Metagenomic data from cerebrospinal fluid permits tracing the origin and spread of Neisseria meningitidis CC4821 in China

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Metagenomic next-generation sequencing (mNGS) is useful for difficult to cultivate pathogens. Here, we use cerebrospinal fluid mNGS to diagnose invasive meningococcal disease. The complete genome sequences of Neisseria meningitidis were assembled using N. meningitidis of ST4821-serotype C isolated from four patients. To investigate the phylogeny, 165 CC4821 N. meningitidis genomes from 1972 to 2017 were also included. The core genome accumulated variation at a rate of $4.84 \times 10^{-8}$ substitutions/nucleotide site/year. CC4821 differentiated into four sub-lineages during evolution (A, B, C, and D). While evolving from sub-lineage A (early stage) to sub-lineage D (late stage), the ST and CC4821 serotype converged into the ST4821-serotype C clone. Most strains of sub-lineage D were isolated from invasive meningococcal disease, with increasing resistance to quinolones. Phylogeographic analysis suggests that CC4821 has spread across 14 countries. Thus, the selective pressure of quinolones may cause CC4821 to converge evolutionarily, making it more invasive and facilitating its spread.

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Meningitis is a serious life-threatening inflammation of the meninges and subarachnoid space that occurs on a global scale in individuals of all ages, causing high rates of morbidity and mortality. *Neisseria meningitidis* are one of the important pathogens causing bacterial meningitis. The upper respiratory tract is the only reservoir of *N. meningitidis*, although some individuals develop invasive diseases. Once this bacterium invades the blood, human bactericidal antibodies, complement, and phagocytes are activated to eliminate the bacteria. However, if the host defense system is unable to clear the bacteria, this infection develops into bacteremia, followed by invasion into the meninges, which then quickly develops into meningitis and pathogenic sepsis. Typical signs of meningitis include a stiff neck, fever, and changes in mental status. Susceptible populations include infants, adolescents, and young individuals (under the age of one). In addition, individuals with complement system defects and asplenia are also at high risk. Meningococcal meningitis has a mortality rate between 5% and 15%, which can rise to 50% if left untreated. Over one-third of individuals who develop invasive meningococcal disease survive but experience major complications, with 9% suffering from long-lasting sequelae.

Abnormal results of general laboratory tests for meningococcal meningitis cerebrospinal fluid include high opening pressure, increased white blood cell count, increased protein concentration, and decreased glucose concentration. Clinicians can make preliminary clinical diagnoses of meningococcal meningitis based on the symptoms and abnormal laboratory test results to promptly treat affected patients. However, to make a precise pathogenic diagnosis, it is necessary to isolate or detect *N. meningitidis* in the patient’s cerebrospinal fluid, blood, or skin lesions. Pathogenic detection methods for *N. meningitidis* include Gram staining, culture, and polymerase chain reaction (PCR). The majority of meningococcal meningitis patients (85%) are diagnosed using Gram staining. In meningococcal meningitis, the positive rate of *N. meningitidis* cultured in blood culture is approximately 50%, while that in the cerebrospinal fluid is 75%. Compared with the culture method, PCR can be used for faster detection of *N. meningitidis* and non-viable bacteria. Loop-mediated isothermal amplification can be used to detect *N. meningitidis* more accurately than culture and PCR. In recent years, metagenomic next-generation sequencing (mNGS) has started to be applied in the detection of infectious disease pathogens. In the present study, mNGS was used to successfully detect *N. meningitidis* in the cerebrospinal fluid of four patients with suspected meningococcal meningitis, and the genome of *N. meningitidis* was assembled for further research.

The incidence of invasive meningococcal disease has been declining since the early 2000s. In 2017, the incidence of invasive meningococcal disease in Europe was 0.45 to 1.33 cases per 100,000 population, with a case fatality rate of 9.7%. Meningococcal B (MenB) (51%) is currently the most common serotype in Europe, followed by MenW (17%) and MenC (16%). In the United States, invasive meningococcal disease has declined sharply in recent decades, and by 2017, the incidence was only 0.13/100,000. The distribution of serotypes from 2006 to 2015 was as follows: MenB, 38%; MenY, 30%; MenC, 24%; and MenW, 7%. In Central and South America, the current incidence of invasive meningococcal disease is less than 1 in 100,000, with MenC being the most common serotype. Historically, the meningitis belt in North Africa has had the highest incidence of invasive meningococcal disease in the world. MenA used to be the most common serotype in sub-Saharan Africa, giving rise to several outbreaks of invasive meningococcal disease. Since the large-scale vaccination of individuals with MenA conjugate vaccine MenAfriVac in 2010, the incidence of invasive meningococcal disease has fallen rapidly, resulting in a shift of the epidemiology of the invasive meningococcal disease. The current most common serotypes are mainly MenC, MenW, and MenX.

In Australia, the incidence of the invasive meningococcal disease dropped after MenC conjugate vaccine vaccination, but gradually increased (1.5/100,000) from 2013 to 2017. Currently, MenW is the most common serotype. In New Zealand, MenB is the most common serotype of invasive meningococcal disease. After a MenB strain-specific OMV vaccine (MeNZB) inoculation, the incidence of invasive meningococcal disease dropped from 17.4/100,000 to 1.2/100,000. Before the 1980s, the meningococcal disease was a very serious public health problem in China, reaching a peak of 403/100,000 in 1967, with MenA being the dominant serotype. However, since the widespread vaccination against MenA was started in the early 1980s, the rates of meningococcal disease have dropped markedly and are currently below 1/100,000. Since then, MenC belonging to the cloning complex (ST4821) has become the most dominant serotype. In this study, the *N. meningitidis* detected in the cerebrospinal fluid of four pediatric patients with mNGS also belonged to ST4821-MenC. We downloaded all the genome sequences of *N. meningitidis* belonging to CC4821 from http://pubmlst.org/neisseria, performed phylogenetic analysis, analyzed its evolutionary rate, traced its origin time, and investigated its evolutionary trajectory in the world.

## Results

### Epidemiology of CC4821

The CC4821 clone of *N. meningitidis* was mainly prevalent in China (127/169, 75.1%), followed by the UK (20/169, 11.8%), with sporadic cases in Brazil, Colombia, Greece, India, Ireland, Italy, Japan, Malta, New Zealand, Spain, Sweden, and the USA (Fig. 1). Serotype B (92/169, 54.4%) was the most prevalent serotype of CC4821, followed by serotype C (65/169, 38.5%) and serotype W (10/169, 5.9%). The CC4821 clones included in this study had 49 ST types, of which ST4821 (45/169, 26.6%) was the dominant ST type, followed by ST3200 (31/169, 18.3%), ST5664 (14/169, 8.3%), ST8491 (9/169, 5.3%), ST3436 (7/169, 4.1%), ST5798 (6/169, 3.6%), and ST9454 (5/169, 3.0%). Among 45 ST4821s, serotype C (38/45, 84.4%) was the most prevalent serotype, followed by serotype B (7/45, 15.6%). ST3200 was the opposite of ST4821, with serotype B (29/31, 93.5%) being the dominant serotype, while serotype C accounted for only 6.5% (2/31). Four ST5798s were from Shanghai, all of which were serotype B. The geographical distribution of ST3200 was as follows: UK (16 serotype B, 1 serotype C), China (7 serotype B, 1 serotype C), Brazil (2 serotype B), Ireland (1 serotype B), Italy (1 serotype B), Greece (1 serotype B), and Malta (1 serotype B).

### Phylogenetic reconstruction of the CC4821 populations

After the recombination regions were excluded, a total of 169 core genomes were used to reconstruct the evolutionary history of *N. meningitidis* CC4821 (Fig. 2 and Supplementary Figure 3). These data revealed that the core genome accumulated variation at a rate of $4.8 \times 10^{-8}$ substitutions per nucleotide site per year [95% highest probability density (HPD), $4.2 \times 10^{-8}$ to $5.7 \times 10^{-8}$]. Phylogeny analysis showed that CC4821 differentiated into four sub-lineages during evolution: sub-lineage A, B, C, and D (Fig. 2). These four sub-lineages shared the most recent common ancestor (MRCA), dating back to approximately 1941 (Fig. 2; 95% HPD 1924–1954). From sub-lineage A (early stage) to sub-lineage D (late stage), the serotype of CC4821 converged, mainly into the serotype C clone (Fig. 2). A cluster of serotype W isolates emerged in sub-lineage C (Fig. 2). In the CC4821 phylogenetic tree, isolates from different locations were found to be intermingled (Fig. 2), indicating that frequent transmission had
occurred between different locations. In sub-lineage A, more strains were isolated from carriers than invasive diseases, while all other strains were isolated from invasive disease, except for nine strains in sub-lineage D (Fig. 2).

Phylogeographic reconstruction of CC4821 populations in the world. The worldwide spread of CC4821 was reconstructed using Bayesian phylogeography. As a result, CC4821 was found to spread to 14 countries (Fig. 3). This cross-country and cross-continent spread may be related to globalization.

The evolutionary dynamic of the accessory genome of CC4821 populations. We extracted the accessory genomes of 169 N. meningitidis CC4821 isolates and found that the accessory genomes were quite different (Supplementary Figure 4). This suggests that CC4821 underwent some changes in its accessory genome during its evolution, which is worthy of attention.

During the evolution of CC4821, antimicrobial resistance did not change. The vast majority of CC4821 carried farB and had 23S rRNA mutation. Several isolates carried farA, mtrD, tet(B), tetR, and rpsJ and had penicillin-binding protein (PBP) and gyrA mutations. FarA and farB are components of the FarAB efflux complex, which are related to the resistance of antibacterial free fatty acids. The 23S rRNA mutation confers resistance to macrolide antibiotics. As a component of MtrCDDE efflux complex, mtrD is related to macrolide resistance. Tet(B) and tetR are the tetracycline efflux proteins expressed in many Gram-negative bacteria. They confer resistance to tetracycline, doxycycline, and minocycline, but not tigecycline. The rpsJ gene encodes tetracycline-resistant ribosomal protection protein, which is associated with tetracycline. The PBP2 mutation is related to β-lactam resistance, while gyrase (encoded by gyrA), the target site of quinolones, mutated, giving rise to quinolone resistance. During the evolution from early sub-lineage A to late sub-lineage D, the resistance to quinolones increased quickly, and most sub-lineage D strains carried the amino acid change (T91I) in gyrA, which confers quinolone resistance (Fig. 4).

Over time, the virulence genes carried by CC4821 were found to not have changed much. Most isolates carried several virulence genes, which are related to adherence, immune evasion, immune modulator, invasion, iron uptake, protease, and stress adaptation (Fig. 5). Moreover, no differences were observed in virulence genes between carrier strains and invasive strains, the reason being that some virulence genes were located on plasmids, which were not sequenced.

Discussion

At present, the immunization schedule for children with meningococcal disease in China is as follows: children between 6–18 months of age receive two doses of serotype A capsular polysaccharide vaccine, followed by one dose of serotype A plus C capsular polysaccharide vaccine at 3 and 6 years of age. Although China has generally implemented meningococcal disease immunizations in children, sporadic cases of meningococcal disease are still being reported. Meningococcal meningitis is more common in children, and the four cases evaluated in this study were in patients between the ages of 9 and 13 years. The isolation of N. meningitidis from the cerebrospinal fluid is the most direct evidence for the diagnosis of meningococcal meningitis. However, none of the four cases in this study were cultured with N. meningitidis. As such, mNGS was used to confirm the etiological diagnosis, although only in four cases, indicating that the sensitivity of mNGS may be higher than that of traditional culture methods in the etiological diagnosis of central nervous system infections. mNGS has been used in the diagnosis of central nervous system infections13–16. When meningococcal meningitis is clinically suspected but the culture is negative, mNGS can be used to confirm the etiological diagnosis in as little as 24 h in order to obtain early etiological evidence and enable rapid treatment.

There were five epidemics of meningococcal serotype A in China between 1938 and 1977, during which the incidence reached a peak of 403/100,000 in 196720. However, since China began to vaccinate using the MenA capsular polysaccharide vaccine in the 1980s, the incidence of MenA has fallen markedly. Subsequently, the expansion of the highly virulent clone complex (CC) 4821 led to a change in the epidemiology of meningococcal disease, from MenA to MenC23. Previous surveillance data showed that MenC CC4821 had an outbreak in China between...
Fig. 2 Maximum clade credibility (MCC) tree and distributions of serotype, sequence type (ST), and disease type amongst CC4821 isolates. MCC tree of CC4821 population based on BEAST analysis. The tips of the tree were colored according to the location of which isolates were sampled. The time scale was shown below the tree. The four colored boxes in the MCC tree represented the four lineages of CC4821, sub-lineages A, B, C, and D. The distributions of serotype, sequence type (ST), and disease types were plotted against the core genome phylogeny.
Italy

CC4821 has spread to new countries, including France 2003 and 200524, and the MenC strain has been the dominant clone between 2005 and 201225. The N. meningitidis strains of the four pediatric cases evaluated in this study were all ST4821, belonging to the CC4821 clone complex. Therefore, we conducted further studies on the phylogeny of N. meningitidis CC4821. This study also suggests that CC4821 has undergone a process of converging evolution, from the early polyclonal to the late single clone ST4821-MenC, which indicates that the outbreak of CC4821 is caused by the expansion of the MenC clone.

Also worth noting is the evolution of CC4821, from the early carrier and invasion strains coexisting (sub-lineage A) to the recent tendency to be more prone to invasion (sub-lineage D). This phenomenon has also been found in other clones. Compared with the N. meningitidis ST-41/44 CC isolated between 1971-2000, the strains isolated between 1988-2015 are more prone to invasive disease26. However, the mechanism underlying the tendency of CC4821 to be more likely to occur in invasive diseases during evolution requires further study.

In this study, the number of quinolone-resistant strains in CC4821 gradually increased over time, and most of the strains in the late lineage (sub-lineage D) were resistant to quinolones. Quinolone resistance in N. meningitidis in China has evolved over time, from the era of no quinolone resistance (before ≈1985) to the era of quinolone resistance (none versus >70%), especially in the highly virulent CC4821 and CC5 lineages24. In this study, the results of phylogenetic analysis suggest that quinolone resistance plays an important role in the evolution of CC482123, and fluoroquinolone selective pressure is the main reason for the outbreak of CC4821 in China. A recent study has shown that over half of quinolone-resistant N. meningitidis isolates obtained quinolone resistance genes from three commensal Neisseria species, N. lactamica, N. cinerea, and N. subflava, through horizontal gene transfer27. Nonetheless, in the present study, quinolone resistance was found to be caused by a target mutation, which was related to the selective pressure of quinolones.

To the best of our knowledge, this study is the first to deduce the evolutionary rate and transmission of CC4821. Recently, CC4821 has spread to new countries, including France (2009–2011)28, the UK (2011-2014)28, USA (2007–2016)28, Canada (2013–2015)29, Japan (2017)30, and India (2017)31. This reveals that the geographical spread of CC4821 is increasing, which is worthy of continued attention.

**Methods**

**Patients and samples.** Patient 1: A 9-year-old previously healthy boy presented with fever and confusion of half a day to the Children’s Hospital of Hebei Province. Upon examination, the patient was found to be febrile (36.7 °C), with a pulse of 120 beats per minute and a respiratory rate of 38 breaths per minute. Laboratory examination revealed peripheral leukocytosis with an increased neutrophil count and elevated C-reactive protein levels. Lumbar punctures revealed increased pressure in the cerebrospinal fluid. Elevated cell counts and protein levels but lower glucose levels were also observed. A cerebrospinal fluid smear was positive for Gram-negative diplococcus. N. meningitidis was identified using mNGS in a cerebrospinal fluid sample. Upon detection, the patient was administered a treatment of meropenem and 15 days of penicillin, and he was discharged from the hospital after his complete recovery.

Patient 2: A 13-year-old boy presented to the Children’s Hospital of Hebei Province with a two-day history of fever and a one-day history of headache and lethargy. Upon examination, the patient was found to have a body temperature of 36.7 °C, with a pulse of 128 beats per minute and a respiratory rate of 22 breaths per minute. A lumbar puncture was performed on day 2 of hospitalization after the administration of revealed pleocytosis (199858 white blood cell µL⁻¹), 3.95 g L⁻¹ protein content, and 0.16 mmol L⁻¹ glucose content. Although this patient’s cerebrospinal fluid and blood cultures were negative, he was diagnosed with invasive meningococcal disease by mNGS. He was then treated with meropenem and penicillin for two weeks and recovered.

Patient 3: A 13-year-old boy was admitted to the Children’s Hospital of Hebei Province with a two-day history of fever, headache, emesis, and confusion. Upon examination, the patient was found to have a temperature of 36.4 °C, with a pulse of 72 beats per minute, a blood pressure of 107/67 mmHg, and a respiratory rate of 20 breaths per minute. The first laboratory test indicated hematologic abnormalities of the white blood cell (18.7 × 10⁹ L⁻¹) and polymorphonuclear neutrophils (87.0%). The results showed cerebrospinal fluid pleocytosis (59440 white blood cell µL⁻¹), elevated protein content (6.1 g L⁻¹), and decreased glucose content (0.03 mmol L⁻¹). Although the results of infectious disease cultures were negative, N. meningitidis was detected by mNGS in his cerebrospinal fluid. The patient was then treated with chloramphenicol and penicillin for two weeks, but was discharged from the hospital with incomplete recovery.

Patient 4: A 12-year-old girl presented to the Children’s Hospital of Hebei Province with a two-day history of fever and headache, a one-day history of emesis, and a half-day history of confusion. Upon examination, she was found to be febrile (37.5 °C), with a pulse of 104 beats per minute, a blood pressure of 111/64 mmHg, and a respiration rate of 28 breaths per minute. The cerebrospinal fluid evaluation...
Fig. 4 Presence or absence of antimicrobial resistance (AMR) genes amongst CC4821 isolates. The distribution of AMR genes was plotted against the core genome phylogeny. The names of different types of AMR genes were displayed in different colors.
Fig. 5 Presence or absence of virulence genes amongst CC4821 isolates. The distribution of virulence genes was plotted against the core genome phylogeny. The names of different types of virulence genes were displayed in different colors.
revealed cerebrospinal fluid pleocytosis (19858 white blood cell μL⁻¹), protein (3.95 g L⁻¹), and glucose (0.16 mmol L⁻¹). Although the results of infectious disease evaluation were negative, N. meningitidis was detected by mNGS in a cerebrospinal fluid sample. The patient was then administered a course of meropenem, and her symptoms resolved before discharge.

*N. meningitidis* identified by mNGS in the cerebrospinal fluid specimens of these four patients all belonged to ST4821-serotype C. To investigate the phylogeny of CC4821, we also included the genome of 165 CC4821 *N. meningitidis* from the PubMLST Neisseria genome database (http://pubmlst.org/neisseria)22. Among the 169 *N. meningitidis* isolates, 127 were from China, 20 were from the UK, four were from India, three were from Italy, three were from USA, two were from Brazil, two were from France, one was from Colombia, one was from Greece, one was from Japan, one was from Malta, one was from New Zealand, one was from Spain, and one was from Sweden (Fig. 1). This study has included all the sequenced CC4821 genomes downloaded from the PubMLST Neisseria genome database (http://pubmlst.org/neisseria)22 (Supplementary Data 1).

**Metagenomic DNA-seq** DNA was extracted from the cerebrospinal fluid using QIAamp DNA MicroBee Kit (Qiagen). Paired-end libraries were prepared with the Vazyme TruePrepTM DNA Library Prep Kit (Vazyme). Sequencing was performed using the NextSeq 550 System (Illumina), resulting in paired reads of 150 bp in length. Taxonomy was evaluated using Kraken2 v2.0.8beta -db $database -threads 6 -unclassified -min_length. Taxonomy was evaluated using Kraken2 v2.0.8beta -db $database -threads 6 -unclassified -min_length. Taxonomy was evaluated using Kraken2 v2.0.8beta -db $database -threads 6 -unclassified -min_length. Taxonomy was evaluated using Kraken2 v2.0.8beta -db $database -threads 6 -unclassified -min_length. Taxonomy was evaluated using Kraken2 v2.0.8beta -db $database -threads 6 -unclassified -min_length.

**Code availability** The software used is described in the Methods section. The open source software includes the following external tools: Kraken2 v2.0.8beta, Prokka v1.12, Roary v3.11.2, and BEAST v2.4.7. Descriptions of all open source code and software are included in the Methods section, and further details are available on request.

**Data availability** The sequence data of the clinical *N. meningitidis* isolates were deposited under BioProject ID PRNA722594. Other relevant data that support the findings of this study are available in the PubMLST Neisseria genome database (https://doi.org/10.12688/wellcomeopenres.14826.1) (ref. 22).

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Author contributions
H.W. and H.C. designed and supervised the study. M.L. and Y.G. obtained the samples and clinical details. S.T., X.Z., X.W., Y.Z., and C.Z. assisted in extracting the DNA and performing the mNGS. H.C. performed the data analysis. H.C. and M.L. wrote the manuscript. H.C. was a major contributor to writing the manuscript. All authors read, edited, and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by the research ethics board at Peking University People's Hospital (2019PHB134).

Competing interests
The authors declare no competing interests.

Additional information
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