Molecular characterization of serogroup C *Neisseria meningitidis* isolated in China

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An increase in the number of serogroup C meningococcal disease cases occurred in China from September 2003 to January 2006 as a result of several successive outbreaks. In addition, the proportion of serogroup C *Neisseria meningitidis* isolates from sporadic cases and carriers has also increased. In this study, 113 serogroup C meningococcal isolates were characterized by multilocus sequence typing (MLST) and PorA typing. These isolates comprised those from outbreak cases and their close contacts, the national carriage survey conducted during the same period and some historical isolates from 1966–2002. Twenty MLST sequence types (STs) and 21 PorA variable region (VR) types were identified in the collection. The ST-4821 complex, a newly identified lineage, was the most prevalent lineage (95/113). These data also showed a high level of diversification of serogroup C isolates, as indicated by the number of variants of the ST-4821 clone and the VR types present. There were ten PorA VR types among the ST-4821 isolates, and certain VR types (P1.7-2,14, P1.12-1,16-8) were associated with isolates from outbreak cases. The results of this study allow us to draw a profile of the molecular characteristics of serogroup C strains in China. These data are helpful for monitoring the spread of virulent strains and will provide valuable information for the prevention of bacterial meningitis in China.

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INTRODUCTION

Neisseria meningitidis infects only humans and is capable of causing meningitis, bacteraemia and some less common syndromes (Rosenstein *et al.*, 2001). In China during the last century, serogroup A meningococci were the main cause of meningitis and were responsible for more than 95% of cases and all of the outbreaks, whilst serogroup B and C strains caused mostly sporadic cases (Hu, 1991; Wang *et al.*, 1992; Zhu *et al.*, 1995). According to updated data in 2005, the incidence of meningococcal disease declined to 0.18–0.20 per 100 000 of the population and the

number of clinical cases was about 2000 nationwide per year (unpublished data). This change was due to the national immunization campaign initiated in the 1980s; about 80 million individuals were immunized with the MenA vaccine or the MenA+C vaccine each year (unpublished data). Another noticeable change is that the incidence of serogroup C meningococcal infections has increased due to several outbreaks in Anhui province that spread to other provinces after 2003 (Shao *et al.*, 2006). To monitor the circulation of serogroup C meningococci in China, a nationwide survey, comprising 20 000 individuals from 26 provinces, was performed by the Chinese Center for Disease Control and Prevention during 2004–2005. This nationwide carriage survey showed that serogroup C meningococci have spread to 11 provinces in China and are apparently continuing to spread (data not shown). Of the

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Abbreviations: MLST, multilocus sequence typing; ST, sequence type; VR, variable region.

113 meningococci collected for molecular characterization, 104 were isolated in China between September 2003 and January 2006. In addition, nine isolates from China prior to 1992 were included to compare the molecular subtypes of serogroup C meningococci.

Multilocus sequence typing (MLST) is currently the most widely used approach for studying genetic variations of *N. meningitidis* based on the rapid diversification of meningococcal clones by frequent, localized recombinational exchanges among lineages (Holmes *et al.*, 1999; Jolley *et al.*, 2005; Maiden *et al.*, 1998). However, MLST has not been sufficiently discriminatory for some outbreak investigations (Sacchi *et al.*, 2002); therefore, PorA variable region (VR) typing by sequencing of the *porA* gene has been used for further subtyping (Russell *et al.*, 2004). Monitoring the molecular epidemiology of serogroup C meningococci is important not only to attempt to curtail the possible spread of these organisms worldwide but also to provide useful information for the prevention of bacterial meningitis.

METHODS

Meningococcal isolates. A total of 104 isolates of *N. meningitidis* serogroup C obtained from 14 provinces in the Chinese mainland from September 2003 to January 2006 were included in this study. Among 26 patient isolates, 11 were collected from cases associated with eight outbreaks, which occurred during 2003–2006 starting in Anhui and then Sichuan province, whilst 15 were sporadic cases collected during this same time period. Carrier isolates were from two types of subject: close-contact isolates were household members, classmates or roommates of patients (39 isolates), whilst healthy carriers had no signs or symptoms. An additional 39 healthy-carrier isolates were collected in 11 provinces from the nationwide carriage survey, which included approximately 20 000 healthy individuals in 26 provinces from 2004 to 2005. For historical comparison, nine isolates (four from cases and five from carriers) from 1966–2002 were also examined. In our collection, patient isolates were from cerebrospinal fluid, whilst carrier isolates and close-contact isolates were from throat swabs. All isolates were characterized by Gram staining and biochemical tests. The meningococcal serogroup was determined by slide agglutination (Slidex immunological agglutination tests; bioMérieux).

Meningococcal isolates were stored at -80°C in brain heart infusion broth with 10% glycerol and recovered by plating on heated-blood Mueller–Hinton agar. For each isolate, the growth obtained from the surface of a single Petri dish after overnight incubation in an atmosphere of 5% CO_2 was used to prepare an opaque cell suspension in 1 ml deionized water. Meningococcal DNA was extracted from 100 μl cell suspension using a nucleic acid extraction kit (Promega).

MLST. Each isolate was characterized by MLST (Maiden *et al.*, 1998), which included seven housekeeping genes: *abcZ*, *adk*, *aroE*, *gdh*, *pdhC*, *pgm* and *fumC*. Fragments of these genes were amplified by PCR from chromosomal DNA. PCR products were sequenced using an ABI Prism 3730 system (Applied Biosystems). Sequences were assembled with SEQMAN II (DNASTAR). Nucleotide sequences were submitted to the MLST website database (Jolley *et al.*, 2004; <http://pubmlst.org/neisseria/>) to acquire the genotype of each isolate. If an allelic profile or an allele was novel, it was submitted to the curator of the database

using the appropriate template provided on the website. Novel sequence types (STs) or new alleles were assigned by the curator.

PorA VR typing. Chromosomal DNA was used as template for PCR amplification of the *porA* gene using the previously described primers 210 and 211, and sequencing reactions were carried out with primers 8L and 8U (Feavers & Maiden, 1998). Sequence assembly was performed as described above and determination of PorA VR types was performed through the PorA typing website (<http://www.neisseria.org/nm/typing/pora>).

Data analysis. After the assignment of STs to clonal complexes, founder STs were identified with the aid of heuristic methods such as the BURST algorithm, implemented in the computer programs START and SplitsTree (Huson, 1998; Jolley *et al.*, 2001). The relationships of STs were analysed using eBURST (Feil *et al.*, 2004). All of these programs are available for electronic download (<http://pubmlst.org/>, <http://www.splitstree.org/>, <http://www.megasoftware.net/>). The relationships among clonal complexes were determined by reference to the *Neisseria* MLST database (<http://pubmlst.org/neisseria/>).

RESULTS

Distribution of STs

MLST analysis showed that 113 serogroup C isolates belonged to 20 different STs, 17 of which had not been detected in China before this study (Table 1). Only three STs belonged to lineages that were already known: ST-7, ST-658 and ST-2146, belonging to the ST-5, ST-11 and ST-198 complexes, respectively. ST-7 was represented by eight isolates, two from sporadic cases (one in 1992 and one in 2006) and six from close contacts of patients from an outbreak that occurred in a middle school in Sichuan province in January 2006. Although strains could not be isolated from patients, infection with serogroup C meningococci was determined by latex agglutination test. The ST-658 isolate was from a sporadic case, whilst ST-2146 was from a healthy carrier, both isolated in 2005.

Among the 17 novel STs first recognized in China, 11 (ST-4820, ST-4821, ST-4823, ST-4831, ST-4832, ST-4833, ST-4837, ST-4894, ST-4895, ST-4896 and ST-4978) were represented by 96 isolates obtained during 2002–2006 (Table 1). Among the isolates up to 2002, there were eight STs (ST-4821, ST-4822, ST-4830, ST-4831, ST-4979, ST-4980, ST-4981 and ST-5081). Only ST-4821 and ST-4831 were isolated during both time periods. As shown in Table 1, six STs (ST-4820, ST-4821, ST-4831, ST-4896, ST-4822 and ST-4979) were isolated from cases, three STs (ST-4820, ST-4821 and ST-4823) from carriers who were close contacts and 12 STs (ST-4820, ST-4821, ST-4832, ST-4833, ST-4837, ST-4894, ST-4980, ST-5081, ST-4830, ST-4895, ST-4978 and ST-4981) from healthy carriers. ST-4820 and ST-4821 were present in patient, contact and healthy-carrier isolates.

Based on a search of the MLST database, nine of the 17 novel STs belonged to the ST-4821 complex, whilst the remaining eight novel STs could not be assigned to any known complex. Each of the STs not assigned to a complex

Table 1. Molecular characterization of serogroup C strains showing temporal and geographical distribution in China

Year of isolation	Province	Complex	ST	PorA type	Epidemiological information*	Patient type (no. of isolates)		
						Case	Contact	Carrier
1966	Unknown	None	4822	P1.7-4,13-20	Sporadic case	1		
1978	Shanxi	4821	4980	P1.20,23-7				1
		None	4981	P1.22,14				1
1979	Beijing	None	4830	P1.12-12,23-8				1
	Jiangxi	4821	5081	P1.20,23-2				1
1980	Henan	4821	4821	P1.20,23-7				1
1992	Guangdong	5	7	P1.20,9	Sporadic case	1		
		None	4979	P1.21-2,28	Sporadic case	1		
2002	Guangxi	4821	4831	P1.20-3,23	Sporadic case	1		
2003	Anhui	4821	4821	P1.20,23-6	Outbreak 1		6	
		4821	4821	P1.7-2,14	Outbreak 2	1	15	
2004	Anhui	4821	4821	P1.7-2,14	Outbreak 3	1	6	
		4821	4837	P1.18,34				1
		None	4823	P1.20,23	Outbreak 4		1	
2005	Beijing	4821	4821	P1.12-1,16-8	Sporadic case	1		
	Anhui	4821	4820	P1.7-2,14	Outbreak 5		2	
		4821	4821	P1.20,23				1
		4821	4821	P1.20,23-1	Outbreak 6	1	2	
		4821	4821	P1.7-2,14	Outbreak 7	8	1	1
	Beijing	4821	4821	P1.18,25-11				1
		4821	4821	P1.20,23-1				2
		4821	4821	P1.20-2,23-9				1
		4821	4821	P1.7-2,14	Sporadic case	1		2
		4821	4832	P1.20,23-1				2
		None	4894	P1.21-2,4				1
	Fujian	4821	4821	P1.12-1,16-8	Sporadic case	1		
	Gansu	4821	4821	P1.12-1,16-8				1
	Guangdong	4821	4831	P1.20-3,23	Sporadic case	1		
		4821	4833	P1.18,25				1
	Hebei	4821	4821	P1.12-1,16-8				3
		4821	4821	P1.20,23-7				1
	Jiangsu	4821	4820	P1.7-2,14	Sporadic case	2		1
		4821	4821	P1.12-1,16-8	Sporadic case	1		
		4821	4821	P1.20,23-7				1
		4821	4821	P1.20,23-9	Sporadic case	2		
		None	4895	P1.21-2,28				1
	Jiangxi	198	2146	P1.18,25-13				1
		4821	4821	P1.20,9				16
	Qinghai	5	7	P1.20,9	Sporadic case	1		
	Shanghai	11	658	P1.12-6,2-35	Sporadic case	1		
		4821	4821	P1.20,23-7	Sporadic case	1		
	Shanxi	4821	4821	P1.7-2,14	Sporadic case	1		
	Yunnan	None	4978	P1.21-2,28				1
	Zhejiang	4821	4821	P1.7-2,14	Sporadic case	1		
		4821	4896	P1.12-1,16-8	Sporadic case	1		
2006	Sichuan	5	7	P1.20,9	Outbreak 8		6	
Totals						30	39	44

*An outbreak was defined as three or more confirmed or probable cases of the same serogroup in less than 3 months among people who had a common affiliation. The incidence of sporadic cases in China is approximately 0.15 cases per 100 000 of the population.

was represented by only a single isolate. Of the eight STs belonging to the ST-4821 complex (not including ST-4821 itself), four were single-locus variants of ST-4821 and four

were double-locus variants of ST-4821. This complex was the most prevalent, representing 83.3 % (25/30) of cases, 82.1 % (32/39) of close-contact carriers and 86.4 % (38/44)

of healthy-carrier isolates. Although case isolates were not available from all eight outbreaks in this study, six outbreaks were apparently caused by the ST-4821 complex. The founder genotype, ST-4821, represented 81 (77.9 %) of the 104 isolates isolated during 2003–2006, including 20/26 case isolates (76.9 %). ST-4821 was seen in only one of nine isolates, but four isolates of this complex were found prior to 2002.

During 2003–2006, the ST-4821 complex was disseminated to 11 provinces in south-east China, whereas the other genotypes were observed in only three of these provinces and in three additional provinces. Among the 104 isolates, 50 were from eight outbreaks occurring in Anhui or Sichuan province, of which were 11 were case isolates (all ST-4821) and 39 were close-contact isolates (30 ST-4821, two ST-4820, one ST-4823 and six ST-7).

At present, the emergence of the ST-4821 complex has occurred only in China and Taiwan according to the MLST isolate database. Strains of this lineage related to four STs (ST-3200, ST-3441, ST-3469 and ST-3470) were isolated in Taiwan during 1996–2004, and almost all of these were serogroup B. The relationships of these STs are illustrated in Fig. 1.

Diversity of PorA VR types

porA genes were amplified and the VRs sequenced from all isolates. A total of 21 PorA VR types was found (Table 1). Among these VR types, ten novel PorA VR sequences were identified comprising three VR1 types (12-12, 20-2, 20-3) and seven VR2 types (23-8, 2-35, 23-6, 23-7, 23-9, 23, 13-20). Of all the isolates, 72.6 % had one of only four PorA VR types: P1.7-2,14 was the most prevalent type (43 isolates), followed by 24 with P1.20,9, eight with P1.12-1,16-8 and seven with P1.20,23-1.

Among the 21 PorA VR types, eight were seen in isolates from before 2003 and 17 were in isolates identified during or after 2003. Only four types (P1.20,9; P1.20,23-7; P1.20-

3,23 and P1.21-2,28) were observed in both periods. The data also showed that the 30 case isolates belonged to ten PorA VR types. The 39 contact isolates comprised five PorA types, whilst 44 isolates of healthy carriers were more diverse, comprising 16 PorA types. There appeared to be less diversity in outbreak-related isolates, as only four PorA VR types were seen, with P1.7-2,14 causing four of the eight outbreaks. The P1.7-2,14 serosubtype also caused sporadic cases and all were ST-4821 or ST-4820. This serosubtype was also seen in the carriage isolates.

DISCUSSION

Serogroup C meningococci are common throughout Europe and are often associated with more severe disease and higher mortality (Maiden & Begg, 2001). Although many different clones of serogroup C are responsible for sporadic cases of invasive meningococcal disease in these countries, outbreaks and epidemics are generally associated with a few virulent clones (Snape & Pollard, 2005). The ST-8/A4 complex and the ST-11/ET-37 complex are two hypervirulent meningococcal lineages involved in a significant proportion of serogroup C disease worldwide (Achtman, 1995). Isolation of serogroup C of the ST-4821 complex has increased due to a number of serogroup C disease outbreaks in China (Shao *et al.*, 2006). The ST-4821 complex remains the prevalent lineage of serogroup C meningococci in China, accounting for 87.5 % (91/104) of isolates in this study. The recent national carriage survey also showed that this complex was present in 11 provinces of China in 2005 (Table 1). Therefore, the spread of this complex has apparently continued.

Although the expansion of this complex occurred recently, ST-4821 complex isolates were observed as early as 1980 (an ST-4821 isolate from a carrier in Henan). Among nine strains obtained up to 2002, four isolates were assigned to four STs (ST-4821, ST-4831, ST-4980 and ST-5081) of the ST-4821 complex. It should be pointed out that ST-4821 was

Fig. 1. Relationships among STs belonging to the ST-4821 complex of *N. meningitidis* shown by an annotated splits graph: alleles shown along branches represent the loci that differ between ST-4821 and its variants: for example, ST-4820 differs from ST-4821 by having *abcZ-3*; ST-4831 differs from ST-4821 by having *abcZ-8* and *aroE-4*; and ST-3469 differs from ST-4821 by having *fumC-261*, *gdh-263* and *pgm-13*.

not the founder genotype of the ST-4821 complex, as the occurrence of this genotype was sporadic during this period. Six STs of the ST-4821 complex were isolated during 2003–2006 in China. The relationship among these genotypes may help us to understand the evolution of the ST-4821 clone.

In addition to the ST-4821 complex, two other hypervirulent lineages also emerged in China during 2003–2006. ST-658 isolates belonging to the ST-11 complex and ST-7 isolates of the ST-5 complex were obtained from two sporadic cases and six close contacts in an outbreak of meningococcal disease in 2006. ST-7 isolates belonging to the ST-5/subgroup III complex of serogroup A emerged in China during the mid-1980s and have existed worldwide, as well as in China, for the last 10 years (Zhu *et al.*, 2001).

Transformation and recombination-mediated switching of meningococcal capsular types has been reported *in vivo*, putatively by transformation of the capsule operon (Dolan-Livengood *et al.*, 2003; Swartley *et al.*, 1997). This is a mechanism for meningococci to escape vaccine-induced or natural protective immunity (Kriz *et al.*, 1999). In addition to serogroup C isolates, the ST-4821 complex has also been seen in serogroup B meningococcal isolates from healthy carriers on the Chinese mainland (unpublished data) and from invasive cases in Taiwan (Chiou *et al.*, 2006). The emergence of an ST-7 isolate with the PorA VR type P1.20,9 as serogroup C may be an example of an A to C switch. As the ST-7 strain with P1.20,9 belonging to serogroup A has been circulating in China in recent years (unpublished data), recombination between these two serogroup strains would lead to capsule switching.

N. meningitidis expresses different porins in its outer membrane. PorA is a class 1 outer-membrane protein, and sequencing of the *porA* gene has revealed that antigenic variation occurs within two VRs of the porin, VR1 and VR2, which are responsible for the generation of the serosubtype specificities (Barlow *et al.*, 1989; Suker *et al.*, 1994). In China, there was a high degree of *porA* gene variability among the ST-4821 isolates (ten VR types), probably related to continual selection imposed by host immune responses (Russell *et al.*, 2004).

The expansion of a virulent clone, and some closely related strains, of the ST-4821 complex with PorA type P1.7-2,14 has occurred in China, resulting in an increase in the number of serogroup C outbreaks and also an increase in isolation of these bacteria from sporadic disease cases. This clonal complex has also been observed in carriers and has spread to at least 11 provinces, suggesting further geographical dissemination in the future. The increase in serogroup C disease cases due to the prevalence and spread of the ST-4821 complex is indicative of the need for better surveillance of the epidemiology of meningococcal meningitis in China.

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