

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Wu HM, Harcourt BH, Hatcher CP, et al. Emergence of ciprofloxacin-resistant *Neisseria meningitidis* in North America. *N Engl J Med* 2009;360:886-92.

SUPPLEMENT TO: Wu HM, Harcourt BH, Hatcher CP, et al. Emergence of Ciprofloxacin-Resistant *Neisseria meningitidis* in North America. N Engl J Med 2009;360.

SUPPLEMENTARY METHODS

Carriage survey

Close contacts of Patient 3 that participated in the survey provided oral informed consent and were interviewed to obtain information on demographics and meningococcal carriage risk factors. Recruited participants from the universities and pub provided written informed consent and were given a questionnaire regarding demographic and meningococcal carriage risk factors, which included questions on gender, age, recent antibiotic use, student status and year of study, smoking, living arrangements, history of meningococcal vaccination, and pub/nightclub attendance.

Real-time PCR detection of *N. meningitidis* serogroups

Real-time PCR was used to detect the capsule transport gene and serogroup specific genes (A, B, C, X, W135, and Y) as previously described¹ with the following modifications to the probe sequences:

ctrA: AACCTTGAGCAA"TTCCATTTATCCTGACGTTCT,

sacB (MenA): CTAAAAG"TTAGGAAGGGCACTTTGTGGCATAAT

siaD (MenC): TTTCAATGC"TTAATGAATACCACCGTTTTTTTTGC

synF (MenY): TATGGTG"TTACGATATCCCTATCCTTGCCTATAAT

The presence of a “T” indicates that the black hole quencher (BHQ1) was moved from the 3’ end to an internal position.

Multilocus sequence typing and typing of the porA, porB, and fetA alleles

Multilocus sequence typing and typing of *porA*, *porB*, and *fetA* were performed as described by the cited references in the article text; however, *fetA* amplification was performed under the following conditions:

95°C for 5 minutes, followed by 40 cycles of 95°C for 1 minute, 55°C for 1 minute, 72° for 2.5 minutes followed by 72°C for 7 minutes.

Amplification and sequencing of the quinolone resistance determining region of the gyrA and parC genes

The quinolone resistance determining region (QRDR) of *gyrA* and *parC* were PCR amplified and sequenced using primers derived from regions of homology between *Neisseria meningitidis* (MC58, FAM18, and Z2491; GenBank accession numbers: AE002098, AM421808, and AL157959, respectively), *Neisseria gonorrhoeae* (FA1090; accession number AE004969), and *Neisseria lactamica* (Sanger Institute sequence² used with permission of Dr. Julian Parkhill) that bracket the region. The QRDR of *gyrA* was amplified using the forward primer 5’ -¹ATGACCGACGCAACCATCCGC²¹ and the reverse primer 5’ -⁵³⁹GACGAGCCGTTGACGAGCAGTG⁵¹⁸ to produce a 539-base amplicon that was sequenced with the primers 5’ -⁵⁹GCCTTGAAGACGAAATGC⁷⁶ and 5’ -⁴⁸⁴GTTCGCTACCGTCGTAGTTC⁴⁶⁵. The QRDR of *parC* was amplified using the forward primer 5’ -⁴⁸GCTCGGCCGATACGCCGAACG⁶⁸ and the reverse primer 5’ -

⁵⁹⁸CAATCGCCGCCTGCGTGACTTC⁵⁷⁷ to produce a 530-base amplicon that was sequenced using the primers 5'-⁷⁹GAATACGCCATGAGCGTG⁹⁶ and 5'-⁵⁶⁵GCGACGGAATCTCGGTTCG⁵⁴⁸. Numbers denote base position in *gyrA* of AE002098.

gyrA and *parC* QRDR amplification were performed under the following conditions: 94°C for 4 minutes followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by 72°C for 5 min.

Whole gyrA gene amplification and sequencing:

The *gyrA* gene was amplified in two reactions:

Reaction 1: Forward primer, ¹ATGACCGACGCAACCATCCGC²¹ and reverse primer, ¹⁶¹⁹CAATGTCGCCGCCGAACGGGTTG¹⁵⁹⁶.

Reaction 2: Forward primer, ¹³⁸³GAGCGAGATTCAGGCAGATGC¹⁴⁰³ and reverse primer, ²⁷⁵¹TCAGTTCTCGGCTTCCGGTTC²⁷³¹.

The amplification conditions for the both of the reactions above were:

95°C for 5 minutes followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min followed by 72°C for 7 min.

DNA sequencing was performed using a set of 16 primers derived from regions of homology from the organisms listed above.

SUPPLEMENTARY RESULTS**Supplementary Table 1. Characteristics of Carriage Survey Participants.**

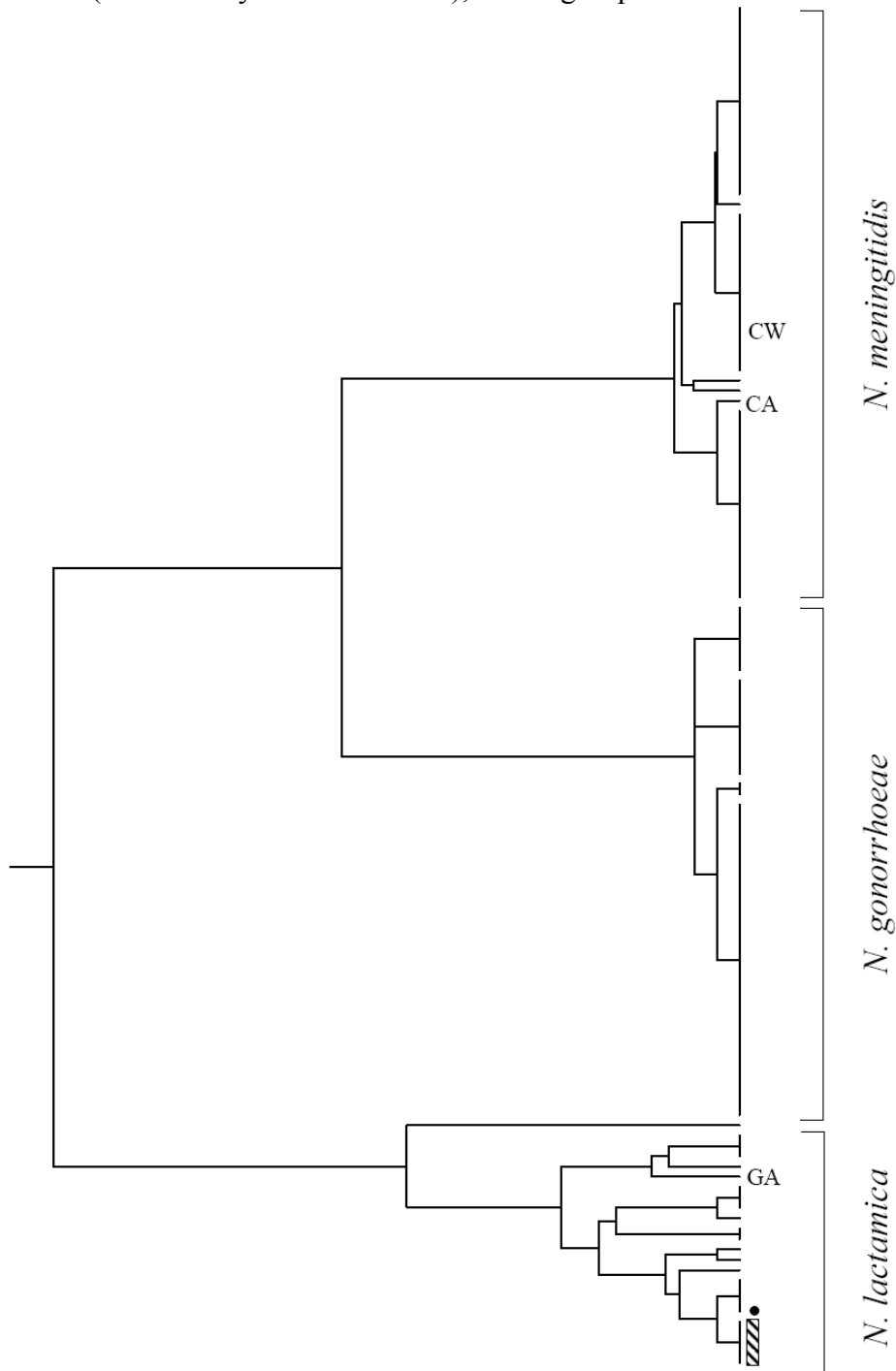
Participant demographics and questionnaire responses from the North Dakota-Minnesota pharyngeal carriage survey, 2008.

	Number (percent), total = 530^a
Close contacts of Patient 3	5 (1)
University participants	513 (97)
University A	142 (28)
University B	122 (24)
University C	249 (49)
Participants recruited at the pub	12 (2)
Currently a student	496 (95)
First year student	133 (25)
Female sex	261 (54)
Antibiotic use in the past 30 days	64 (12)
Smoked in the last 30 days	67 (13)
Reported meningitis vaccination in the past 3 years	144 (28)
Current residence in a dormitory	170 (33)
Bar or pub attendance once a week or more	114 (22)

^aTotals for specific questionnaire items vary due to missing values.

Supplementary Figure 1. Phylogenetic Analysis of *gyrA* Quinolone Resistance Determining Regions from Ciprofloxacin-Resistant *Neisseria meningitidis* and Other *Neisseria* Strains.

Phylogenetic analysis^a of the quinolone resistance determining regions (QRDR) of *gyrA* (nucleotides 73-518) from 62 *N. meningitidis*, 50 *N. gonorrhoeae*, and 20 *N. lactamica* isolates revealed that the sequences cluster by species, except for the sequences from ciprofloxacin-resistant *N. meningitidis* isolates from Patients 1-3 and the close contact of Patient 3 (indicated by the hatched bar), which group within the *N. lactamica* cluster.



Footnote for Supplementary Figure 1

^aPhylogenetic analysis using Unweighted Pair Group Method with Arithmetic Mean Averages (UPMGA) was performed using MEGA3.³

Supplementary Figure 1 Symbol Legend:

CW: QRDR sequence amplified from the cerebrospinal fluid (CSF) of the child-care worker who died from a probable case of meningococcal disease (North Dakota, 2006). No isolate was cultured from the CSF.

CA: Ciprofloxacin-resistant *N. meningitidis* isolate from California (2008).

GA: Ciprofloxacin-resistant *N. lactamica* isolate. Susceptibility testing was performed on a convenience sample of 9 *N. lactamica* isolates from a 2007 adolescent pharyngeal carriage survey conducted in Georgia (unpublished results). One isolate (GA) was found to have an elevated MIC against ciprofloxacin (0.5 µg per milliliter) by Etest. This isolate was also found to have the same T91I mutation found in the ciprofloxacin-resistant *N. meningitidis* isolates from North Dakota, Minnesota, and California, indicating that this mutation can be harbored by *N. lactamica*.

- Ciprofloxacin-sensitive *N. lactamica* North Dakota-Minnesota carriage isolate. The *gyrA* sequence from this isolate is depicted in the article Figure 2, panel C.

▨ North Dakota-Minnesota ciprofloxacin-resistant *N. meningitidis* isolates from Patients 1-3 and the close contact of Patient 3.

Supplementary Tables 2-4 list GenBank accession numbers (strain designations) and information on sequences used for UPMGA phylogenetic analysis in Supplementary Figure 1.

Supplementary Table 2. *N. meningitidis* accession numbers.

EU780465*	EU780515	EU780537
EU780466†	EU780516	EU780538
EU780467	EU780517	EU780539
EU780468	EU780518**	EU780540
EU780469	EU780519	EU780541
EU780470	EU780520	EU780542
EU780471‡	EU780521	EU780543
EU780472	EU780522	EU780544
EU780474§	EU780523	EU780545
EU780477	EU780524	EU780546
EU780478	EU780526	EU780547
EU780479	EU780527	EU780548
EU780480	EU780528	EU780549
EU780481	EU780529	EU780551
EU780482	EU780530	EU780552
EU780483	EU780531	EU780556
EU780484	EU780532	CP000381¶
EU780485	EU780533	AE002098 (MC58)
EU780486	EU780534	AL157959 (Z2491)
EU780504	EU780535	AM421808 (FAM18)
EU780514	EU780536	

* Ciprofloxacin-resistant isolate from the close contact of Patient 3.

† Ciprofloxacin-resistant isolate from Patient 1.

‡ *N. meningitidis* DNA amplified from the child-care worker who died from a probable case of meningococcal disease (North Dakota, 2006).

|| Ciprofloxacin-resistant isolate from Patient 2.

§ Ciprofloxacin-resistant isolate from Patient 3.

** Ciprofloxacin-resistant isolate from a patient with pneumonia (California, 2008).

¶ Genome from an isolate from China which has the T91I mutation in GyrA.⁴

Supplementary Table 3. *N. gonorrhoeae* accession numbers.

EU780487*	AY605900	AY443516†
EU780488*	AY443500†	AY443517†
EU780489*	AY443501†	AY443518†
EU780490*	AY443502†	AY443519†
EU780491*	AY443503†	AY443520†
EU780492*	AY443504†	AY443521†
EU780493*	AY443505†	AY443522†
EU780494*	AY443506†	AY443523†
EU780495*	AY443507†	AY443524†
EU780496*	AY443508†	AY443525†
EU780497*	AY443509†	AY443526†
EU780498*	AY443510†	AY443527†
EU780499*	AY443511†	AY443528†
EU780500*	AY443512†	AY443529†
EU780501*	AY443513†	AY443530†
EU780502*	AY443514†	AE004969 (FA1090)
EU780503*	AY443515†	

* Sequences EU780487-503 were from a convenience sample of 17 ciprofloxacin-resistant *N. gonorrhoeae* isolates collected by the Minnesota Department of Health from 2006 and 2007. These isolates were ciprofloxacin-resistant as tested by Etest and broth microdilution by the Minnesota Department of Health.

† Sequences AY443500-530 obtained by Sultan and coworkers.⁵

Supplementary Table 4. *N. lactamica* accession numbers.

EU780505	EU780525
EU780506	EU780550
EU780507	EU780553
EU780508	EU780554
EU780509	EU780555
EU780510*	EU780473
EU780511	EU780475
EU780512	EU780476
EU780513	

* Ciprofloxacin-resistant isolate from a 2007 adolescent pharyngeal carriage survey conducted in Georgia (unpublished results).

SUPPLEMENTARY REFERENCES

1. Mothershed EA, Sacchi CT, Whitney AM, et al. Use of real-time PCR to resolve slide agglutination discrepancies in serogroup identification of *Neisseria meningitidis*. *J Clin Microbiol* 2004;42:320-8.
2. Wellcome Trust Sanger Institute. (Accessed February 6, 2009 at <ftp://ftp.sanger.ac.uk/pub/pathogens/nl/>.)
3. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 2004;5:150-63.
4. Peng J, Yang L, Yang F, et al. Characterization of ST-4821 complex, a unique *Neisseria meningitidis* clone. *Genomics* 2008;91:78-87.
5. Sultan Z, Nahar S, Wretlind B, Lindback E, Rahman M. Comparison of mismatch amplification mutation assay with DNA sequencing for characterization of fluoroquinolone resistance in *Neisseria gonorrhoeae*. *J Clin Microbiol* 2004;42:591-4.