pbp2b* and *pbp2x*, and with *HinfI* or *HaeIII* and *DdeI* for *pbp1a* (Gherardi et al., 2000). The genomic DNA was digested with restriction endonuclease *SmaI* for PFGE. Products were separated by electrophoresis in a GenePath apparatus (CHEF-DR III; Bio-Rad) with pulse times of 5 to 30 s for 23 h at 14 °C and with voltage of 6 V/cm. PulseMarker 50 to 1000 kb (Sigma, St. Louis, MO) was included in each gel as a molecular weight standard. PFGE fingerprint profiles were interpreted according to standard guidelines (Tenover et al., 1995). A roman number was assigned to each of the *pbps* RFLP patterns, as well as PFGE patterns. The PCR fragments of the 7 MLST genes — *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* — were amplified from chromosomal DNA. Then, the products were sent to BGI Company (Beijing, China) for sequencing on both strands. Sequence types (STs) were determined by comparing the allelic profile to the recognized STs at the MLST website (<http://spneumoniae.mlst.net>). New alleles and allelic profiles found in the present study were submitted to the MLST database for ST assignment. The eBURST version 3 software (available at <http://www.mlst.net>) was used to estimate the relationships among the isolates and to assign strains to a clonal complex (CC) using the stringent group definition of 6 of 7 shared alleles (Feil et al., 2004).

2. Results

2.1. IPD Isolates

Among the 171 IPD isolates, 19 (11.1%) were identified as serogroup 6, of which 8 (42.1%) were assigned to subtype 6B-I, 8 (42.1%) to 6B-II, and 3 (15.8%) to 6A. No strain was typed as the new serotypes 6C and 6D. The penicillin intermediate rate for serogroup 6 was 63.2% (12/19). No resistance against the tested antibiotics was detected except for resistance against erythromycin (100%) and cefuroxime (6/19, 36.8%). 6B-II showed higher penicillin resistance than 6B-I with higher intermediate rate (8/8 versus 1/8), MIC₅₀ (0.016 and 0.5 µg/mL), and MIC₉₀ (0.5 and 1 µg/mL). The 3 serotype 6A isolates were all intermediate to penicillin with maximum MIC of 1 µg/mL.

2.2. Distribution of the serotypes among non-IPD isolates

The type and subtype distribution of the non-IPD serogroup 6 *S. pneumoniae* isolates according to isolation year are shown in Table 1. Overall, 210 of the 1662 non-IPD isolates were identified as serogroup 6 (12.6%). The rates of serotypes and subtypes 6A, 6B-I, 6B-II, 6C, and 6D were 42.4%, 21.0%, 29.1%, 4.8%, and 2.9%, respectively. The rate of serogroup 6 isolates in the respective collections decreased over time. Although some fluctuations occurred during the period, a decreasing trend was shown for serotypes 6A, 6B-I, 6C, and 6D, but not for subtype 6B-II. It is noticeable that more than half of serotypes 6C and 6D were isolated before 2000 (6/10 and 4/6, respectively).

2.3. Antimicrobial susceptibility of the types of non-IPD isolates

The antibiotic susceptibility of 210 serogroup 6 *S. pneumoniae* isolates to tested antimicrobials is presented in Table 2. Among the isolates, 6.2% were resistant to penicillin and 28.1% were of intermediate susceptibility. Of the β-lactam antibiotics tested, amoxicillin–clavulanic acid was the most active (susceptibility rate: 99.0%), followed by ceftriaxone (96.2%) and cefuroxime (69.5%). Almost all isolates (93.3%) were resistant to erythromycin with MIC₉₀ >256 µg/mL. As expected, serogroup 6 isolates were highly susceptible to imipenem and levofloxacin, and fully susceptible to vancomycin.

Table 1
Frequencies of serotype or subtype of noninvasive serogroup 6 *S. pneumoniae* isolated from Chinese children during 1997–2008

Years	Total of isolates	No. of group 6 (% of all isolates)	No. of isolates of different type (% of group 6)				
			6A	6B-I	6B-II	6C	6D
1997–1999	309	46 (14.9)	26 (56.5)	9 (19.6)	1 (2.2)	6 (13.0)	4 (8.7)
2000–2002	825	106 (12.8)	32 (30.2)	27 (25.5)	44 (41.5)	2 (1.9)	1 (0.9)
2003–2005	369	44 (11.9)	25 (56.8)	6 (13.6)	11 (25.0)	2 (4.5)	0 (0)
2006–2008	159	14 (8.8)	6 (42.9)	2 (14.3)	5 (35.7)	0 (0)	1 (7.1)
Total	1662	210 (12.6)	89 (42.4)	44 (21.0)	61 (29.1)	10 (4.8)	6 (2.9)

Table 2

Antimicrobial susceptibilities of 210 Chinese isolates of *S. pneumoniae* serogroup 6

Serotype/subtype	No.	Drug	R (%) ^b	I (%) ^c	MIC ₅₀ (μg/mL)	MIC ₉₀ (μg/mL)	MIC range (μg/mL)
Total (group 6)	210	Penicillin	6.2	28.1	0.032	1	0.001–4
		AMC ^a	0	1	0.032	1	0.004–4
		Ceftriaxone	1.9	1.9	0.032	1	0.004–12
		Cefuroxime	28.6	1.9	0.064	4	0.004–16
		Erythromycin	93.3	0	>256	>256	0.064–>256
		Imipenem	0	6.2	0.032	0.125	0.001–0.5
		Levofloxacin	0	1.4	2	2	0.032–4
		Vancomycin	0	0	0.5	1	0.016–1
6A	89	Penicillin	3.4	7.9	0.032	0.125	0.006–4
		AMC	0	0	0.016	0.125	0.004–2
		Ceftriaxone	1.1	1.1	0.032	0.25	0.004–12
		Cefuroxime	6.7	2.2	0.064	0.25	0.004–16
		Erythromycin	88.8	0	>256	>256	0.064–>256
6B-I	44	Penicillin	0	13.6	0.016	0.5	0.002–1
		AMC	0	0	0.016	0.5	0.004–1
		Ceftriaxone	0	2.1	0.032	0.25	0.004–0.5
		Cefuroxime	9.1	0	0.032	0.5	0.004–4
		Erythromycin	93.2	0	>256	>256	0.125–>256
6B-II	61	Penicillin	16.4	73.8	1	2	0.004–2
		AMC	0	3.3	1	2	0.004–4
		Ceftriaxone	9.8	29.5	0.5	1	0.004–4
		Cefuroxime	80.3	3.3	2	4	0.016–8
		Erythromycin	98.4	0	>256	>256	0.125–>256
6C	10	Penicillin	0	0	0.032	0.064	0.016–0.064
		AMC	0	0	0.016	0.032	0.016–0.032
		Ceftriaxone	0	0	0.032	0.064	0.008–0.047
		Cefuroxime	0	0	0.064	0.125	0.008–0.125
		Erythromycin	100	0	>256	>256	8–>256
6D	6	Penicillin	0	0	0.016	0.064	0.001–0.064
		AMC	0	0	0.016	0.032	0.016–0.032
		Ceftriaxone	0	0	0.064	0.125	0.016–0.125
		Cefuroxime	0	0	0.064	0.5	0.016–0.5
		Erythromycin	100	0	>256	>256	2–>256

^a AMC = Amoxicillin–clavulanic acid.^b Resistance rate (percentage).^c Intermediate rate (percentage).

Analysis of the resistance according to serotype and subtype showed noticeable differences (Table 2). All of the 6C and 6D isolates were susceptible to penicillin and other β-lactam antibiotics. Subtype 6B-II strains were more resistant to penicillin (Fig. 1) and other β-lactam antibiotics than 6B-I and other types, as demonstrated by the resistance and intermediate rates, MIC₅₀, MIC₉₀, and maximum MIC value.

Only 1 isolate expressing a 6A capsule was found nonsusceptible (intermediate plus resistant) to penicillin (MIC 4 μg/mL) when the interpretive breakpoints of Clinical Laboratory Standards Institute (2009) were used.

2.4. Molecular epidemiology of serotypes 6C and 6D

Among the 4 STs found in the MLST analysis among 10 serotype 6C isolates, 3 STs were new assignments (ST4173, ST4174, and ST4175) (Table 3). The new STs represented novel combinations of known alleles. Each of the new STs included only a single strain, each with a unique PFGE pattern. Two of them were isolated in Beijing (ST4173 and

ST4174) and 1 in Guangzhou (ST4175). Another ST2912 contained 7 isolates, all from Beijing, and included 4 PFGE patterns. Although the resistance pattern was always penicillin susceptible and erythromycin resistant for 6C isolates, the *pbps* and *ermB/mefA* profiles showed some diversity, even between isolates with identical ST and PFGE pattern (Table 3).

Two STs were found among the serotype 6D isolates, one of which was a new assignment (ST4190). ST4190 was characterized by a novel combination of known alleles and represented only a single strain with a unique PFGE pattern (Table 3). The other, ST982, contained 5 strains, 4 of which were isolated in Beijing except 1 in Guangzhou. The 5 ST982 strains showed 2 PFGE patterns. The resistance pattern for 6D isolates was mainly penicillin susceptibility and erythromycin resistance. The *pbps* and *ermB/mefA* profiles also showed some diversity for the serotype 6D ST982 isolates (Table 3).

The STs found in the present study were singletons when examined by eBURST analysis together with serotype 6C or 6D strains in the MLST database. Then, all *S. pneumoniae*

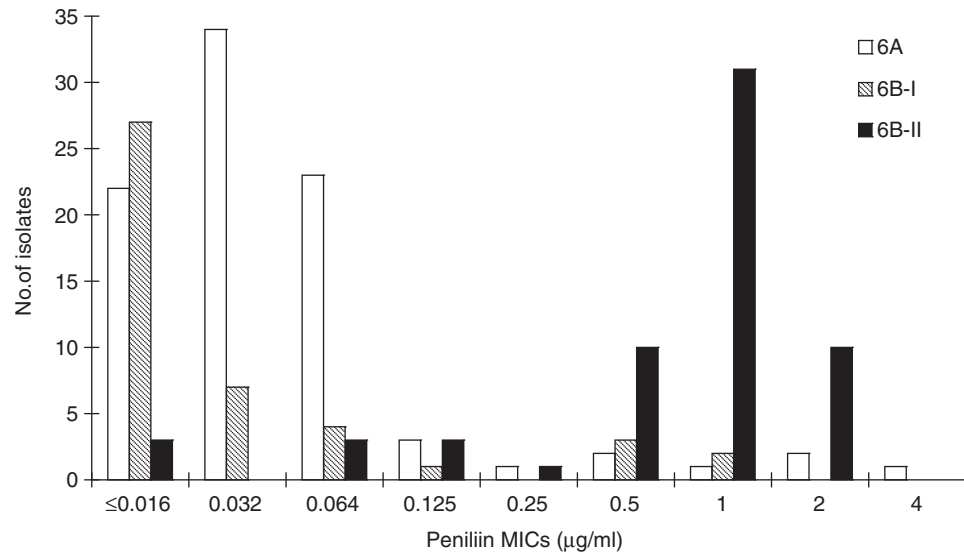


Fig. 1. The penicillin MIC distribution of *S. pneumoniae* serotype 6A, subtypes 6B-I and 6B-II, examined in the present study.

isolates in the MLST database were included in this analysis. The results showed that 2 STs belonged to some CC. ST4173 belonged to CC176, which comprised mainly serotype 6B isolates. It was noticed that ST4173 had only 1 single locus variant (SLV), ST4542, which covered 1 serotype 6A strain in the MLST database. The isolates that were SLVs of ST4542 (ST171, ST2954, ST3480, and ST4173), however, were serotype 6B except for ST4173. The other, ST982, could be a primary founder that had 3 SLVs (ST4407, ST4408, and ST4384). The 4 strains covered by the 3 SLVs were all serotype 6A, and ST982 itself covered 2 type 6A isolates and 1 6D isolate. Other STs identified in the present

study were still singletons when all isolates in the MLST database were included in the eBURST analysis.

3. Discussion

Serotype 6C was described in 2007, but it has been indicated that this serotype emerged as early as 1962 (Lambertsen and Kern, 2010) and could be identified among isolates collected from many locations in the United States (Jacobs et al., 2009) and other countries (Campos et al., 2009; Hermans et al., 2008; Jin et al.,

Table 3
Genetic characters of *S. pneumoniae* serotype 6C and 6D isolates identified in the present study

Serotype/ ST	MLST allelic profile							CC ^a	PFGE group	<i>pbps</i>	<i>ermB</i> / <i>mefA</i> ^b	Susceptibility to penicillin and erythromycin ^c	No. of isolates	Year and city
	<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>							
6C														
ST2912	2	8	9	38	17	1	9	^d	I	I	+/-	S/R	2	1997, Beijing (1) 2004, Beijing (1)
									II	I	-/+	S/R	1	1997, Beijing
										II	+/-	S/R	1	2004, Beijing
									III	I	+/	S/R	1	1997, Beijing
										II	+/	S/R	1	1997, Beijing
									IV	I	-/+	S/R	1	1997, Beijing
ST4173	7	13	1	6	6	6	271	176	V	II	+/	S/R	1	1997, Beijing
ST4174	137	12	62	1	10	121	120	^d	VI	III	+/	S/R	1	2002, Beijing
ST4175	7	8	4	10	15	58	271	^d	VII	IV	+/	S/R	1	2000, Guangzhou
6D														
ST982	8	13	9	60	78	119	6	982	VIII	V	+/-	S/R	2	1997, Beijing
									VIII	V	+/+	S/R	1	2006, Guangzhou
									IX	V	+/-	S/R	1	1997, Beijing
									IX	VI	+/+	S/R	1	1997, Beijing
ST4190	5	8	4	39	6	104	271	^d	X	VII	+/-	I/R	1	2002, Beijing

^a Clonal complex.

^b + and - mean *ermB/mefA* PCR test was positive and negative, respectively.

^c S, I, and R mean susceptible, intermediate, and resistant, respectively.

^d STs not assigned to any CC by eBURST.

2009a; Leach et al., 2009; Nahm et al., 2009; Nunes et al., 2009; Tocheva et al., 2010). The reports show that the prevalence of serotype 6C isolates was low among carriage isolates (0.3–3.2%) or IPD isolates (2.3%) in countries before administration of PCV7. Absence of cross-protection against serotype 6C was noted after immunization with PCV7, which includes serotype 6B. Several studies showed that the incidence or prevalence of serotype 6C IPD increased significantly in postvaccine years (Jacobs et al., 2009; Nahm et al., 2009; Park et al., 2008; Tocheva et al., 2010). One study found that antibiotic resistance among nonvaccine types was associated with serotype 6C in recent years (Sá-Leão et al., 2009). Recently, a study in Fiji suggested that serotype 6D may also have a selective advantage after immunization with PCV7 (Jin et al., 2009a).

In the present study, serogroup/serotype was determined for a total of 1833 *S. pneumoniae* isolates. The 2 new serotypes 6C and 6D were not detected among the IPD isolates from Chinese children. This result may be associated with the collection years (2006–2008) for IPD isolates, because the distribution, over time, of these 2 types among non-IPD isolates suggests that they were becoming rare after 2004. Serotypes 6C and 6D were not frequent among the non-IPD isolates (0.6% and 0.4%, respectively). The frequency of 6C was similar to previously reported data from before PCV7 use. The 6D frequency (5% of previously identified as 6B) is far lower than those reported from the Fiji islands and Korea, which were 41% (Jin et al., 2009a) and 18% (Bratcher et al., 2010), respectively. Most serotypes 6C and 6D were isolated before 2000, which might suggest that these “newfound” serotypes were in fact more common in the 1990s or even earlier.

Although some fluctuations were observed, a decreasing trend was shown for the serogroup 6 serotypes except for subtype 6B-II, which was associated with higher drug resistance. Such changes occurred before the administration of PCV7, which became available in China from September 2008. Considering the common use of penicillin and cephalosporin in China (Zhang et al., 2008), it is conceivable that the observed changes in serotype distribution are driven, in part, by antibiotic use. Antibiotic use can restrict the spread of serotypes with lower resistance and provides a selective advantage for serotypes with higher resistance such as subtype 6B-II. The capsule locus of *S. pneumoniae* is flanked by the *pbp2x* and *pbp1a* genes, coding for penicillin-binding proteins, enzymes involved in cell wall synthesis that are targets for β -lactams (Trzciński et al., 2004). These molecular characters should be studied in the future. It is yet unclear whether some serologic difference exists between subtype 6B-I and 6B-II that may potentially influence their epidemiology.

In Western countries, both serotypes 6A and 6B decreased in prevalence, while 6C increased after PCV7 administration (Klugman and McGee, 2007; Nahm et al.,

2009; Park et al., 2008). The present study showed a different trend in China for serotypes 6C and 6B. Such discrepancy means that common antibiotic use and universal immunization with PCV7 may differentially affect the serotype distribution. However, the potential cumulative effect of the 2 selection mechanisms is of even more concern. We noticed that an increase of serotype 19A was reported in Western countries (Klugman and McGee, 2007), Korea (Choi et al., 2008), and China (Gao et al., 2009; Xue et al., 2010; Yao et al., 2009), whereas serotype 6A decreased. This suggests that both universal immunization and antibiotic use may provide selective advantage for the nonvaccine serotypes with higher resistance (such as the well-known serotype 19A) and both may have restricted the spread of the vaccine or vaccine-related serotypes with lower resistance (such as 6A). The cumulative effect may have accelerated the global spread of some serotypes, especially of resistant clones (<http://www.sph.emory.edu/PMEN/>).

The ST profiles of 6C or 6D isolates present in the MLST database showed that the isolates were genetically diverse. Most STs identified in the present study were singletons even when analyzed together with the whole *S. pneumoniae* MLST dataset. ST2912 was the main identified clone among serotype 6C isolates, which was a singleton even when the group definition was relaxed to 5 out of 7 alleles for all serogroup 6 isolates in the MLST database (data not shown). There was no serotype 6A isolate sharing ST2912 in the MLST database. ST1692, ST395, ST1379, and ST1390, which were the main cause of the increased prevalence of serotype 6C in Western countries (Jacobs et al., 2009; Nunes et al., 2009; Tocheva et al., 2010), were not identified in the present study.

Analysis of ST profiles revealed that ST4173 was a SLV of ST4542 covering type 6A isolates in the database and belonged to CC176. CC176 is closely related to CC138, which was the major group of serotype 6B in a previous report (Jacobs et al., 2009). In the MLST database, the SLVs of ST4241 covering 1 6D isolate had 10 SLVs which covered mostly the serotype 6B or 6A isolates. These findings suggest a capsular switching from serotype 6A and 6B to serotype 6C and 6D. On the other hand, ST982, which was identified among serotype 6D isolates, could be a primary founder with 3 SLVs covering 4 serotype 6A strains. Another ST covering 6D isolates was noted in the MLST database: ST282, which belonged to CC81. ST81 was a predicted founder and covered 212 isolates expressing at least 9 serotypes (23F, 19F, 19A, 6A, 14, 2, 1, 9V, 23B, and nontypeable). Besides ST282, among other SLVs of ST81, only 2 STs included serotype 6A isolates: ST1476 and ST2842. Except for ST81, there were 5 SLVs of ST282 in the MLST database. None of them included serogroup 6 isolates. Furthermore, ST282 itself included 7 isolates expressing serotypes 6A ($n = 3$), 6D ($n = 2$), 6B ($n = 1$), and 23F ($n = 1$). These suggested that some serotype 6D strains could be derived from other serotypes besides serotype 6B clones and that capsular

switching from serotype 6D to 6A, even from other serotypes, may be possible.

In summary, the present study highlights the presence of serotypes or subtypes of group 6 *S. pneumoniae* in China and the decreasing frequency of the group over time. All the recognized serotypes and subtypes of serogroup 6 could be found. It may be of important epidemiologic significance that the subtype 6B-II isolates were more drug resistant. The main STs of serotype 6C and 6D found in the present study did not show any genetic association with the STs spreading in other countries. It deserves attention that a serotype replacement occurred after the PCV7 administration in China. It should be noted that serotype 6A may return because some of the remaining clones have higher resistance in the present results and that the new type 6C clones which had acquired higher resistance in another country (Sá-Leão et al., 2009) may spread into China.

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