Genetic analysis of capsular status of meningococcal carrier isolates

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SUMMARY

The meningococcal capsule is the primary virulence factor with systemic isolates requiring full expression of the capsule but with capability to down-regulate the capsule in order to invade. The meningococcal capsular operon is composed of a number of genes that are involved in capsular synthesis and transport. Differences in capsular synthesis genes may allow discrimination between meningococcal serogroups whereas absence of genes for either synthesis or transport imply that the meningococcus is unencapsulated. Although mechanisms such as slipped-strand mispairing and acquisition of insertion sequences have been demonstrated to be involved in regulation of capsular expression, few studies have addressed the mechanisms of capsular expression in carrier isolates. Following a community-based intervention programme for an outbreak of meningococcal disease, we collected meningococcal carrier isolates from the intervention area and control areas. We undertook genetic analysis of the capsular operon and the mechanisms of capsular regulation, together with an investigation of the potential of capsular genes to identify the genogroup of non-serogroupable isolates. Use of the *siaD* gene allowed the discrimination of 30/89 (34%) non-serogroupable isolates into B, C, W135 and Y with a *siaA* gene PCR permitting the characterization of a further 6 isolates whose capsules contained sialic acid. Slipped-strand mispairing was evident in only 4 of 13 genogroupable B isolates and the insertion sequence IS*1301* was found in 2 of 36 *siaA*-positive isolates. Of 51 non-genogroupable isolates 25 (49%) were shown to be *ctrA* negative. There was a higher percentage of *ctrA*-positive isolates ($P < 0.001$) amongst meningococcal strains obtained from those sampled in non-intervention schools than those sampled at intervention schools. The *ctrA*-negative isolates warrant further investigation of their genotypic organization since such avirulent strains may be important in conferring natural protection against invasive disease. We found that after mass antibiotic prophylaxis, recolonization occurs preferentially with non-pathogenic meningococcal strains. This has implications for assessment of the benefits of mass antibiotic and vaccination programmes for outbreak control. Previously expressed concerns of increased risk due to removal of protective flora may have been overstated.

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INTRODUCTION

Meningococcal capsules are critical in pathogenesis and are considered the primary virulence factor. This is evidenced by the exclusive recovery of capsulate organisms from the blood or cerebrospinal fluid (CSF) of patients with invasive meningococcal disease whereas those from the nasopharynx comprise a mixture of both capsulate and acapsulate bacteria [1]. Adherence to epithelial cells is enhanced in the absence of capsule suggesting that long-term carriage may be aided by loss of capsule expression [2, 3]. Meningococci may range from fully virulent encapsulated strains to those that are terminally down-regulated and avirulent. Community carriage rates of 10–20 % may increase to 80 % or more in institutional settings such as military recruit training camps, boarding schools and prisons [4]. Avirulent meningococci may represent important immunizing agents generating natural protective immunity against invasive strains. To date, no studies of the genetic basis of capsule expression of meningococcal carrier isolates have been performed.

The virulence status of the organism depends upon the possession of the full complement of the capsular operon (cps) including those involved in regulation, transport and biosynthesis [5]. Capsular expression may be regulated by loss or phase variation of one or more of these major components. Variation in capsular expression has been demonstrated as a consequence of slipped-strand mispairing within the sialyltransferase (siaD) gene of serogroup B meningococci [6] and also by the insertion or excision of an IS element (IS1301) in the siaA gene, essential for the synthesis of sialic acid [7]. Recently, transcriptional control mechanisms have been demonstrated in meningococci for capsular expression [8, 9]. In this study, the capsular transport gene ctrA, which is also widely used as a PCR gene target for non-culture case confirmation of meningococcal disease [10, 11], was chosen as a surrogate marker of capsular status and hence virulence status of meningococcal isolates. The ctrA gene occurs exclusively in N. meningitidis and is part of the capsular polysaccharide biosynthesis locus. Although the 3′ end of the ctrA gene is highly conserved, the 5′ end varies between different serogroups [12]. It encodes a conserved meningococcal outer membrane protein (OMP) involved in the transport of the capsular polysaccharide [12].

The main group of isolates that we examined were obtained from a carriage study that took place following a community based outbreak of invasive

serogroup C disease in Rotherham, United Kingdom, over the winter of 1995/6 [13]. Eight cases of meningococcal serogroup C disease were observed within a single locality and within a 6-week period. Nasopharyngeal swabbing was performed in 4 schools, 2 following outbreak intervention and 2 'control' (i.e. non-intervention) schools. The non-intervention schools served wards (populations defined for electoral purposes) with Jarman scores (a measure of economic deprivation) closest to the intervention schools [13]. Nasopharyngeal swabs were taken from pupils 6 and 11 months following intervention. The intervention consisted of meningococcal AC polysaccharide vaccine and either rifampicin or ciprofloxacin.

Phenotypic analysis by the Public Health Laboratory Service Meningococcal Reference Unit (PHLS MRU) confirmed 80 meningococcal isolates from the 1858 swabs taken 6 months post-intervention, of which 37 were serogroupable and 43 were non-serogroupable. Of the 2457 swabs collected 11 months following intervention, N. meningitidis was cultured from 178, of which 89 strains were serogroupable and 89 non-serogroupable [13].

The isolates obtained from the outbreak provided an opportune collection within which to study the genetic basis for capsular expression in meningococcal carrier isolates, the ability of using genetic techniques to genogroup non-serogroupable meningococci and the effect of outbreak control intervention.

MATERIALS AND METHODS

Strains

The 89 non-serogroupable isolates collected 11 months following intervention from the Rotherham outbreak were investigated. The isolates were identified at the PHLS MRU as being non-serogroupable as they did not co-agglutinate using the available rabbit polyclonal serogroup-specific antisera as described previously [14]. The control strains used in the investigations were: serogroup B, B:15:P1.7,16 (H44/76); serogroup C, C:16:P1.7-1,1 (MENC11); serogroup W135, W135:NT:P1.3,6 (M96.256130); serogroup Y, Y:NT:P1.2,5 (M96.256142). All isolates were stored on PROTECT beads (ProLab Diagnostics, Ontario, Canada) and grown on Columbian blood agar at 37 °C in an atmosphere of 5% CO_2. The isolates were extracted using Isoquick kits (Orca Research Inc., Bothell, WA, USA).

For the investigation into presence/absence of the ctrA gene the 43 non-serogroupable isolates obtained

6 months following intervention were analysed in addition to the 89 isolates obtained 11 months after intervention.

The *ctrA* genes of eight different serogroups of meningococci were sequenced. The serogroups and serotypes of the isolates sequenced were: A:21:P1.9 (M97.250767); B:4:P1.4 (M97.252573); C:2a:P1.7 (M97.252560); W135 NT:P1.3,6 (M96.256130); Y: NT:P1.5 (M97.252568); 29E:4z:P1.5 (M97.252331); H:1:P1.6 (M97.252572) and Z:NT:P.1.5 (M97. 250718). These isolates were obtained from the collection of meningococcal isolates held at the PHLS MRU.

DNA extraction method

DNA was extracted from isolates using Isoquick kits (Orca Research Inc.).

Genogrouping of *N. meningitidis* isolates using the *siaD* PCR ELISA

The *siaD* PCR amplifications and ELISA were performed as described previously [15, 16].

SiaA gene PCR for investigation of presence of IS*1301*

The *siaA* PCR amplification was performed in a final volume of 50 μl containing 1·5 units of *Taq* DNA polymerase, 2 mM MgCl$_2$, 5 μl of 10 × PCR buffer, 200 μM of each dNTP, 1 μM of each primer, 0·02 % W1 detergent and 5 μl of Isoquick extract. *SiaA* amplifications were performed using the following cycling parameters: denaturation of 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. A 4 min extension at 72 °C was included at the end of the last cycle. Primers were designed using nucleotide sequence data deposited in GenBank, accession no M95053.1, and from Hammerschmidt et al. [7] and were synthesized by Oswel DNA Services, Southampton, UK.

siaA forward primer:
5′-[174]ATGAAAAGAATTCTTTGCATTAC[196]-3′,
siaA reverse primer:
5′-[1305]TTAAAGATTCAAATCGATAAAAC[1285]-3′.

Presence of IS*1301* was demonstrated by an increase in the size of the *siaA* PCR product by 844 bp [7].

Determination of the presence/absence of the *ctrA* gene

The samples investigated for the presence or absence of the *ctrA* gene included the 89 isolates from the Rotherham study that were obtained 11 months' post-intervention and 43 isolates that were obtained 6 months' post-intervention. The *ctrA* PCR amplification was performed in a final volume of 50 μl containing 2 units of *Taq* DNA polymerase, 1 mM MgCl$_2$, 5 μl of 10 × PCR buffer, 200 μM of each dNTP, 0·5 μM of each primer, 0·02 % W1 detergent and 5 μl of Isoquick extract. *CtrA* amplifications were performed using the following cycling parameters: denaturation of 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min. A 10 min extension at 72 °C was included at the end of the last cycle. Primers were designed using nucleotide sequence data deposited in GenBank accession no. M80593 and were synthesized by Oswel DNA Services.

ctrA forward primer:
5′-[609]ATGCGGGTGGCTGCGGTAGGT[628]-3′,
ctrA reverse primer:
5′-[1142]CCGGCGAGAACACAAACGACAAG[1120]-3′.

Investigation of slipped-strand mispairing within the *siaD* gene of serogroup B meningococci

The samples investigated for slipped-strand mispairing were the 16 isolates that were non-serogroupable, yet genogroupable using the *siaD* serogroup B PCR ELISA. The *siaD* promoter region PCR amplification was performed in a final volume of 100 μl containing 4 units of *Taq* DNA polymerase, 3 mM MgCl$_2$, 10 μl of 10 × PCR buffer, 200 μM of each dNTP, 1·0 μM of each primer, 0·02 % W1 detergent and 5 μl of Isoquick extract. *SiaD* promoter region PCR amplifications were performed using the following cycling parameters, denaturation of 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. A 10 min extension at 72 °C was included at the end of the last cycle. The PCR primers were designed using nucleotide sequence data deposited in GenBank accession no. M95053.1 and were synthesized by Oswel DNA Services.

siaD PR (B) forward primer:
5′-[3121]ACATACAATAATGCTAAGAAAATA[3145]-3′,
siaD PR (B) reverse primer:
5′-[3355]CTTAGGCATTTTTAAGTTTTTAGAA[3331]-3′.

PCR products were purified using GFX PCR DNA and Gel Band Purification kits (Amersham Pharmacia Biotech, Hertfordshire, UK) as according to the manufacturers' instructions. All *siaD* PCR products were sequenced using the ABI Prism 310 Genetic Analyser (PE Applied Biosystems, Warrington, UK). The sequencing primers used were those previously used for the *siaD* serogroup B promoter region PCR. The sequencing chemistry used was the ABI PRISM BigDye Terminator. Cycle sequencing reactions were performed in a final volume of 10 μl: The purified PCR product was diluted to a concentration of approximately 10 ng/μl. For each sequencing reaction the following mix was prepared in a 0·2 ml PCR tube: 0·5 μl of primer (5 μM), 4 μl of BigDye reaction mix, 2 μl of DNA template and 3·5 μl of double-distilled (dd)H$_2$O. The reaction(s) were incubated at 95 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min for 25 cycles. The sequencing reactions were ethanol precipitated and resuspended according to the manufacturers' instructions. The sequences were analysed using Sequencher^{TM} (Gene Codes Corporation, Michigan, USA).

Determination of the nucleotide sequence of the *ctrA* gene

As the *ctrA* sequences for the majority of the isolates were not known it was not possible to sequence the gene in the conventional manner. Instead a reverse primer was designed, using known sequence (GenBank accession no. M80593), and the genomic DNA was sequenced directly. Once the sequence had been determined a new primer was designed to continue sequencing the gene, this process was continued until the complete gene had been sequenced.

ctrA reverse 01:
5′-GCTCAGACATCTTAATTACTC-3′,
ctrA reverse 02:
5′-CGTAAAGGTATAGGGATTG-3′,
ctrA reverse 03:
5′-GCATAACCATCGCCAAACT-3′.

All sequencing reactions were performed on the ABI310 Genetic Analyser, using BigDye Terminator sequencing chemistry. For each sequencing reaction the following mix was prepared in a 0·2 ml PCR tube: 16 μl of BigDye reaction mix, 4 μl of primer (5 μM), 5 μl of Isoquick extraction and 15 μl of ddH$_2$O. The reaction(s) were incubated at 95 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min for 99 cycles. The sequencing

reactions were ethanol precipitated and resuspended according to the manufacturers' instructions. Once the sequence had been determined in the reverse direction a forward primer was designed for each of the eight different serogroups. Using the sequence determined via gene-walking it was also possible to design internal sequencing primers. The *ctrA* PCR amplification was performed in a final volume of 50 μl containing 1·5 units of *Taq* DNA polymerase, 3 mM MgCl$_2$, 10 μl of 10× PCR buffer, 200 μM of each dNTP, 0·4 μM of each primer, 0·02 % W1 detergent and 2·5 μl of specimen. *CtrA* promoter region PCR amplifications were performed using the following cycling parameters: denaturation of 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. A 10 min extension at 72 °C was included at the end of the last cycle.

The reverse primer was the same for all serogroups (*ctrA* reverse 01). Forward primers were designed for the serogroups A, H, Z and 29E whilst, due to the conserved nature of the *ctrA* gene in the sialic acid containing serogroups, one forward primer was designed that amplified serogroups B, C, W135 and Y:

ctrA A forward:
5′-TTTCACGATGCTGTGCAGTA-3′,
ctrA H forward:
5′-GTTGAAATAGTCACGAAAGAA-3′,
ctrA Z forward:
5′-GGATAACATACAATTAGCTC-3′,
ctrA 29E forward:
5′-TACATAACTTCCGTCGATGA-3′,
ctrA B/C/W135/Y forward:
5′-CTATAATTAGGCCATTTATA-3′.

As well as the primers used for gene-walking and PCR (*ctrA* reverse 01, *ctrA* reverse 02 and *ctrA* reverse 03) and the appropriate forward primer, two internal sequencing primers were designed:

ctrA forward 01:
5′-ATTGGGAAGCGCCGCCAGC-3′,
ctrA forward 02:
5′-CTATGGGTGCGGTGGGGAGA-3′.

Sequencing reactions were performed as previously described for the sequencing of the *siaD* promoter region. The *ctrA* sequences were assembled using Sequencher^{TM}.

A serogroup-specific *ctrA* PCR assay was also designed for serogroup X meningococci. The isolate used for the assay was serogroup X: X:21:P1.6 (M99.243709). PCR conditions were the same as for
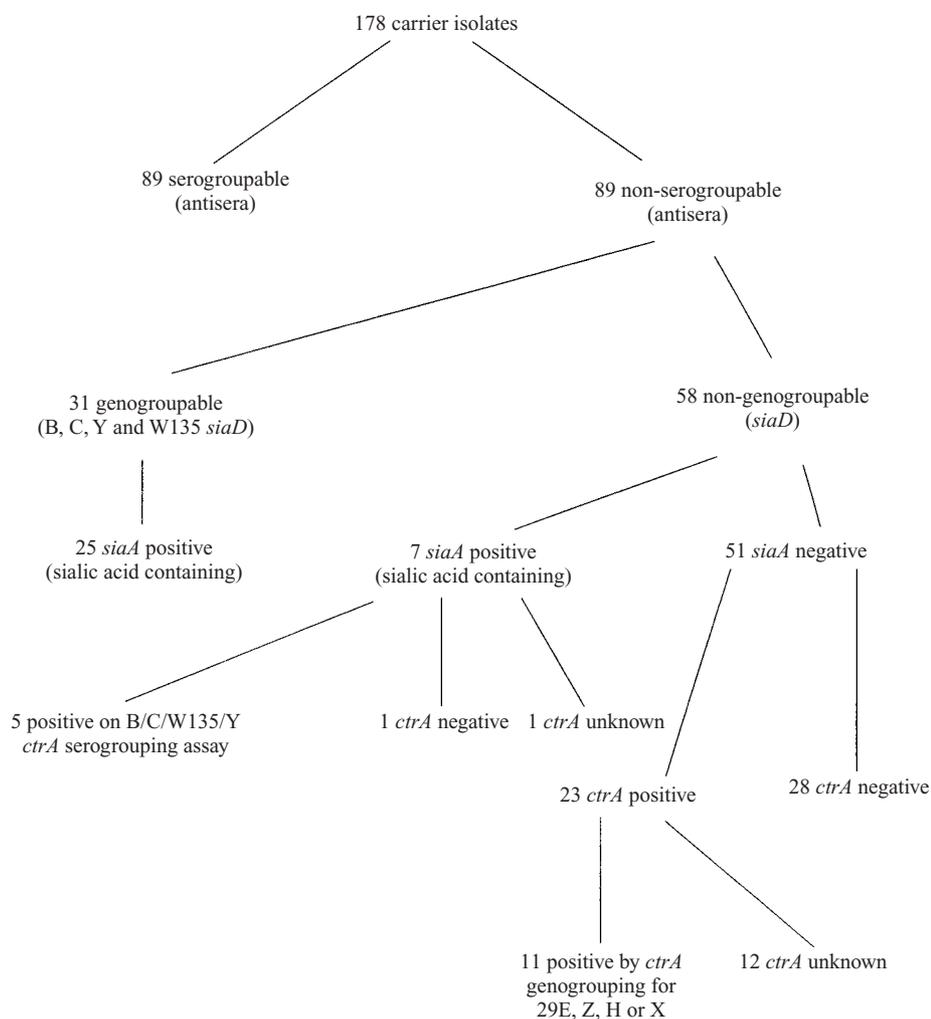
Fig. 1. Flow chart of capsular status of meningococcal carrier isolates collected 11 months following prophylaxis in the intervention and control schools.

the previously described *ctrA* PCRs and used the following primers:

ctrA X forward:
5′-ATGTCAACCATGCTGTGGCGC-3′,
ctrA X reverse:
5′-TAATTTAGTTTGCTGTGCGCGCTACCC-3′.

Serogroup determination of non-serogroupable isolates using the *ctrA* PCR

The *ctrA* PCRs (as described previously) used for the determination of the nucleotide sequence of the eight different serogroups of the gene were used to determine the serogroup of the isolates that were non-sero-groupable. The 89 non-serogroupable isolates were amplified using the six different sets of PCR primers (serogroup-specific for A, H, Z, 29E, X and one PCR to identify if a meningococcus is a B, C, W135 or Y).

Statistics

The χ^2 test (11 months' data) and Fisher's exact test (6 months' data) were used to evaluate the significance of differences between the proportions of *ctrA*-positive isolates from students in intervention and non-intervention schools.

RESULTS

The capsular status of the meningococcal carrier isolates is shown in Figure 1. Of the 178 isolates collected, 50 % (89/178) were serogroupable using conventional antisera with 89 remaining non-serogroupable. When tested using the *siaD* PCR ELISA 31 isolates were genogroupable for B ($n = 16$), C ($n = 5$), Y ($n = 4$) and W135 ($n = 6$) (Fig. 2).

On testing the 89 non-serogroupable isolates for the presence of the *siaA* gene, 25 of the 31 (81 %) *siaD*

Table 1. *Percentage of* ctrA-*positive* N. meningitidis *isolates 6 and 11 months' post-intervention*

School	6 months' post-intervention ctrA positive (%)	11 months' post-intervention ctrA positive (%)
Intervention schools	(17/22)* 77%	(71/96)† 74%
Non-intervention schools	(54/58)‡ 93%	(78/82)§ 95%
P value (intervention vs. non-intervention)	P = 0·105	P < 0·001

* 11 of the 22 isolates were serogroupable using antisera.
† 45 of the 96 isolates were serogroupable using antisera.
‡ 26 of the 58 isolates were serogroupable using antisera.
§ 44 of the 82 isolates were serogroupable using antisera.
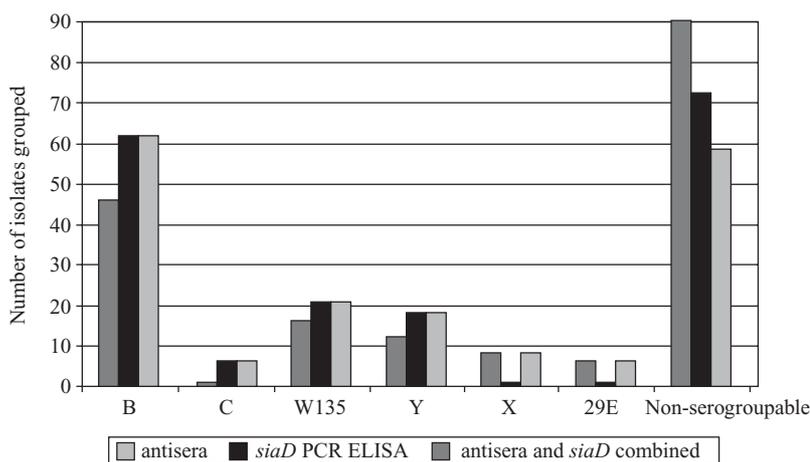
Fig. 2. Enhanced epidemiological surveillance achieved using the *siaD* PCR ELISA following the Rotherham outbreak investigation.

genogroupable isolates were positive, as were an additional 7 isolates which were negative on the *siaD* PCR ELISA. The presence of the insertion sequence IS*1301* within the *siaA* gene was found in 2 isolates of the 32 *siaA* positives.

The 31 isolates that were positive using the *siaD* PCR ELISA were all found to possess a *ctrA* gene. One isolate which was not genogroupable, yet positive using the *siaA* PCR assay did not possess a *ctrA* gene. Of the 51 isolates that were negative for the both *sia* genes, 23 were *ctrA* positive. Therefore of the total number of carrier isolates 16% (29/178) were *ctrA* negative.

Comparisons of *ctrA* status were made between isolates obtained from the intervention and non-intervention schools. Table 1 illustrates the numbers of *ctrA*-positive and -negative isolates found in both the intervention and non-intervention schools 6 and 11 months' post-intervention.

The percentages in Table 1 represent the number of *ctrA*-positive isolates divided by the total number of

isolates. At 11 months, the non-intervention schools had a significantly higher proportion of *ctrA*-positive isolates (P < 0·001) than the intervention schools. The difference was not significant at 6 months (P = 0·105).

Of the 16 genogroupable B meningococci, only 13 were amplified using the *siaD* promoter region PCR. When this promoter region was sequenced, 9 isolates were found to contain a run of 7 cytidine residues (the known number of cytidine residues required for capsule expression), 3 isolates had a run of 8 cytidine residues and 1 isolate had a run of 4 cytidine residues. One of the isolates, which displayed 7 cytidine residues, possessed the insertion sequence IS*1301* within the promoter region of the *siaD* gene. Therefore of the genogroupable B isolates, which could be amplified using the *siaD* promoter region PCR, 38% (5/13) were down-regulated.

Nucleotide sequence data obtained for the *ctrA* gene was derived from isolates representing eight different meningococcal serogroups, as detailed in Figure 3.

```
serogroup 29E      #1      -T---AG-AA AAAAAA-A-C TTC-TTT--C A::-C-G--G TTT-GT-::C :~:-AT-A:C
serogroup A        #1      TAATGAGATT T--TAT---- TGC-T--G-- AA-GT--T-- ATT-:T-::- :-:--C--:-
serogroup B        #1      -:-------- ---------- ---------- ---------- ----:----- ----------
serogroup C        #1      -:-------- ---------- ---------- ---------- ----:----- ----------
serogroup H        #1      G:-------- ---------- ---------- ---------- ----:----- ----------
serogroup W135     #1      -:-------- ---------- ---------- ---------- ----:----- ----------
serogroup Y        #1      -:-------- ---------- ---------- ---------- ----:----- ----------
serogroup Z        #1      -T---AG--A AAAAAA-A-C TTC-TTT--G A-T---G--G ::T-CTG::T :-:CGC-A:-
                           .......... .......... .......... .......... .......... ..........
consensus sequence #1      A:GGATTTGG CTTGTGTTTA AAGTGAAATT TTATATTCGT CACG:CAGTA TTATTATTGT
                           ********** ****** * * *** **** * ***** ** * *** ****** * **** ***

serogroup 29E      #61     -::::C:::- ::GACG:GC- --TGGA--C- -------C-- ---T--A-GT --T-----G-
serogroup A        #61     T-:T--T--- GGGAAGTGC- --TAG---T- ----C--C-- T-----A-GT ---------G
serogroup B        #61     ---------- ---------- ---------- ---------- --------GC ----------
serogroup C        #61     ---------- ---------- ---------- ---------- --------GC ----------
serogroup H        #61     ---------- ---------- ---------- ---------- --------GC ----------
serogroup W135     #61     ---------- ---------- ---------- ---------- --------GC ----------
serogroup Y        #61     ---------- ---------- ---------- ---------- --------GC ----------
serogroup Z        #61     -::::G:::- ::-ACG:GC- --TAG---T- ----C--C-- C-----T-GT -G--------
                           .......... .......... .......... .......... .......... ..........
consensus sequence #61     GTGGAAGTTT AATTGTAGGA TGCTCTGCGA TTCCTTCATC AGGCCCCAGC GCAAAAAAAA
                           ********** ******* *  **** *     * *   * * * * *   *     **

serogroup 29E      #121    -------C-- ---------G --C----C-- ---------- -------T-- -----C--C-
serogroup A        #121    -------A-- G--------G -------C-- ---------- ---A---T-- -----C--C-
serogroup B        #121    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #121    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #121    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #121    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #121    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #121    ---------- ---------- ---------- ---------- ---------- ----------
                           .......... .......... .......... .......... .......... ..........
consensus sequence #121    TTGTCTCTTT AGGGCAACAA TCTGAAGTTC AAATTCCTGA AGTGGAGCTG ATTGATGTGA
                                 *   *      * *   *     *                     * *       * *

serogroup 29E      #181    -----G-T-- G--G----CT --G-----A- -------G-- ------T--T --C-------
serogroup A        #181    -----G-T-- A--G----CT --G-----A- -----G-G-- ------T--T --C-------
serogroup B        #181    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #181    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #181    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #181    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #181    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #181    ---------- ---------- ---------- ---------- ---------- ----------
                           .......... .......... .......... .......... .......... ..........
consensus sequence #181    ATCATACGGT TGCTCAGTTA TTATATAAGG CTCAGATAAA TCAGTCATTC ACTCAGTTTG
                             * *    * *    *  **    *      *       * *      * *     *

serogroup 29E      #241    ---------- ------TA-C ---------- ---------- ---------- ----------
serogroup A        #241    ---------- ------TA-C ---------- ---------- ---------- ----------
serogroup B        #241    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #241    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #241    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #241    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #241    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #241    ---------- ---------- ---------- ---------- ---------- ----------
                           .......... .......... .......... .......... .......... ..........
consensus sequence #241    GCGATGGTTA TGCTTCGGCT GGTACGCTAA ATATTGGTGA TGTATTGGAT ATTATGATTT
                                        ** *

serogroup 29E      #301    ---------- ---A--G--- ---------- ---------- ---------- ----------
serogroup A        #301    ---------- ---A--G--- ---------- ---------- ---------- ----------
serogroup B        #301    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #301    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #301    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #301    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #301    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #301    ---------- ---------- ---------- ---------- ---------- ----------
                           .......... .......... .......... .......... .......... ..........
consensus sequence #301    GGGAAGCGCC GCCGGCAGTA TTGTTTGGTG GTGGCCTTTC TTCGATGGGC TCGGGTAGTG
                                        * *
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Fig. 3. For legend see page 68.

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serogroup 29E      #361    ----A----- C--------G -----AC--- -G-------- ---------- ----------
serogroup A        #361    ----A----- C--------G -----AC--- -G-------- ---------- ----------
serogroup B        #361    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #361    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #361    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #361    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #361    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #361    ---------- ---------- ---------- ---------- ---------- ----------
                           ................................................................
consensus sequence #361    CGCATCAAAC TAAGTTGCCA GAGCAGTTGG TCACGGCACG TGGTACGGTT TCTGTGCCGT
                              *          *          *         **        *


serogroup 29E      #421    ---------- ---------- --G------- ----A--G-- ---------- --C--C----
serogroup A        #421    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #421    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #421    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #421    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #421    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #421    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #421    ---------- ---------- --G------- ----A--G-- ---------- --C--C----
                           ................................................................
consensus sequence #421    TTGTTGGCGA TATTTCGGTG GTCGGTAAAA CGCCTGGTCA GGTTCAGGAA ATTATTAAAG
                                                     *              *  *              *  *


serogroup 29E      #481    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #481    ---------- ---------- --------G- ---------- ---C------ ----------
serogroup B        #481    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #481    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #481    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #481    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #481    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #481    ---------- ---------- ---------- ---------- ---------- ----------
                           ................................................................
consensus sequence #481    GCCGCCTGAA AAAAATGGCC AATCAGCCAC AAGTGATGGT GCGTTTGGTG CAGAATAATG
                                                         *                   *


serogroup 29E      #541    ---------- ---------- -----A--C- ---------- ---------- ----------
serogroup A        #541    ----A----- A--------- --C--A--C- ---------- -------T-- ----------
serogroup B        #541    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #541    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #541    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #541    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #541    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #541    ---------- ---------- -----A--C- ---------- ---------- ----------
                           ................................................................
consensus sequence #541    CGGCGAATGT GTCGGTGATT CGTGCTGGGA ATAGTGTGCG TATGCCGCTG ACGGCAGCCG
                              *          *          *  *  *                   *


serogroup 29E      #601    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #601    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #601    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #601    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #601    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #601    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #601    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #601    ---------- ---------- ---------- ---------- ---------- ----------
                           ................................................................
consensus sequence #601    GTGAGCGTGT GTTGGATGCG GTGGCTGCGG TAGGTGGTTC AACGGCAAAT GTGCAGGATA


serogroup 29E      #661    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #661    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #661    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #661    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #661    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #661    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #661    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #661    ---------- ---------- ---------- ---------- ---------- ----------
                           ................................................................
consensus sequence #661    CGAATGTGCA GCTGACACGT GGCAATGTAG TACGAACTGT TGCCTTGGAA GATTTAGTTG
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Fig. 3. (*cont.*)

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serogroup 29E      #721    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #721    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #721    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #721    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #721    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #721    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #721    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #721    ---------- ---------- ---------- ---------- ---------- ----------
                           ..........................................................
consensus sequence #721    CAAATCCGCG ACAAAATATT TTGCTGCGTC GCGGTGATGT GGTTACCATG ATTACCAATC


serogroup 29E      #781    ---------- ---------- ---------- ---------- ---------- --------T-
serogroup A        #781    ---------- ---------- ---------- ---------- ---------- --------T-
serogroup B        #781    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #781    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #781    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #781    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #781    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #781    ---------- ---------- ---------- ---------- ---------- ----------
                           ..........................................................
consensus sequence #781    CCTATACCTT TACGTCTATG GGTGCGGTGG GGAGAACACA AGAAATCGGT TTTTCAGCCA
                                                                                          *


serogroup 29E      #841    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #841    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #841    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #841    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #841    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #841    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #841    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #841    ---------- ---------- ---------- ---------- ---------- ----------
                           ..........................................................
consensus sequence #841    GAGGCTTATC GCTTTCTGAA GCCATTGGCC GTATGGGCGG TTTGCAAGAT CGCCGTTCTG


serogroup 29E      #901    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #901    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #901    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #901    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #901    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #901    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #901    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #901    ---------- ---------- ---------- ---------- ---------- ----------
                           ..........................................................
consensus sequence #901    ATGCGCGTGG TGTGTTTGTG TTCCGCTATA CGCCATTGGT GGAATTGCCG GCAGAACGTC


serogroup 29E      #961    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #961    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #961    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #961    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #961    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #961    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #961    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #961    ---------- ---------- ---------- ---------- ---------A- ----------
                           ..........................................................
consensus sequence #961    AGGATAAATG GATTGCTCAA GGTTATGGCA GTGAGGCAGA GATTCCAACG GTATATCGTG
                                                                                *


serogroup 29E      #1021   ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #1021   ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #1021   ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #1021   ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #1021   ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #1021   ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #1021   ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #1021   ---------- ---------- ---------- ---------- ---------- ----------
                           ..........................................................
consensus sequence #1021   TGAATATGGC TGATGCGCAT TCGCTATTTT CTATGCAGCG CTTTCCTGTG AAGAATAAAG
```

Fig. 3. (*cont.*)

```
serogroup A        #1081    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #1081    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #1081    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #1081    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #1081    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #1081    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #1081    ---------- ---------- ---------- ---------- ---------- ----------
                            ..........................................................
consensus sequence #1081    ATGTATTGTA TGTGTCGAAT GCGCCGTTGG CTGAAGTGCA GAAATTCTTG TCGTTTGTGT


serogroup 29E      #1141    ---------- ---------- ---------- ---------- ---------- ----------
serogroup A        #1141    ---------- ---------- ---------- ---------- ---------- ----------
serogroup B        #1141    ---------- ---------- ---------- ---------- ---------- ----------
serogroup C        #1141    ---------- ---------- ---------- ---------- ---------- ----------
serogroup H        #1141    ---------- ---------- ---------- ---------- ---------- ----------
serogroup W135     #1141    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Y        #1141    ---------- ---------- ---------- ---------- ---------- ----------
serogroup Z        #1141    ---------- ---------- ---------- ---------- ---------- ----------
                            ..........................................................
consensus sequence #1141    TCTCGCCGGT TACCAGTGGC GCGAACAGTA TTAATAATTT AACTAATTAA TGTGAGTAAT


serogroup 29E      #1201    ---------- ----
serogroup A        #1201    ---------- ----
serogroup B        #1201    ---------- ----
serogroup C        #1201    ---------- ----
serogroup H        #1201    ---------- ----
serogroup W135     #1201    ---------- ----
serogroup Y        #1201    ---------- ----
serogroup Z        #1201    ---------- ----
                            ...............
consensus sequence #1201    TAAGATGTCT GAGC
```

Fig. 3. *ctrA* nucleotide sequence data. * denotes a base difference, : denotes a deletion.

A *ctrA* grouping PCR developed using the nucleotide sequence provided by the sequencing of the entire *ctrA* gene, was used to identify 6 isolates as serogroup 29E, 2 as Z, 1 as H and 2 as X. The primers used for the amplification of serogroups H and 29E were designed using sequence data obtained upstream of the *ctrA* gene. None of the isolates were amplified using the serogroup A-specific primers. The percentage homologies of the *ctrA* genes, when compared to that of serogroup B, were determined to be: A (92·5%); Z (94·2%); 29E (91·4%); H (99·9%); W135 (100%); Y (100%) and C (100%). The nucleotide sequence of the *ctrA* gene for each serogroup was submitted to GenBank (accession nos. AF520902–AF520909).

DISCUSSION

We have shown that of 178 meningococcal carrier isolates analysed, the capsular status could be elucidated in the majority, with only 16% (28/178) remaining unassignable by capsular gene analysis. The capacity of genogrouping to characterize carrier isolates that are otherwise non-serogroupable isolates (35% (31/89) positive by *siaD* PCR ELISA [15, 16] for serogroups B, C, Y and W135) illustrates the potential of this technique for enhanced surveillance. Further discrimination of the 58 isolates which were non-genogroupable using the *siaD* PCR ELISA can be

undertaken using DNA sequencing of the *ctrA* gene that displays variation in its 5′ end [12]. The *ctrA* serogrouping PCR assays yielded 11 further isolates that were genogroupable (29E, Z, H or X). Seven of the 58 (12%) non-serogroupable isolates were shown to be positive for sialic acid by *siaA* PCR [7], and 6 of these isolates were shown to be either serogroup B, C, W135 or Y using the *ctrA* genogrouping assay, suggesting that these isolates have sequence variation within the *siaD* gene or do not possess a *siaD* gene.

Apart from the 16% of carrier isolates that are obviously deficient in part or all of their capsular operon as evidenced by the *ctrA* PCR, the mechanisms for down-regulation of capsule have only been demonstrated for a minority of isolates. These include 3 isolates with slipped-strand mispairing in the *siaD* promoter region, the presence of the IS element IS*1301* in 3 isolates (either in *siaD* or *siaA*) and 1 isolate which was positive for the *siaA* gene yet negative for the *ctrA* gene. In the study of Hammerschmidt et al. [6] that first demonstrated the reversible slipped-strand mispairing in the promoter region of serogroup B meningococci, the carrier isolates were all collected as part of a community outbreak of serogroup B disease [1] and were all related to the outbreak strain [17]. These isolates were potentially virulent as they were able to turn their capsule status on or off by this mechanism. In our study the isolates examined were not related and were

less likely to be potentially virulent. Recently, it has been shown that putative promoters for the capsular transport and biosynthesis operons are located in the intergenic region between these operons [8, 9]. Transcription regulation through sequence variation in these promoters is therefore another important potential control mechanism for meningococci that incorporate sialic acid as part of their capsule.

Sequencing of the *ctrA* gene can distinguish between meningococci whose capsules contain sialic acid, (which have similar percentage homologies), and those, whose capsules do not contain sialic acid. We found that serogroup H *ctrA* sequence was strongly homologous to serogroups B, C, W135 and Y, indicating that it probably contains sialic acid in its capsular polysaccharide. However, the non-serogroupable isolate which was identified as serogroup H using geno-grouping did not possess a *siaA* gene and was not amplified using the *ctrA* serogroup B, C, W135 and Y PCR assay. The low homologies of serogroups A, 29E and Z compared to the sialic acid containing meningococci illustrate the divergence of these sero-groups, as previously reported.

Also of interest is the existence of meningococcal isolates with unknown *ctrA* genes. Meningococcal isolates of rare serogroups have been reported namely D, L, K, H and I and it is possible that these isolates belong to one of these serogroups as antisera are not available for these serogroups at the PHLS MRU. Another explanation could be that these isolates represent a novel serogroup yet to be identified.

When analysed by school and by intervention (meningococcal AC polysaccharide vaccination and antibiotics), non-intervention schools had a higher proportion of *ctrA*-positive isolates than intervention schools at 11 months. This difference was not significant at 6 months' post-intervention due to the small numbers of isolates at this time-point. This suggests that following prophylaxis, strains that do not express capsular polysaccharide may have the capability to recolonize their hosts more rapidly than capsulated strains. The percentages of *ctrA*-positive isolates were similar at 6 and 11 months (77 and 74%, respectively).

If recolonization occurs preferentially with acapsulate (and thus putatively avirulent) isolates, there may be implications for control of community outbreaks of meningococcal disease. Mass use of antibiotics has been criticized and is not standard practice in all parts of the world [18], even though induction of resistance in meningococci is rare. No strains in

this outbreak were resistant to either rifampicin or ciprofloxacin although rifampicin resistance was detected after mass usage in Finnish army recruits [19]. These findings will help to allay concerns that protective nasopharyngeal flora may be eliminated by antibiotic prophylaxis used to control clusters and outbreaks of meningococcal disease, by exposing treated individuals to the risk of colonization by pathogenic or antibiotic-resistant bacteria. Though based on a small sample, our results provide some evidence to suggest that these concerns may have been overstated.

This investigation demonstrates the additional information that may be obtained through analysis of meningococcal capsular genes during carriage studies and illustrates that the majority (84%) can be characterized by genetic analysis.

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