Original Article

Molecular Characteristics of *Neisseria meningitidis* Isolated during an Outbreak in a Jail: Association with the Spread and Distribution of ST-4821 Complex Serogroup C Clone in China*

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Abstract

Objective To characterize the meningococcal strains isolated from cases and close contacts with meningococcal disease associated with an outbreak in a jail in May 2010 by investigating the national distribution of hyperinvasive ST-4821 serogroup C clone associated with this outbreak.

Methods The cases were described based on the clinical symptoms and laboratory results. Pharyngeal swabs were cultured for *N. meningitidis* from men in the jail. Meningococcal isolates were identified by serogrouping, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), respectively. Four hundred and sixteen serogroup C *N. meningitidis* strains were collected from 27 provinces between 2003 and 2010 for a nationwide survey and analyzed by PFGE and MLST.

Results Three persons in a jail system were infected with invasive *N. meningitidis* serogroup C. All isolates tested had matching PFGE patterns and belonged to the multilocus sequence type (ST) 4821 clonal complex. All 47 *N. meningitidis* strains were identified from the pharyngeal swabs of 166 peoples in the jail, and 26 of them belonged to ST-4821 serogroup C clone, and 90.14% (375/416) serogroup C strains identified in the nationwide survey belonged to the ST-4821 complex. The ST-4821 serogroup C clone was spread nationwide, distributed in 24 provinces, especially in eastern provinces between 2003 and 2010.

Conclusion Endemic transmission and carriage rate of ST-4821 serogroup C clone are high in this jail system. The ST-4821 serogroup C clone is spreading in China and nationwide distributed despite the existence of some effective vaccines.

Key words: Pulsed-field gel electrophoresis; Multilocus sequence typing; *Neisseria meningitidis*; ST-4821; Serogroup C

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INTRODUCTION

Neisseria meningitidis is a Gram-negative bacterium found only in human beings and a major cause of life-threatening diseases, such as meningitis and septicemia[1-2]. 100 000 cases of *N. meningitidis* infection are reported worldwide each year, despite existence of some effective vaccines[3]. More than ten serogroups have been classified on the basis of the chemical and serological properties of capsular polysaccharide. It is generally accepted that most cases of invasive disease are caused by serogroups A, B, C, Y and W135[1,4-5].

Some molecular methods for typing *N. meningitidis* have been developed and used in epidemiological studies. Among these subtyping techniques, multilocus sequence typing (MLST) is a very useful molecular typing method for long-term genetic analysis of *N. meningitidis* and for identification of lineages with an increased propensity to cause meningococcal disease[3,6-7,9-12] and pulsed-field gel electrophoresis (PFGE) is usually used in short-term studies and outbreak investigations[13-15]. Combined MLST and PFGE is a widely adopted epidemiological tool for cluster designation, disease outbreak investigation, and tracking the national and global spread of meningococcal clones[9-11,15-16]. The population structure of *N. meningitidis* is effectively panmictic as a result of frequent horizontal genetic exchanges[17]. However, groups of *N. meningitidis* with highly related sequence types (STs) can persist for decades and spread as clonal populations. These groups are referred to as clonal complexes. The majority cases of meningococcal disease are caused by a limited number of clonal complexes, termed hyperinvasive lineages and distinguished by using MLST[8,18]. Unique ST-4821 clone serogroup C *N. meningitidis* is a new hyperinvasive lineage first identified in Anhui Province, China, during 2003-2004 and a dominant hyperinvasive lineage circulating in China, causing several outbreaks of serogroup C meningococcal disease and an increase in serogroup C cases[15,19].

Meningococcal polysaccharide vaccines A and C were used for routine immunization after the initial serogroup C disease outbreak in 2003. Since then, strains of ST-4821 complex have caused only sporadic cases. However, in 2010, an outbreak of ST-4821 serogroup C meningococcal infections occurred in a jail in Jinan City, China. Three patients with meningococcal disease, reported in five days, were inmates sharing the same cell and administered chemoprophylaxis. Furthermore, a mass polysaccharide vaccination was undertaken for serogroup A plus C in people closely contacted with the patients. An investigation focusing on the meningococcal carriage of the inmates was conducted. In this study, the cases, the carriage status among the men in the jail and the molecular characteristics of *N. meningitidis* strains isolated during this outbreak were described, and the molecular characteristics of 416 serogroup C *N. meningitidis* strains isolated from 27 provinces in China between 2003 and 2010 and their distribution were analyzed.

MATERIALS AND METHODS

Meningococcal Disease and Bacterial Strains

A case of meningococcal disease was diagnosed on the basis of clinical symptoms and laboratory results of *N. meningitidis* isolated from a normally sterile site or detected from the meningococcal special genomic fragment in cerebrospinal fluid (CSF). Bacteria were identified by Gram-staining, oxidase reaction, and standard biochemical tests using API® NH test kits (bioMérieux, Lyons, France). *N. meningitidis* strains were serogrouped by slide agglutination using polyclonal antisera (Difco, Fisher Scientific, Paris, France) and PCR.

Study of Pharyngeal Carriage

The point prevalence of meningococcal carriage was determined in a sample of 166 people containing 16 cellmates of the patients and 150 inmates in other cells of the jail. Pharyngeal swabs were placed onto a blood agar plate (BAP) with 0.3% vancomycin and 0.4% polymyxin B and transferred to a carbon dioxide incubator.

Molecular Subtyping of Strains

PFGE and MLST were used for the molecular characterization of strains based on the PulseNet 1-day standardized PFGE protocol[14-15]. The cell suspension in a polystyrene tube (Falcon; 12 by 75 mm) was adjusted to an optical density of 4.8-5.2 by using bioMérieux DENSIMAT. *N. meningitidis* slices were digested with 40 U per slice *Nhe*I (New England Biolabs, Ipswich, MA, USA) for 4 h at 37 °C. The electrophoresis was performed in the CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA, USA).

Images were captured on the Gel Doc 2000 system (Bio-Rad) and converted to TIFF files for computer analysis. PFGE patterns and cluster analysis were compared using the BioNumerics version 5.1 software (Applied Maths, Kortrijk, Belgium). A PFGE pattern with one or more DNA bands different from the others was taken as a unique PFGE pattern and a new PFGE pattern code was assigned. The similarity of DNA fragment patterns was estimated using the Dice coefficient. Cluster analysis of fingerprints was performed with the unweighted-pair group method and arithmetic averages (UPGMA).

MLST was done by sequencing the gene fragments of *abc*Z, *adk*, *aro*E, *fum*C, *gdh*, *pdh*C, and *pgm* and using *Neisseria* primers and the method on the MLST website (http://pubmlst.org/neisseria/)[20]. Briefly, the 7 gene fragments were amplified by PCR with Pyrobest DNA polymerase (TaKaRa Bio, Dalian, China), purified, and sequenced using an ABI Prism 3730 DNA sequencer (Applied Biosystems, Foster City, USA). The sequence data were assembled using the SeqMan II of the DNASTAR program (DNASTAR, Wisconsin, USA). The sequences were compared with existing alleles on the MLST website, in order to determine the number of alleles and STs, and to classify the isolates into correct clonal complexes.

Strains Used in Nationwide Survey

Four hundred and sixteen serogroup C *N. meningitidis* strains were collected from 27 provinces in China from 2003 to 2010 in order to study the molecular characteristics of serogroup C *N. meningitidis* and the distribution of ST-4821 complex clone. The strains were identified as serogroup C *N. meningitidis* and subtyped by PFGE. One or more strains (at least 30%) of each PFGE pattern were analyzed by MLST.

RESULTS

Meningococcal Disease Cases in Jail

Three patients admitted to hospitals in Jinan City, Shandong Province, China, in May 2010, were inmates of the jail. The first two were 24 years old and the third was 18 years old. The 3 patients were suspected of *N. meningitidis* infection according to their clinical symptoms, which was confirmed by laboratory results. The patients complained of headache, nausea, vomiting, neck stiffness and loss of consciousness. Physical examinations showed positive Kernig's and Brudzinski's signs in the first patient and high temperature (>38 °C) in 3 patients. Cerebrospinal fluid samples were turbid. *N. meningitidis* was found 24 h after CSF and blood from the first two patients were cultured. Isolates were identified as serogroup C using specific antiserum (Remel, Lenexa, KS, USA). PCR showed positive *N. meningitidis* serogroup C in CSF samples from the 3 patients.

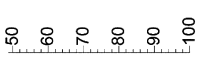
Pharyngeal Carriage among the People in Jail

The point prevalence of meningococcal carriage was investigated in 166 subjects containing 16 cellmates of the patients and 150 inmates in other cells of the jail. Of the identified, 47 *N. meningitidis* strains, 29 were serogroup C strains, 10 were serogroup B strains, 2 were serogroup A strains, 1 was serogroup W135 strain and 4 were non-grouped strains. Of the 16 cellmates of patients, 10 carried *N. meningitidis* strains including 6 serogroup C strains, 1 serogroup B strain, 1 serogroup W135 strain and 2 non-grouped strains, with a carriage rate of 62.5%. Of the other 150 inmates, 37 carried *N. meningitidis* strains including 23 serogroup C strains, 9 serogroup B strains, 2 serogroup A strains, 1 serogroup Y strain, and 2 non-grouped strains, with a carriage rate of 24.6%. The prevalence of *N. meningitidis* with pharyngeal carriage was significantly higher in inmates sharing a cell with the patients than in those sharing other cells (P<0.01, Mantel-Haenszel chi-square test).

Molecular Characteristics of Outbreak-related Strains

The *N. meningitidis* isolates were characterized by PFGE and MLST. Forty-nine strains isolated from 21 PFGE patterns were analyzed (Figure 1). Of which, 4 belonged to the NMNh.CN0001 pattern (a pulse type associated with the serogroup C outbreak in 2003-2004), 24 (2 from 2 patients, 6 from cellmates of the patients and 16 from inmates in other cells of the jail) showed an identical new pulse type designated as NMNh.CN0244 with a high similarity (87.0%) to NMNh.CN0001. Two bands (96.200 Kb and 45.480 Kb) present in NMNh.CN0001 were absent in NMNh.CN0244 and their total molecular weight was approximate to the molecular weight of one band (147.160 Kb) present in NMNh.CN0244 but absent in NMNh.CN0001, suggesting that there is a digesting site of *Nhe*I different between NMNh.CN0001 and NMNh.CN0244. The *Nhe*I digesting site and molecular weight of fragments in strain 053442 were analyzed using the DNASTAR 5.01 software (DNASTAR, Inc., WI). Strain 053442

was a complete genome-sequenced strain with pulse type NMNh.CN0001[21]. A digesting site of *Nhe*I cutting 2 fragments of 102.956 Kb and 47.779 Kb was similar to the molecular weight of 2 bands in NMNh.CN0001. The digesting site was located in 960032 (noncoding region) of complete genomic of 053442 (NC-010120). Primers were designed to amplify and sequence the base sequences of *Nhe*I



Strain ID	PFGE pattern	MLST type	MLST complex	Source of sample	Serogroup with agglutination	Serogroup with PCR
371036	NMNh.CN0245			Other	NG	other
371040	NMNh.CN0248	ST-9236		Other	NG	A
371047	NMNh.CN0255			Roommate	NG	other
371061	NMNh.CN0257	ST-8489		Other	C	C
371033	NMNh.CN0179			Roommate	W135	W135
371054	NMNh.CN0244	ST-4821	ST-4821 complex	Patient	C	C
371055	NMNh.CN0244	ST-4821	ST-4821 complex	Other	C	C
371056	NMNh.CN0244			Other	C	C
371058	NMNh.CN0244			Other	C	C
371060	NMNh.CN0244			Other	C	C
371062	NMNh.CN0244			Other	C	C
371013	NMNh.CN0244			Other	C	C
371018	NMNh.CN0244			Other	C	C
371020	NMNh.CN0244	ST-4821	ST-4821 complex	Roommate	C	C
371021	NMNh.CN0244	ST-4821	ST-4821 complex	Other	NG	C
371024	NMNh.CN0244			Roommate	NG	C
371028	NMNh.CN0244			Roommate	NG	C
371029	NMNh.CN0244	ST-4821	ST-4821 complex	Roommate	C	C
371031	NMNh.CN0244			Other	C	C
371032	NMNh.CN0244			Other	C	C
371039	NMNh.CN0244			Other	NG	C
371041	NMNh.CN0244	ST-4821	ST-4821 complex	Roommate	C	C
371042	NMNh.CN0244			Other	NG	other
371044	NMNh.CN0244	ST-4821	ST-4821 complex	Other	C	C
371045	NMNh.CN0244			Other	C	C
371046	NMNh.CN0244			Roommate	C	C
371049	NMNh.CN0244			Other	NG	C
371050	NMNh.CN0244	ST-4821	ST-4821 complex	Other	NG	C
371012	NMNh.CN0244	ST-4821	ST-4821 complex	Patient	C	C
371052	NMNh.CN0001			Other	NG	C
371053	NMNh.CN0001	ST-4821	ST-4821 complex	Other	C	C
371016	NMNh.CN0001			Other	NG	C
371019	NMNh.CN0001	ST-4821	ST-4821 complex	Other	NG	C
371057	NMNh.CN0258			Other	NG	B
371026	NMNh.CN0261			Other	B	B
371034	NMNh.CN0262	ST-9237		Other	NG	B
371037	NMNh.CN0259	ST-2146	ST-198 complex	Other	NG	A
371043	NMNh.CN0260	ST-8492		Other	C	C
371030	NMNh.CN0252	ST-6927		Other	NG	B
371035	NMNh.CN0254	ST-92	ST-92 complex	Other	NG	Y
371023	NMNh.CN0250			Other	NG	B
371025	NMNh.CN0250			Other	NG	B
371017	NMNh.CN0249			Other	NG	other
371038	NMNh.CN0251			Roommate	NG	other
371022	NMNh.CN0247			Other	NG	B
371027	NMNh.CN0247	ST-8490		Roommate	B	B
371015	NMNh.CN0256			Other	NG	B
371014	NMNh.CN0253	ST-32	ST-32 complex	Other	NG	C
371051	NMNh.CN0246			Other	B	B

Figure 1. PFGE showing 49 patterns of *Neisseria meningitidis* strains.

digesting site in 960032 of strain 053442. The 3 strains of NMNh.CN0001 and NMNh.CN0244 were sequenced. A→G and T→C mutations were detected in 11 bases away from the digesting site of NMNh.CN0244 strains different from NMNh.CN0001 strains (Figure 2). Twenty strains containing 9 NMNh.CN0244 strains, 2 NMNh.CN0001 strains and 9 other pulse types were selected for MLST analysis. The MLST results were highly consistent with those of the PFGE analysis. The 11 NMNh.CN0244 and NMNh.CN0001 strains formed the sequence type -4821 (ST-4821 complex), and a high MLST diversity was found between the strains of other pulse types. The carriage rate of the ST-4821 clone and strains was 15.7% in close contacts. The carriage rate of ST-4821 clone strains was significantly higher in cellmates than in inmates in other cells (37.5% *vs* 13.3%, $P<0.05$, Mantel-Haenszel chi-square test).

Molecular Characteristics and Nationwide Distribution of Serogroup C N. meningitidis

A total of 416 serogroup *C N. meningitidis* strains was collected from 27 provinces in China between 2003 and 2010 for analyses, where 132 strains were from patients, 150 from close contacts of patients, and 134 from healthy individuals. All the 416 strains were analyzed by PFGE, producing 53 pulsetypes. Pulsetypes NMN06.CN0001 and NMN06.CN0002 were the most dominant two, containing 252 and 51 strains, respectively. There was only one band different between NMN06.CN0001 and NMN06.CN0002, giving a similarity value of 95.65%. Other 113 strains belonged to 52 different pulsetypes containing 1-7 in each. For the 226 strains, 1-110 from each pulsetype were selected to be analyzed by MLST and produced 29 STs (Figure 3). In the MLST analysis, 87.61% (198/226) of serogroup C strains belonged to ST-4821 complex, including 180 strains belonging to ST-4821 and other 46 strains belonging to 5 SLVs, 4 double-locus variants (DLVs) and 1 three-locus variants (TLVs) from ST-4821. The other 12.39% (28/226) of serogroup C strains belonged to 18 different STs containing 1-7 in each.

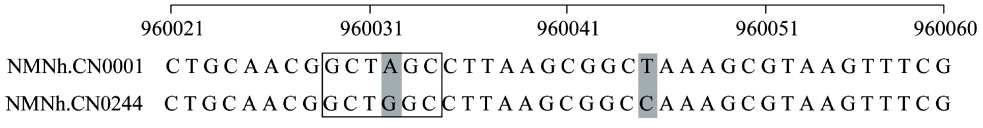


Figure 2. Genetic basis for difference in PFGE patterns of *Neisseria meningitidis* strains.

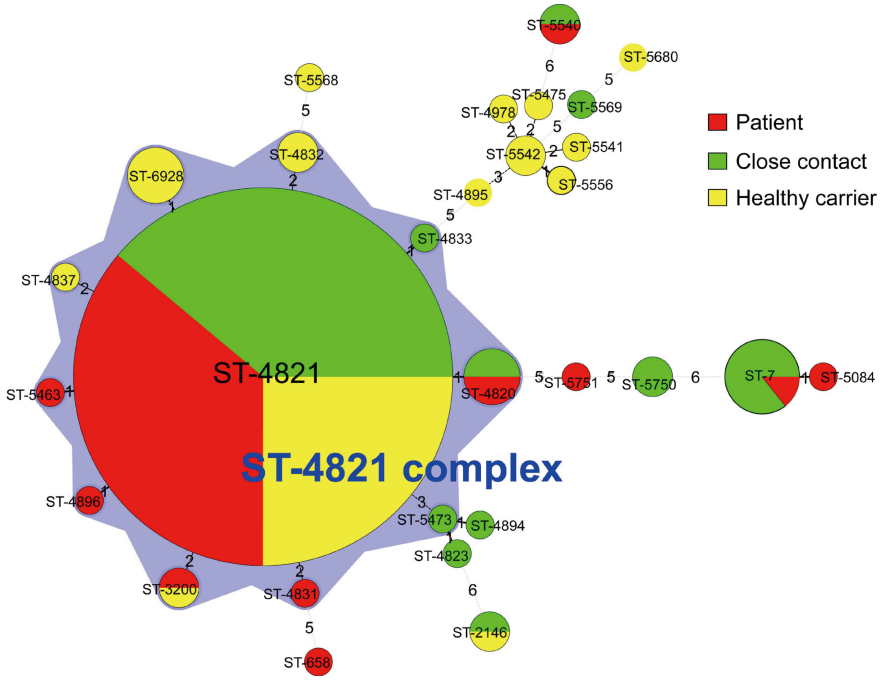


Figure 3. Minimum spanning tree analysis showing serogroup C *Neisseria meningitidis* strains according to their sequence type (ST) in 2003-2010.

MLST and PFGE results showed high consistency and we presumed the strains with the same PFGE patterns also had the same STs. According to combined analyses by PFGE and MLST, 375 (90.14%) strains belonged to ST-4821 complex, containing 126 (95.45%) strains from patients, 132 (88.00%) from close contacts of patients, and 117 (87.31%) from healthy individuals. The ST-4821 complex was observed each year that was studied, and was always the dominant lineage of serogroup C strains from 2003 to 2010. The isolates from the ST-4821 complex that were firstly identified in Anhui Province in 2003 had spread to and got distributed in 24 provinces between 2003 and 2010, and was mainly concentrated in the eastern provinces (Figure 4).

Figure 4. Distribution of analyzed serogroup C and ST-4821 complex *Neisseria meningitidis* in China.

DISCUSSION

In this study, PFGE and MLST analysis showed that the meningococcal disease was caused by the same strain in 3 patients in a male prison. Isolates from other inmates in close contact with the patients could be divided into strains related or not related with disease by PFGE and MLST, suggesting that these two methods can identify the types of meningococcal clones circulating in a specific place and determine the range of an outbreak. The pharyngeal carriage rates of *N. meningitidis* and pathogenic strain were significantly higher in cellmates of patients than in inmates of the jail.

In China, clusters of meningococcal diseases are usually found in schools and communities[15,22]. No cluster of meningococcal diseases was found in a jail of China, although indirect exposure to the jail population is a strong risk factor for meningococcal disease[23]. A cluster of meningococcal diseases in a jail system described in this study may be a risk factor for meningococcal disease in China.

The population structure of serogroup C *N. meningitidis* is of high genetic diversity, and the invasive meningococcal disease is caused by a limited number of clonal groups, such as ST-11, ST-41/44, ST-8, and ST-4821 complexes[24-26]. Studies on pharyngeal carriage during outbreaks of clonal serogroup C disease demonstrated that the carriage

rate of pathogenic strain is low (0.3%-1.7%)[27-29]. However, the carriage rate of ST-4821 clone was higher in close contact inmates in this study, suggesting that the ST-4821 clone is highly transmissible and spreads quickly in healthy populations under certain conditions.

The *N. meningitidis* serogroup C ST-4821 clone was first identified in Anhui Province, China, in 2003-2004 and caused several outbreaks. A serogroup A+C vaccination campaign has been implemented in China since 2004 and no similar outbreak has occurred since then. However, the sporadic meningococcal cases caused by ST-4821 have been occurring continually, and almost all serogroup C invasive meningococcal cases in China were caused by this clone[15,19]. The ST-4821 serogroup C clone is an important clone in China at present and 90.14% of the serogroup C strains isolated between 2003 and 2010 belong to ST-4821 complex containing strains from 24 provinces as was confirmed in this study. The PFGE patterns of ST-4821 complex strains from other provinces were identical or similar to those of outbreak isolates from Anhui Province. ST-4821 complex serogroup C meningococci are a major hyperinvasive clone in China as observed in the past 10 years. Further study is needed to monitor the incidence of disease outbreaks caused by this virulent meningococcal clone and its spread, and the carriage status of ST-4821 clonal complex strains in healthy populations.

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