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Multilocus Sequence Typing of *Neisseria meningitidis* Directly from Clinical Samples and Application of the Method to the Investigation of Meningococcal Disease Case Clusters

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Infections associated with *Neisseria meningitidis* are a major public health problem in England, Wales, and Northern Ireland. Currently, over 40% of cases are confirmed directly from clinical specimens using PCR-based methodologies without an organism being isolated. A nested/seminested multilocus sequence typing (MLST) system was developed at the Health Protection Agency Meningococcal Reference Unit to allow strain characterization beyond the serogroup for cases confirmed by PCR only. This system was evaluated on a panel of 20 meningococcus-positive clinical specimens (3 cerebrospinal fluid and 17 blood samples) from different patients containing various concentrations of meningococcal DNA that had corresponding *N. meningitidis* isolates. In each case, the sequence type generated from the clinical specimens matched that produced from the corresponding *N. meningitidis* isolate; the sensitivity of the MLST system was determined to be less than 12 genome copies per PCR. The MLST system was then applied to 15 PCR meningococcus-positive specimens (2 cerebrospinal fluid and 13 blood samples), each from a different patient, involved in three case clusters (two serogroup B and one serogroup W135) for which no corresponding *N. meningitidis* organisms had been isolated. In each case, an MLST sequence type was generated, allowing the accurate assignment of individual cases within each of the case clusters. In summary, the adaptation of the *N. meningitidis* MLST to a sensitive nested/seminested format for strain characterization directly from clinical specimens provides an important tool for surveillance and management of meningococcal infection.

Meningococcal infection is a major public health problem in the United Kingdom (20, 28, 30) and elsewhere (1, 2, 9, 21, 31, 44), with a wide spectrum of disease, from benign meningococcemia and meningococcal meningitis to rapidly fulminant fatal septicemia. During the 1990s, the discrepancy between the numbers of laboratory-confirmed cases of meningococcal infection and the numbers of cases reported to the Office of National Statistics for England and Wales (28) widened progressively. The impact of improved clinician awareness and the growing practice of early antibiotic treatment, before hospital admission or immediately upon arrival at the emergency department, led to a reduction in the numbers of culture- and therefore laboratory-confirmed cases (8, 32). In order to improve the ascertainment of meningococcal infection and to resolve this discrepancy, methods (including both serological and nucleic acid amplification methods) for the detection of meningococcal infection directly from clinical specimens, such as blood and cerebrospinal fluid (CSF), were developed and implemented in England, Wales, and Northern Ireland by the Meningococcal Reference Unit (MRU) of the Health Protection Agency (HPA) (5, 6, 10, 11, 16, 17). The development of nucleic acid-based detection using PCR assays was highly successful and had a major impact on the laboratory confirmation of meningococcal infection and improved case ascertainment (20).

The only effective long-term prevention strategy for the overall control of meningococcal infection is vaccination. In 1999, the enhanced surveillance of meningococcal disease (ESMD) scheme was initiated throughout England, Wales, and Northern Ireland to obtain accurate incidence data and to develop a robust surveillance system (30). The ESMD scheme was established to monitor the impact of the meningococcal serogroup C conjugate (MCC) vaccine during and following its introduction in the United Kingdom from November 1999. The ESMD scheme included improved case ascertainment data for meningococcal infection directly from clinical specimens.

Between 2000 and 2005, over 45% of all laboratory-confirmed cases of meningococcal infection in ESMD-covered areas were by PCR detection alone, with the remainder established by culture or by both culture and PCR methodologies. Enhanced surveillance requires accurate strain determination, but the conventional phenotypic characterization of meningococci by serotyping and serosubtyping can only be carried out on isolates. The large numbers of cases confirmed by PCR directly from clinical specimens in ESMD regions has meant that there are only genogroup (35) data available in many cases, limiting the effectiveness of the enhanced surveillance system. Several molecular-typing techniques have been developed to type *N. meningitidis* beyond the serogroup/genogroup

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TABLE 1. Oligonucleotide primers for *Neisseria meningitidis* MLST and *porA* nested/seminested PCR

Gene	Protocol[a]	Forward primer	Reverse primer	Amplicon size (bp)	Thermal-cycling conditions[b]
abcZ	*abcZ*-CS1st	*abcZ*-P1d 5′-GCTGGCGGCGCAGYTCTTCC-3′	*abcZ*-P2d 5′-ATGGGCGGCATCATTATTGTTTCC-3′	985	MLSTN3
	abcZ-CS2nd	*abcZ*-P1C 5′-TGTTCCGCTTCGACTGCCAAC-3′	*abcZ*-SNP1A 5′-CGGTAAAATCCAAACGGTAACTG-3′	802	MLSTN1
adk	*adk*-CS1st	*adk*-P1b 5′-CCAAGCCGTGTAGAATCGTAAACC-3′	*adk*-P2b 5′-TGCCCAATGCGCCCAATAC-3′	708	MLSTN1
	adk-CS2nd	*adk*-SNP1 5′-GCATTCCGCAAATCTCTACCG-3′	*adk*-P2b 5′-TGCCCAATGCGCCCAATAC-3′	570	MLSTN4
aroE	*aroE*-CS1st	*aroE*-P1 5′-ACGCATTTGCGCCGACATC-3′[c]	*aroE*-P2 5′-ATCAGGGCTTTTTTCAGGTT-3′[c]	911	MLSTN1
	aroE-CS2nd	*aroE*-P1b 5′-TTTGAAACAGGCGGTTGCGG-3′	*aroE*-P2b 5′-CAGCGGTAATCCAGTGCGAC-3′	835	MLSTN2
fumC	*fumC*1-CS1st	*fumC*-A1 5′-CACCGAACACGACGATGG-3′[d]	*fumC*-A2 5′-ACGACCAGTTCGTCAAACTC-3′[d]	1,350	MLSTN2
	*fumC*1-CS2nd	*fumC*-P1b 5′-TCCCCGCCGTAAAAGCCCTG-3′	*fumC*-P2b 5′-GCCCGTCAGCAAGCCCAAC-3′	820	MLSTN2
	*fumC*2-CS1st	*fumC*-P1b 5′-TCCCCGCCGTAAAAGCCCTG-3′	*fumC*-P2b 5′-GCCCGTCAGCAAGCCCAAC-3′	820	MLSTN2
	*fumC*2-CS2nd	*fumC*-SNP1 5′-GTCAAAATCGGCCGCACCCAC-3′	*fumC*-P2b 5′-GCCCGTCAGCAAGCCCAAC-3′	798	MLSTN6
gdh	*gdh*-CS1st	*gdh*-P2 5′-GGTTTTTCATCTGCGTATAGAG-3′[c]	*gdh*-P1 5′-ATCAATACCGATGTGGCGCGT-3′[c]	678	MLSTN1
	gdh-CS2nd	*gdh*-P2 5′-GGTTTTTCATCTGCGTATAGAG-3′[c]	*gdh*-P2b 5′-TGTTGCGCGTTATTTCAAAGAAGG-3′	726	MLSTN1
pdhC	*pdhC*-CS1st	*pdhC*-P1b 5′-CCGGCCGTACGACGCTGAAC-3′	*pdhC*-P2b 5′-GATGTCGGAATGGGGCAAACAG-3′	818	MLSTN2
	pdhC-CS2nd	*pdhC*-NP1A 5′-TGCGCCGTATGTATGCCAATAATG-3′	*pdhC*-NP2A 5′-ACAGGCCGTCTGAAACATCAATCA-3′	662	MLSTN1
pgm	*pgm*-CS1st	*pgm*-P2 5′-CGGATTGCTTTCGATGACGGC-3′[c]	*pgm*-P1 5′-CTTCAAAGCCTACGACATCCG-3′[c]	963	MLSTN2
	pgm-CS2nd	*pgm*-NP1A 5′-GGCTTTGAATTGGTTTTGAATCC-3′	*pgm*-NP2A 5′-AATCGGCYTGGCGTTTGAC-3′	796	MLSTN5
porA	*porA*-CS1st	210 5′-ATGCGAAAAAAACTTACCGCCCTC-3′[e]	211 5′-AATGAAGGCAAGCCGTCAAAAACA-3′[e]	1,148	*porA*N1
	porA-VR1	VR1A 5′-CTTACCGCCCTCGTATTG-3′	VR1B 5′-GGCAACGGATACGTCTTG-3′	300	*porA*N2
	porA-VR2	VR2C 5′-TGGCTTCGCAATTGGGTA-3′	VR2E 5′-ACCGGCATAATACACATC-3′	250	*porA*N2

[a] CS1st, first-round PCR; CS2nd, second-round PCR; VR1, second-round PCR for *porA* variable region 1; VR2, second-round PCR for *porA* variable region 2.
[b] Thermal cycling conditions and listed in Table 2.
[c] Primer described previously (23).
[d] Primer described previously (14).
[e] Primer described previously (38).

level directly from clinical samples, including *porA* subtyping (9, 29), *porB* typing (39), 16S rRNA sequencing (43), and multilocus sequence typing (MLST) (13, 22).

MLST is PCR based (23) and has been shown to be useful in discriminating *Neisseria meningitidis* for both long-term epidemiology studies (43) and outbreak/case cluster analysis (14). Genotypic characterization by MLST reveals the clonal relationships of organisms, which can be masked by the serogroup. The important hypervirulent lineage sequence type 11/electrophoretic type 37 (ST-11/ET-37) complex was identified as the major cause of serogroup C meningococcal (MenC) disease in the United Kingdom during the 1990s (28), making the monitoring of such organisms critical during the vaccination program using the MCC vaccine. MLST can definitively identify ST-11/ET-37 complex meningococci, which cannot be done by serogrouping/genogrouping, as the organisms are known to undergo capsular switching from serogroup C to serogroup B (2, 21). Furthermore, due to the large numbers of cases confirmed solely by PCR directly from clinical specimens in the ESMD regions, outbreaks or case clusters frequently involve one or more cases for which no isolate is available. Under such circumstances, routine characterization of solely PCR-positive cases is often carried out only to the genogroup level, which does not always provide enough information to fully inform outbreak/cluster investigations and management.

This paper describes a sensitive nested-MLST protocol evaluated on a sample set of meningococcus-positive clinical specimens for which matched *N. meningitidis* isolates were also available. MLST profiles were generated from both clinical specimens and their corresponding cultures in order to investigate the reproducibility of the scheme. In addition, three case clusters of meningococcal disease that were wholly or partly based on PCR confirmation were investigated using the nested-MLST system and *porA* sequence typing.

(Part of this work was previously presented at the 13th International Pathogenic Neisseria Conference, 1 to 6 September 2002, Oslo, Norway.)

MATERIALS AND METHODS

Meningococcal isolates and meningococcus-positive clinical specimens. All *N. meningitidis* isolates (21 from blood and 12 from throat swabs) and meningococcal PCR-positive clinical specimens (31 whole-blood–EDTA and 5 CSF) used in this study were obtained from the HPA MRU between September 1999 and March 2001. Specimens were collected from 48 patients, 21 of who had provided both meningococcal PCR-positive clinical specimens and *N. meningitidis* isolates. Of the remaining 27 patients, 15 provided meningococcal PCR-positive clinical specimens that were negative on culture, and 12 provided meningococci isolated from throat swabs but did not provide clinical specimens for PCR testing.

DNA extraction from isolates. Total-DNA extraction from *N. meningitidis* isolates was performed as previously described (10). Extraction of RNA-free meningococcal DNA for use in determining the sensitivity of the nested-MLST protocol was obtained using the Nucleon BACC genomic DNA extraction kit (Amersham Biosciences, Little Chalfont, United Kingdom) and RNase A (Sigma-Aldrich Company Ltd., Gillingham, United Kingdom) in accordance with the manufacturers' instructions. The DNA was then quantified using a GeneQuant II RNA/DNA calculator (Pharmacia Biotech, Cambridge, United Kingdom).

DNA extraction from clinical specimens. Whole-blood–EDTA samples (200 μl) were extracted using Generation capture columns (Gentra Systems, MN) in accordance with the manufacturer's instructions. DNA was extracted from CSF samples (100 μl) using DNAzol (Life Technologies, Paisley, Scotland) as previously described (10).

Primer design. Additional/alternative oligonucleotide primers for the nested/seminested PCR at the seven MLST-determining loci (Table 1) were designed

from the genome sequences of *N. meningitidis* (26, 36) using the Lasergene software package Primer Select (DNASTAR Inc., WI).

Detection and grouping of meningococcal DNAs from clinical specimens. Detection and genogrouping of *N. meningitidis* from DNAs extracted from whole-blood–EDTA and CSF samples had previously been carried out on an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA), as previously described (10).

DNA amplification of MLST and *porA* targets. The MLST PCRs carried out on *N. meningitidis* isolates were performed using the primers, reaction mixture, and thermal-cycling conditions previously described (14, 23). For the first round of the nested/seminested touchdown MLST PCR for clinical samples, each reaction mixture was 50 μl in volume and contained 200 μM (each) dATP, dGTP, dCTP, and dTTP (Amersham Biosciences, Little Chalfont, United Kingdom); 1 mM of each primer; 5 μl of 10× buffer (containing 15 mM of $MgCl_2$); 10 μl of Q-Solution (QIAGEN, Crawley, United Kingdom); 5 μl of specimen DNA extract; 9.5 μl of sterile water; and 0.5 μl (5 U/μl) of HotStar*Taq* DNA polymerase (QIAGEN, Crawley, United Kingdom). The second-round MLST nested PCR used the same reaction mixture concentrations and final volume as the first, apart from the fact that 1 μl of PCR product from the first-round reaction was added, along with 13.5 μl of sterile water.

For the *porA* PCRs carried out on bacterial isolates, the total volume of the PCR mixture was 50 μl, and it contained 1.5 mM $MgCl_2$; 200 μM (each) dATP, dGTP, dCTP, and dTTP (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom); 0.5 mM of primers 210 and 211 (Table 2); 5 μl of 10× buffer; 1 μl of 1% W1 (Life Technologies, Paisley, Scotland); 1 μl of extracted sample; 21 μl of sterile water; and 0.15 μl (5 U/μl) of *Taq* DNA polymerase (Life Technologies, Paisley, Scotland). The first round of the nested *porA* PCR carried out on clinical samples used the same reaction mixture as for the isolate *porA* PCR listed above, except that the amount of DNA extract added was increased from 1 μl to 5 μl and the volume of sterile water was decreased to 24.85 μl. For the second round of the nested *porA* PCR, each PCR mixture was 50 μl in volume and contained 1.5 mM $MgCl_2$; 200 μM (each) dATP, dGTP, dCTP, dTTP (Amersham Pharmacia Biotech); 0.1 mM of primers VR1A and VR2B for amplification of variable region 1 and primers VR2C and VR2E for amplification of variable region 2 (personal communication from A. van der Ende, University of Amsterdam, Amsterdam, The Netherlands) (Table 1); 5 μl of 10× buffer; 1 μl of 1% W1; 1 μl of extracted sample; 28.35 μl of sterile water; and 0.15 μl (5 U/μl) of *Taq* DNA polymerase (Life Technologies, Paisley, Scotland).

Thermal cycling was carried out using an MJ PTC 200 thermal cycler (GRI, Braintree, United Kingdom). The multiple PCR cycling conditions used to amplify the MLST and *porA* targets in both nested and seminested formats are listed in Tables 1 and 2. Amplification of the *porA* gene from culture isolates was accomplished using the thermal-cycling conditions listed as *porA*N1 (Table 2).

Sequencing analysis. All MLST PCR products and *porA* PCR products from isolates were purified for sequencing using the MultiScreen PCR cleanup plate (Millipore, Bedford, MA) in accordance with the manufacturer's instructions. Sequencing of the MLST targets was carried out using previously published primers (14, 23) and, for the p*orA* variable regions, primers VR1A, VR1B, VR2C, and VR2E. Identification of the ET-15 clone was carried out as previously described (41). For the *porA* PCR from clinical specimens, the bands were cut directly out of the 2% agarose gels and purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences, Little Chalfont, United Kingdom). Sequencing reactions were performed in 10-μl volumes with a CEQ Dye Terminator cycle-sequencing kit (Beckman Coulter, Fullerton, CA) utilizing the reduced-volume protocol as previously described (3). Thermal-cycling conditions for the sequencing reaction and ethanol cleanup of sequenced products was carried out according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA); the products were then analyzed on a Beckman Coulter CEQ 8000 automated DNA sequencer. The sequences for the seven gene fragments of each sample were assembled from the resultant chromatograms using Sequencher sequence analysis software (Gene Codes Corporation, MI); MLST sequences were assigned allele numbers and STs using the MLST website (http://pubmlst.org/neisseria/), and *porA* variable regions were assigned using the *porA* variable-region database (http://neisseria.org/nm/).

RESULTS

Nested-MLST sensitivity and reproducibility. Using the nested-MLST protocol, all seven MLST targets were successfully amplified and sequenced from 1 ng, 1 pg, 500 fg, 250 fg, 100 fg, 50 fg, and 25 fg of purified *N. meningitidis* DNA per

TABLE 2. Thermal-cycling conditions for *Neisseria meningitidis* MLST and *porA* nested/seminested PCR

*porA*N1	No. of cycles	*porA*N2	No. of cycles	MLSTN1	No. of cycles	MLSTN2	No. of cycles	MLSTN3	No. of cycles	MLSTN4	No. of cycles	MLSTN5	No. of cycles	MLSTN6	No. of cycles
94°C for 2 min	1	94°C for 5 min	1	95°C for15min	1	95°C for15min	1	95°C for15min	1	95°C for15min	1	95°C for15min	1	95°C for15min	1
94°C for 1 min	40	94°C for 1 min	40	94°C for 1min	30	94°C for 1min	30	94°C for 1min	30	94°C for 1min	30	94°C for 1min	30	94°C for 1min	30
68°C for 1 min		46°C for 1 min		63°C for 1min		65°C for 1min		67°C for 1min		63°C for 1min		60°C for 1min		66°C for 1min	
72°C for 2 min		72°C for 1.5 min		−0.5°C per cycle		−0.5°C per cycle		−0.5°C per cycle		− 0.3°C per cycle		−0.5°C per cycle		−0.3°C per cycle	
				72°C for 2min		72°C for 2min		72°C for 2min		72°C for 2min		72°C for 2min		72°C for 2min	
72°C for 2 min	1	72°C for 10 min	1	94°C for 1min	10	94°C for 1min	10	94°C for 1min	10	94°C for 1min	10	94°C for 1min	10	94°C for 1min	10
4°C hold		4°C hold		48°C for 1min		50°C for 1min		52°C for 1min		53°C for 1min		45°C for 1min		56°C for 1min	
				72°C for 2min		72°C for 2min		72°C for 2min		72°C for 2min		72°C for 2min		72°C for 2min	
				72°C for 2min	1	72°C for 2min	1	72°C for 2min	1	72°C for 2min	1	72°C for 2min	1	72°C for 2min	1
				4°C hold		4°C hold		4°C hold		4°C hold		4°C hold		4°C hold	

TABLE 3. Correlation of *N. meningitidis* MLST sequence types directly from clinical specimens and isolates from the same patients

Clinical-sample no.	C_T value[a]	Sample type[b]	Phenotype[c]	Allele nucleotide sequence no.[d]							Sequence type from clinical sample	Sequence type from cultured isolate	ET-37 complex/ET15 clone
				abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*			
1	18.37	CSF	B:4:P1.4	3	6	9	5	9	6	9	41	41	
2	20.05	EDTA	B:NT:P1.4	3	6	9	5	9	6	9	41	41	
3	20.64	CSF	B:15:P1.7,16	4	10	12	4	6	3	8	1100	1100	
4	22.11	EDTA	C:2a:NT	2	3	4	3	8	4	6	11	11	ET-15
5	22.75	EDTA	B:1:P1.4	3	6	9	5	9	6	9	41	41	
6	24.87	EDTA	C:2b:P1.5,2	2	3	7	2	8	5	2	8	8	
7	24.99	EDTA	B:1:P1.4	3	6	9	5	9	6	9	41	41	
8	26.17	EDTA	B:15:P1.7,2	4	10	5	40	6	3	8	259	259	
9	26.53	EDTA	B:4:P1.4	3	6	9	5	11	6	9	154	154	
10	28.04	EDTA	W135:2a:P1.5,2	2	3	4	9	8	4	6	11	11	ET-37
11	28.13	EDTA	B:22:P1.14	7	8	10	19	10	143	2	1102	1102	
12	30.19	EDTA	B:1:NT	3	6	9	5	9	6	9	41	41	
13	30.39	EDTA	B:2a:P1.5	2	3	4	3	8	4	6	11	11	ET-15
14	32.02	EDTA	C:2a:P1.5,2	2	3	4	3	8	4	6	11	11	ET-15
15	32.30	EDTA	C:2a:P1.5	2	3	4	3	8	4	6	11	11	ET-15
16	32.36	CSF	C:2a:NT	2	3	4	3	8	4	6	11	11	ET-15
17	32.42	EDTA	B:NT:P1.7	4	10	15	9	8	11	9	269	269	
18	32.72	EDTA	C:2a:P1.5,2	2	3	4	3	8	4	6	11	11	ET-15
19	34.00	EDTA	C:2a:P1.5	2	3	4	3	8	4	6	11	11	ET-15
20	34.19	EDTA	C:2a:P1.5	7	5	1	13	36	53	15	213	213	

[a] The C_T value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target sequence; a value of >45.0 is deemed to be negative.
[b] CSF, cerebrospinal fluid; EDTA, blood sample collected in a BD Vacutainer (Becton Dickinson, Plymouth, United Kingdom) containing ethylenediaminetetraacetic acid.
[c] Serogroup:serotype:serosubtype.
[d] Allele nucleotide sequence number in the MLST database (http://pubmlst.org/neisseria).

first-round PCR. These DNA samples were also tested in the *ctrA* PCR assay with cycle threshold (C_T) values ranging from 18.55 for 1 ng to 35.09 for 25 fg per PCR. The C_T value is the PCR cycle number at which amplification of the target sequence is identified and is directly proportional to the number of target copies per PCR. Direct quantification of DNA for the 20 clinical specimens was not possible because RNase is not used in routine total nucleic acid extraction from clinical samples submitted to the MRU. The nested/seminested-MLST protocol was used to successfully generate STs from 20 clinical specimens that had been found positive for meningococcal DNA using the *ctrA* real-time PCR with C_T values ranging from 18.37 to 34.19 and for which a corresponding isolate was available (Table 3). Using the original MLST protocol (14, 23), the ST generated from the corresponding isolate was found to match the ST from the clinical specimen.

The *fumC*1 primer set allowed the amplification of the *fumC* gene for all 20 clinical specimens, making it possible to identify the ET-15 clone within the ST-11/ET-37 clonal complex (41). Seven out of the eight clinical specimens that had been identified as containing meningococcal DNA from an ST-11 organism were ET-15 clones. Six of the isolates corresponding to the clinical specimens found to contain ET-15 clone DNA were phenotype C:2a, while the seventh was phenotype B:2a. The isolate corresponding to the single clinical specimen that contained DNA from an ST-11 organism which was not an ET-15 clone was found to have a W135:2a phenotype.

Case cluster A. The first case cluster consisted of four cases of meningococcal disease caused by serogroup B organisms reported over a 2-month period in a town located in northwest England (Table 4); three of the four cases were confirmed by *ctrA* PCR only (A1, A3, and A4). The fourth case was con-

TABLE 4. Case cluster A: MLST results for a cluster of MenB disease in a northwest England town during winter 2000/1

Sample no.	C_T value[a] from *ctrA* screening test	Sample type[b]	*siaD* group[c]	Phenotype[d]	Seqsubtype[e]	Allele nucleotide sequence no.[f]							MLST classification
						abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	
A1	35.39	EDTA	B		P1.7-2,4	3	6	19	5	3	6	9	ST-340 (ST-41/44/lineage 3 complex)
A2i	29.09	EDTA	B		P1.7-2,4	3	6	19	5	3	6	9	ST-340 (ST-41/44/lineage 3 complex)
A2ii		Culture		B:4:P1.4	P1.7-2,4	3	6	19	5	3	6	9	ST-340 (ST-41/44/lineage 3 complex)
A3	35.23	EDTA	B		NT	3	6	9	5	9	6	9	ST-41 (ST-41/44/lineage 3 complex)
A4	20.23	EDTA	B		P1.7-2,4	3	6	19	5	3	6	9	ST-340 (ST-41/44/lineage 3 complex)

[a] The C_T value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target seqence.
[b] EDTA, blood sample collected in a BD Vacutainer (Becton Dickinson, Plymouth, United Kingdom) containing ethylenediaminetetraacetic acid; Culture, organism isolated from blood culture.
[c] Group determined using the *siaD* TaqMan assay.
[d] Serogroup:serotype:serosubtype (determined by monoclonal antibodies).
[e] Subtype determined by the sequencing of *porA* variable regions 1 and 2. NT, not tested.
[f] Allele nucleotide sequence number in the MLST database (http://pubmlst.org/neisseria).

TABLE 5. Case cluster B: MLST results for a cluster of MenB disease in a school during spring 2001

Sample no.	C_T value[a] from *ctrA* screening test	Sample type[b]	Phenotype[c]	*siaD* group[d]	Seqsubtype[e]	Allele nucleotide sequence no.[g]							MLST classification	School yr[f]
						abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*		
B1	32.69	EDTA		B	P1.19-1,15-11	4	10	15	9	8	11	9	ST-269 (ST-269 complex)	10
B2	35.14	EDTA		B	P1.19-1,15-11	4	10	15	9	8	11	9	ST-269 (ST-269 complex)	10
B3		Culture	B:NT:P1.15		P1.19-1,15-11	4	10	15	9	8	11	9	ST-269 (ST-269 complex)	11
B4		Culture	B:NT:P1.15		P1.17-1,23	9	20	9	9	9	6	2	ST-1097 (ST-44 complex)	11
B5		Culture	B:NT:P1.15		P1.19,15-1	12	6	9	9	9	9	9	ST-1169 (ST-44 complex)	7
B6		Culture	B:NT:P1.15		P1.19,15-1	12	6	9	9	9	6	9	ST-43 (ST-44 complex)	7
B7		Culture	NG:NT:P1.15		P1.19-1,15-11	4	10	15	9	8	11	9	ST-269 (ST-269 complex)	9
B8		Culture	NG:NT:P1.15		P1.19-3,15	8	26	9	24	26	20	18	ST-212	8
B9		Culture	NG:NT:P1.15		P1.19,15	7	16	55	10	26	56	142	ST-1186 (ST-962 complex)	Staff
B10		Culture	B:4Z:P1.15		P1.17,16-3	122	6	9	17	9	6	16	ST-1166 (ST-44 complex)	10
B11		Culture	B:4Z:P1.15		P1.22-1,14	4	10	16	9	6	21	3	ST-1167	8
B12		Culture	B:4Z:P1.15		P1.19,15	10	10	9	9	9	6	2	ST-1168 (ST-44 complex)	11
B13		Culture	NG:21:P1.15		P1.18-1,30	16	2	159	92	77	25	112	ST-1117	7
B14		Culture	B:1:P1.15		P1.22,14	7	5	1	13	36	53	15	ST-213	11

[a] The C_T value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target sequence.
[b] EDTA, blood sample collected in a BD Vacutainer (Becton Dickinson, Plymouth, United Kingdom) containing ethylenediaminetetraacetic acid; Culture, organism isolated from throat swab.
[c] Serogroup:serotype:serosubtype (determined by monoclonal antibodies).
[d] Group determined using the *siaD* TaqMan assay.
[e] Subtype determined by the sequencing of *porA* variable regions 1 and 2.
[f] Year 7, age 11 to 12; year 8, age 12 to 13; year 9, age 13 to 14; year 10, age 14 to 15; year 11, age 15 to 16.
[g] Allele nucleotide sequence number in MLST database (http://pubmlst.org/neisseria).

firmed by both *ctrA* PCR (A2i) and culture (A2ii). All four patients were adolescents who attended the same school and were known to socialize together. Two of them were cousins (A3 and A4). The nested-MLST protocol was able to assign STs directly from the clinical specimens, with samples A1, A2i, and A4 found to be ST-340 and sample A3 being ST-41. Both these sequence types belong to the ST-41/44/lineage 3 complex. Subtyping via *porA* sequencing directly from the clinical specimens was successful for samples A1, A2i, and A4, all of which were found to be subtype P1.7-2,4. The clinical specimen from which no subtype could be obtained, A3, contained an ST-41 meningococcus, a different ST from the other three cases. The isolate from patient A2ii was found to be ST-340, subtype P1.7-2,4; this matched the ST and subtype found by sequencing directly from the corresponding clinical specimen (A2i). It is interesting that the two cousins were infected by different strains of *N. meningitidis*, one by an ST-41 organism (A3) and the other by an ST-340 organism (A4).

Case cluster B. The second cluster comprised two cases of meningococcal meningitis diagnosed within 24 h of each other from clinical specimens by *ctrA* PCR and identified as genogroup B organisms using *siaD* PCR (Table 5). Both patients were adolescents who attended the same school. Although both pupils were in the same school year, no close social or family ties were identified. In this instance, the decision was taken to swab the whole school to ascertain the carriage of the putative outbreak strain. Out of a total of 1,596 individuals swabbed, 176 meningococci were isolated.

TABLE 6. Case cluster C: MLST results from W135-positive clinical specimens collected in ESMD regions from April 2000 to March 2001

Sample no.	C_T value[a] from *ctrA* screening test	Sample type[b]	*siaD* group[c]	Seqsubtype[d]	Allele nucleotide sequence no.[e]							MLST classification	ET-37 complex/ ET-15 clone
					abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*		
C1	34.55	EDTA	W135	NT	2	3	4	3	8	4	6	ST-11 (ST-11 complex)	
C2	31.26	EDTA	W135	P1.5,2	2	3	4	3	8	4	6	ST-11 (ST-11 complex)	ET-37
C3	35.74	EDTA	W135	NT	2	3	4	3	8	4	6	ST-11 (ST-11 complex)	ET-37
C4	23.15	EDTA	W135	P1.18-1,3	11	5	18	8	11	24	21	ST-22 (ST-22 complex)	
C5	22.31	CSF	W135	P1.18-1,3	11	5	18	8	11	4	21	ST-184 (ST-22 complex)	
C6	25.08	CSF	W135	P1.18-1,3	11	5	18	8	129	24	21	ST-1101 (ST-22 complex)	
C7	34.68	EDTA	W135	NT	2	3	4	3	8	4	6	ST-11 (ST-11 complex)	
C8	31.1	EDTA	W135	P1.5	2	3	4	3	8	4	6	ST-11 (ST-11 complex)	ET-37
C9	34.54	EDTA	W135	P1.5,2	2	3	4	3	8	4	6	ST-11 (ST-11 complex)	ET-37
C10	30.19	EDTA	W135	P1.5,2	2	3	4	3	8	4	6	ST-11 (ST-11 complex)	ET-37

[a] The C_T value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target seqence.
[b] EDTA, blood sample collected in a BD Vacutainer (Becton Dickinson, Plymouth, United Kingdom) containing ethylenediaminetetraacetic acid; CSF, cerebrospinal fluid.
[c] Group determined using the *siaD* TaqMan assay.
[d] Subtype determined by the sequencing of *porA* variable regions 1 and 2. NT, not tested.
[e] Allele nucleotide sequence number in MLST database (http://pubmlst.org/neisseria).

The nested/seminested-MLST protocol was performed on both clinical specimens, and the meningococci in samples B1 and B2 were identified as belonging to ST-269 (Table 5). In addition to MLST, *porA* subtyping was also carried out directly on the clinical specimens, showing the subtypes to be P1.19-1, 15-11.

Of the 176 meningococcal isolates obtained from the nasopharyngeal swabs taken at the school, only 12 (B3 to B14) were found to be serogroup B or nongroupable and subtype P1.15 on initial testing. Two pupils carried isolates of *N. meningitidis*, which were ST-269, subtype P.1.9-1,15-11 (B3 and B4). However, neither of these pupils was related to or in the same school year as the two patients. The remaining 10 serogroup B and/or serosubtype P1.15 meningococci isolated at the school (B5 to B14) were not identified as ST-269 by MLST or as subtype P.19-1,15-11 by *porA* sequencing.

Case cluster C. The third case cluster consisted of 10 *ctrA* PCR-confirmed *siaD* PCR genogroup W135 infections reported over the period from April 2000 to March 2001 (Table 6). This period covered the two Hajj-related outbreaks of W135 disease seen in pilgrims and their contacts (19). An MLST profile was generated from all 10 of the clinical specimens, 7 of which were ST-11/ET-37 complex, the same ST as that associated with the Hajj outbreak (C1 to C3 and C7 to C10). The remaining three cases were identified as ST-22 clonal complex (C4 to C6). Three of the patients infected with ST-11/ET-37 complex organisms were found to have visited Mecca during the Hajj pilgrimage (C2, C9, and C10); another had been in casual contact with a returned Hajj pilgrim (C3). There was no epidemiological link between the remaining three ST-11/ET-37 complex cases and the three ST-22 clonal complex cases and the Hajj pilgrimage.

Using *porA* sequencing directly from the clinical specimens, six full subtypes were produced from the 10 cases (C2, C4 to C6, and C9 and C10). Only the VR1 region of the *porA* gene was successfully sequenced from sample C8. No subtype data could be generated at all from the remaining three clinical specimens (C1, C3, and C7). Five of the seven clinical specimens containing ST-11/ET-37 complex meningococcal DNA did not belong to the ET-15 clone (C2, C3, and C8 to C10); this could be determined, since their *fumC* alleles had been amplified with the *fumC*1 nested primer set, while the remaining two clinical samples used the *fumC*2 nested primer set, which does not amplify the area of sequence required to enable the identification of ET-15 clones.

DISCUSSION

Currently in England, Wales, and Northern Ireland, the fact that 45% of meningococcal infections have only limited strain characterization due to confirmation directly from clinical specimens by PCR diminishes the effectiveness of the enhanced surveillance program used to monitor major intervention strategies for meningococcal infection, such as the introduction of the MCC vaccine. Although genogroup identification of meningococci from clinical specimens by nucleic acid amplification (35) partially rectifies this problem, full strain characterization for meningococci remains important due to the phenomenon of capsular switching, whereby meningococci can express alternative capsular serogroups due to recombination in the *cps* operon (2, 21, 31, 33, 37, 40). It is important that any method applied to the strain characterization of meningococci directly from clinical specimens be able to provide data that can identify organisms belonging to hypervirulent lineages, such as the ST-11/ET-37 complex, by adapting it for use directly on clinical specimens. MLST fulfils this objective.

In this study, the allelic profiles and STs for 20 *N. meningitidis* organisms were identified from a panel of clinical specimens, with a range of meningococcal DNA concentrations. The STs were confirmed by the concurrent MLST profile obtained for each of the corresponding meningococcal isolates. Previous papers describing direct MLST from clinical specimens have been based on small numbers, usually fewer than 10 samples, for which the epidemiological associations have been unclear (13, 22). Several of the PCR primer sets developed for this nested-MLST protocol have now been adopted as the standard PCR primers for use on *N. meningitidis* isolates (http://pubmlst.org/neisseria/). The sensitivity of the protocol has been demonstrated to be 25 fg, which corresponds to less than 12 genome copies of *N. meningitidis* per PCR and a *ctrA* C_T value of 35.09. This level of sensitivity is similar to that quoted for the *ctrA* PCR (10), where it is stated that a C_T value of 35 correlates to less than 10 genome copies per PCR. The 20 meningococus-positive clinical specimens used for sensitivity/reproducibility (Table 3) had C_T values ranging from 18.37 to 34.19, representing a wide range of meningococcal DNA concentrations. Amplification of PCR products from clinical specimens with C_T values in the 34 range demonstrates that the clinical sensitivity of the nested protocol is comparable to 25 fg of meningococcal DNA per PCR, or approximately 10 genome copies.

In all three of the case clusters described in this article, the nested/seminested-MLST protocol allowed *N. meningitidis* to be characterized beyond the serogroup level directly from clinical samples. This was critical in case clusters B and C, where no organism was isolated from the patients. The role of *N. meningitidis* characterization by MLST directly from clinical specimens in the public health management of case clusters of meningococcal disease is demonstrated by case cluster B, where unnecessary attempts to establish linkage between cases and carriers in school year groups might have been undertaken without the more detailed information provided by molecular characterization, which identified the two index cases as being distinct from all other meningococci isolated at the school. The 2000–2001 Hajj outbreaks were due to W135 ST-11/ET-37 complex meningococci (19), and therefore, three of the patients whose infections were confirmed by PCR directly from clinical specimens might have remained linked with the Hajj outbreak had they not been identified as belonging to the ST-22 clonal complex (the endemic W135 lineage in England and Wales), again demonstrating the importance of the nested-MLST approach for accurate strain characterization for public health management of meningococcal infection. Although only four of the ST-11-positive clinical specimens (C2, C3, C9, and C10) had definitive epidemiological links with the Hajj pilgrimage, the remaining three ST-11-positive clinical specimens (C1, C4, and C8) were also classified as containing meningococci belonging to the outbreak strain (19) due to the time of onset of disease and genotypic characterization.

For all three case clusters, the *porA* subtyping data direct

from clinical samples provided additional identification, giving the same level of discrimination as the MLST data, although the nested *porA* PCR used at the time of this study was found to be less sensitive than the nested/seminested-MLST protocol. In case cluster A, the nested/seminested-MLST result for specimen A3 identified an ST-41 organism, different at two housekeeping gene loci from the ST-340 meningococci identified from the other three cases characterized in the cluster. The different organism identified in patient A3 probably occurred as a contemporaneous sporadic infection.

Several investigations have shown that MLST and *porA* subtyping are not always able to differentiate organisms fully in outbreak situations and have concluded that other molecular-typing approaches have to be combined with MLST and *porA* sequencing to fully characterize *N. meningitidis* (1, 24, 34). However, it is important to remember that some of the alternative characterization techniques, such as pulsed-field gel electrophoresis and multilocus enzyme electrophoresis, cannot be used directly on clinical specimens or in culture-negative cases. In the ESMD regions, it is possible for case clusters to be wholly or partly comprised of infections confirmed by PCR only, as illustrated by case clusters A and B in this study. Since MLST was developed to monitor the long-term epidemiology of organisms, the nested-MLST protocol could be used to examine the approximately 45% of cases of meningococcal infection in the ESMD areas confirmed by PCR only, or a representative portion of them, in order to determine how these organisms are related to the other 55% for which detailed phenotypic strain characterization is available.

Vaccination has been shown to be the most successful prevention strategy for the control of meningococcal infection, as demonstrated by the success of MCC vaccination programs in the United Kingdom (4) and other countries (7, 12), along with the mandatory vaccination since 2002 of all pilgrims traveling to Saudi Arabia for the Hajj with the A, C, Y, W135 quadrivalent vaccine (18, 42). Work is proceeding to develop possible vaccine candidates for serogroup B meningococcal (MenB) disease (15, 25, 27), as it accounts for over 80% of all meningococcal disease in England, Wales, and Northern Ireland. However, unlike MenC disease in these countries, which was mainly due to a single hypervirulent lineage, namely, the ST-11/ET-37 complex, the bulk of serogroup B disease can be attributed to three different hypervirulent lineages: the ST-41/44/lineage 3 complex, the ST-269 complex, and the ST-32/ET-5 complex. The spread of MenB disease involving several hypervirulent lineages highlights the importance of having comprehensive strain characterization of meningococci from both cultured and clinical specimens, as can be provided by MLST, should a MenB vaccine be introduced in the future.

In conclusion, this work demonstrates that the adaptation of MLST for strain characterization of meningococci directly from clinical specimens has an important role in the public health management of meningococcal infection, both in the enhanced surveillance for meningococcal disease following the introduction of a new serogroup-specific vaccine and in the investigation of case clusters/outbreaks. In fact, since 2002, the MRU has successfully used this nested-MLST protocol in numerous case clusters/outbreaks to characterize *N. meningitidis* culture-negative, meningococcal PCR-positive clinical specimens, allowing improved public health management.

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