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Consecutive Use of Two Multiplex PCR-Based Assays for Simultaneous Identification and Determination of Capsular Status of Nine Common *Neisseria meningitidis* Serogroups Associated with Invasive Disease

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We developed two *Neisseria meningitidis* multiplex PCR assays to be used consecutively that allow determination of the serogroup and capsular status of serogroup A, B, C, 29E, W135, X, and Y *cnl-3*/*cnl-1*-like-containing *N. meningitidis* isolates by direct analysis of the amplicon size. These assays offer a rapid and simple method of serogrouping *N. meningitidis*.

Neisseria meningitidis remains an important invasive pathogen worldwide, and nasopharyngeal carriage is common, with some 10 to 25% of the population harboring meningococci at any one time; however, transition from colonization to invasion is relatively rare (3, 30). Carried meningococcal populations are highly diverse (4, 17, 32, 36) and dynamic, with high acquisition/transmission rates among hosts (3, 20). For epidemiological purposes and particularly in the context of a meningococcal immunization program, it is important to establish the distribution of meningococcal serogroups circulating and to monitor changes in this population, allowing detection of the emergence of serogroups associated with disease, potentially belonging to hyperinvasive lineages (28).

Characterization of isolates by serogroup is an integral part of guiding the management of contacts of a case of meningococcal infection. Currently, 13 serogroups (A, B, C, D, 29E [Z′], H, I, K, L, W135, X, Y, and Z) of *N. meningitidis* are recognized, based on the antigenic variation among the different capsular polysaccharides. Isolates associated with serogroups A, B, C, 29E, H, W135, X, Y, and Z have been frequently isolated from healthy carriers and are responsible for most cases of disease worldwide (9, 10, 12, 24, 27). Most of these serogroups may be identified using commercially available antisera, although not all of the serogroup-specific sera are readily available and the sera can be expensive. However, despite using a comprehensive panel of antisera, not all strains yield a serogroup due to poor capsule expression or absence of a capsule (14, 15, 27, 33, 35). Several PCR-based assays have been developed for the detection and identification of the common serogroups, with assays for serogroups A, B, C, W135, and Y in routine use in many diagnostic laboratories (2, 7, 12, 13, 18, 23, 25, 26, 29, 31). Strains of serogroups D, I, K, and L have rarely been detected and are not thought to be disease associated. An additional problem is that some meningococcal strains lack genes encoding a capsule and harbor sequences that have replaced the capsular biosynthesis and transport loci, referred to as the capsule null locus (*cnl*) (1, 5, 8, 27). Such serologically nongroupable (NG) acapsulate strains have frequently been isolated from healthy carriers (5, 27; D. E. Bennett, A. D. Stack, and M. T. Cafferkey, unpublished data). Recent reports documenting invasive disease with *cnl* strains highlight the pathogenic potential of these strains (16, 34).

The use of individual assays to distinguish between isolates of multiple serogroups would necessitate several consecutive PCR assays. Simultaneous identification of serogroup A, B, C, W135, and Y strains was described in one previous report (29), in which subsequent PCR was necessary to distinguish between W135 and Y. The aim of the present study was to establish and to assess a rapid PCR-based method for the simultaneous identification and characterization of *N. meningitidis* isolates, detecting isolates of serogroups A, B, C, 29E, W135, X, and Y; *cnl-3*/*cnl-1*-like-containing NG isolates; and also serogroups H and Z.

All *N. meningitidis* ($n = 124$) and non-*N. meningitidis Neisseria* (including *N. gonorrhoeae* and *N. lactamica*) isolates used in this study were recovered during a meningococcal carriage survey of students (age 17 to 21 years) attending university or other third-level institutions (D. E. Bennett, A. D. Stack, and M. T. Cafferkey, unpublished data). Control strains used in this study were as previously described (1). The *N. meningitidis* isolates were 2 isolates each of serogroups A and H; 20 isolates each of serogroups B, C, W135, and Y; 10 isolates each of serogroups 29E, X, and Z; and 10 isolates designated NG. The capsular status of each isolate was determined using conventional slide agglutination (24) and the individual PCR genogrouping assays as documented previously (1, 13, 27, 29). A two-step multiplex PCR approach was designed to detect and determine the capsular type of each *N. meningitidis* isolate, using the primers listed in Table 1. Primers to distinguish serogroup W135 and Y isolates were designed, as were primers (in conjunction with primers documented by Claus et al. [5]) to detect strains that do not have capsule biosynthesis and transport loci but instead have short intergenic sequences, either

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TABLE 1. Primer sequences used in this study

Primer type and serogroup specificity	Amplicon size (bp)	Primer name	Multiplex assay	Sequence (5′→3′)	Reference
Serogroup-specific capsule biosynthesis gene primers					
A	400	*orf-2* F	First	CGCAATAGGTGTATATATTCTTCC	29
		orf-2 R	First	CGTAATAGTTTCGTATGCCTTCTT	29
B	170	*siaD* BF	First	TGCATGTCCCCTTTCCTGA	13
		siaD BR[a]	First	AATGGGGTAGCGTTGACTAACAA	13
C	442	*siaD* CF[a]	First	GCACATTCAGGCGGGATTAG	13
		siaD CR	First	TCTCTTGTTGGGCTGTATGGTGTA	13
Y	330	*siaD* YF	First	CATCTCAAAGCGAAGGCTTTGG	This study
		siaD YWR	First	TTGGAATTCGTCCAATTCTTTTCG	This study
W135[b]	300	*siaD* W135F	First	GGTGTATGATATTCCAATCGTTGTA	This study
ctrA primers[c]					
B, C, H, W135, Y	89	*ctrA* 1F	First	TTGTGTGGAAGTTTAATTGTAGGATGC	13
		ctrA 1R	First	CAGCTTGTTGCCCTAAAGAGACA	13
A, B, C, 29E, H, W135, X, Y, Z	110	*ctrA* UF	First and second	GCTGCGGTAGGTGGTTCAA	6
		ctrA UR	Second	TTGTCGCGGATTTGCAACTA	6
Other serogroup-specific primers					
X	525	Sg XF3	First	GTCTTTGTATAAGGCCCAAG	1
29E	667	Sg 29EF2	Second	ATTACGCTGACGGCATGTGGA	1
Z	667	Sg ZF	Individual (with *ctrA* UR)	TATGCGGTGCTGTTCGCTATG	1
H	1,363	Sg H *ctrA*F	Individual	GTTGAAATAGTCACGAAAGAA	27
		ctrA rev 01	Individual	GCTCAGACATCTTAATTACTC	27
N. meningitidis-specific primers					
A, B, C, 29E, H, W135, X, Y, Z, NG	251	*porA* 2F	First	GCGGTTTTGCCGGGAACTAT	This study
		porA 15R	First	AGTGGCGGCAATTTCGGTCGTACT	11
Nongroupable isolate-specific primers					
cnl-3/*cnl-1* like[d,e,f]	432	HC344	Second	GGATTGGACGAGCGAGAC	5
		GH26R	Second	GGTCGTCTGAAAGCTTGCCTTGCTC	5
cnl-1 like[d]	103	IGS F	Second	TGCCTCCGTGATGCCGTCTG	This study
cnl-3/*cnl-1* like[e]		IGS R	Second	CCTTTCAGATTGTATCGTGC	This study

[a] Amplification with *siaD* BR and *siaD* CF generates a serogroup C-specific 104-bp band.
[b] Amplification with *siaD* W135F and *siaD* YWR generates the 300-bp serogroup W135-specific product.
[c] Readthrough amplification with *ctrA* 1F and *ctrA* UF generates a 678-bp *ctrA* gene product in serogroup B, C, W135, and Y isolates.
[d] Readthrough amplification with HC344 and IGS F generates an NG *cnl-1*-like-specific 179-bp product.
[e] Readthrough amplification with GH26R and IGS R generates a 377-bp product in both NG *cnl-3*-like and NG *cnl-1*-like isolates, but this is predominantly observed in NG *cnl-3* isolates.
[f] Readthrough amplification with HC344 and GH26R generates a 432-bp product in both NG *cnl-3*-like and NG *cnl-1*-like isolates.

cnl-3 or *cnl-1*,*2*,*4*,*5*,*6* (*cnl-1*-like). A primer pair to detect the *N. meningitidis*-specific *porA* gene target (21, 22) was also designed.

The first multiplex assay contained primers specifically directed against the universal *N. meningitidis porA* gene (*porA* 2F and *porA* 15R) and the *ctrA* gene (*ctrA* 1F, *ctrA* 1R, and *ctrA* UR) to identify *N. meningitidis*, regardless of serogroup, and also primers specific for serogroups A, B, C, W135, X, and Y (*orf-2* F, *orf-2* R, *siaD* BF, *siaD* BR, *siaD* CF, *siaD* CR, *siaD* W135F, *siaD* YWR, *siaD* YF, and Sg XF3). Each 25-μl volume of PCR mix comprised of 1 unit of *Taq* DNA polymerase (Gibco); 7.5 mM $MgCl_2$; 2.5 μl of 10× buffer (1× buffer contains 10 mM Tris-HCl [pH 9 at 25°C], 50 mM KCl, and 0.1% Triton X-100); 1.25 mM each of primers *ctrA* 1F and 1R, *orf-2* F and *orf-2* R, *siaD* CF and *siaD* CR, *siaD* W135 F, *siaD* YF, and *siaD* YWR; 2 mM each of primers *siaD* BF, *siaD* BR, and Sg XF3; 0.5 mM each of the *porA* 2F and *porA* 15R primers; and 0.2 mM deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), to which 1 μl of *N. meningitidis* DNA (100 to 300 ng) purified using MicroLysis solution (Microzone Ltd., United Kingdom) was added. This assay was subjected to the same cycling conditions as described by Bennett et al. (1).

The second multiplex assay contained seven primers and included primers designed to amplify a region of the *ctrA* gene from serogroups A, B, C, 29E, H, W135, X, Y, and Z (*ctrA* UF and *ctrA* UR); a serogroup 29E-specific primer (Sg 29EF2); and primers (IGS F, IGS R, HC344, and GH26R) to differentiate between either *cnl-3*- or *cnl-1*-like-containing isolates (Table 1). Again each 25-μl volume of PCR mix comprised 1 unit of *Taq* DNA polymerase (Gibco), 5.0 mM $MgCl_2$, 2.5 μl of 10× buffer (1× buffer contains 10 mM Tris-HCl [pH 9 at 25°C], 50 mM KCl, and 0.1% Triton X-100), 0.2 mM deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), and 1.5 mM of each primer (*ctrA* UF, *ctrA* UR, Sg 29EF2, IGS F, IGS R, HC344, and GH26R), to which 1 μl of purified DNA

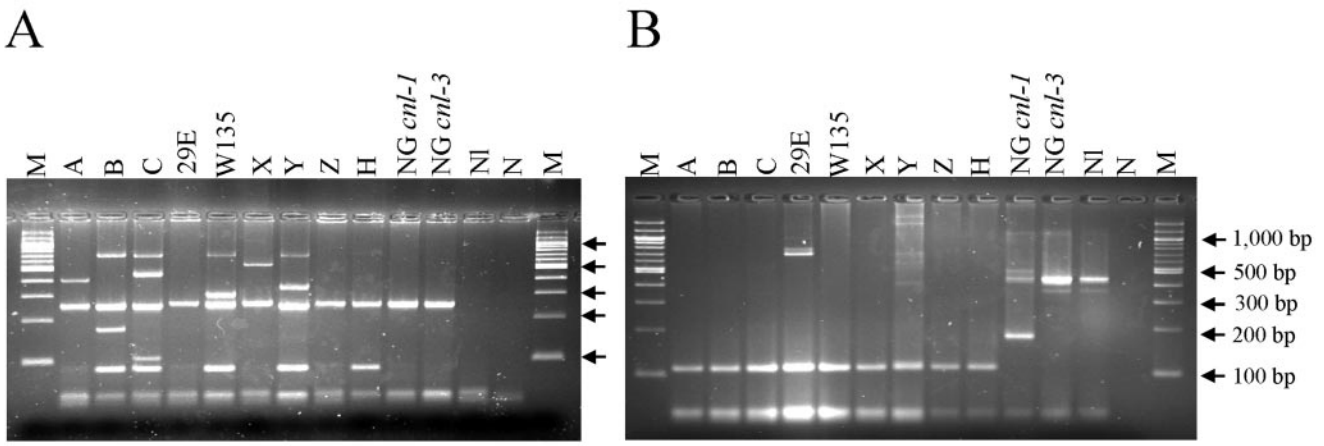

FIG. 1. PCR amplification products obtained with extracts from *N. meningitidis* strains belonging to serogroups A (M96/255449), B (H44/76), C (C11), 29E (3022Y), W135 (3019F), X (3094B), Y (3101L), Z (20355Q), H (21033V), NG *cnl-1*-like (20023K), and NG *cnl-3* (20334Y) and from *N. lactamica* (Nl) after the first (A) and second (B) multiplex PCR assays. Electrophoresis was performed on a 3.5% (wt/vol) agarose gel and products detected by ethidium bromide staining. Lanes M, size markers (the 100-bp ladder from New England Biolabs). No purified *N. meningitidis* DNA was added as template to the PCR mixture used for lane N.

was added. The cycling conditions used for this assay were similar to those used for the first multiplex assay except that an annealing temperature of 56°C instead of 62.5°C was used.

Each multiplex assay was tested and optimized using at least two isolates of each serogroup to be detected; this allowed the accuracy and specificity of each primer pair within each multiplex assay to be evaluated, alone and in combination. Each of the forward primers was tested individually and in combination with its specific reverse primer by using a panel of known serogroups to ensure that a PCR product of the expected size was amplified and that no nonspecific products were generated. Each reverse primer was also checked in this way to ensure that no cross-reactivity occurred. Once the specificity was confirmed, the PCR conditions, buffers, and primer concentrations were optimized to establish conditions in which the primers could be combined into a single PCR mixture. Using the optimized conditions, all primer pairs used were known to be *N. meningitidis* specific for their corresponding serogroup, and despite the number of primers contained in each assay, no cross-reactivity was observed between strains of different serogroups. Furthermore, no cross-reactivity was observed with a panel of non-*N. meningitidis Neisseria* species except with the nongroupable isolate-specific primers (Table 1), but none of these species yielded a *porA* amplicon.

These two assays, developed with the newly designed and previously published primers (1, 5, 6, 11, 13, 29), were evaluated with the 124 *N. meningitidis* isolates whose serogroup/capsular status was already known (D. E. Bennett, A. D. Stack, and M. T. Cafferkey, unpublished data). The product profiles observed following each assay were easy to interpret; each primer pair generated a product that was distinct in size. In the first multiplex assay, the expected sizes of specific products generated were as follows: *porA*, 251 bp; *ctrA* (serogroups B, C, H, W135, and Y), 89 bp; *orf-2* (A), 400 bp; *siaD* (B), 170 bp; *siaD* (C), 442 bp; *siaD* (W135), 300 bp; *siaD* (Y), 330 bp; and *ctrA* (X), 525 bp (Fig. 1A; Table 1). Depending on the *N. meningitidis* isolate examined, the products amplified varied. *porA*, *ctrA*, and *siaD* products of the expected sizes were amplified with DNA from either a serogroup B, C, W135, or Y isolate (Fig. 1A). However, a 678-bp *ctrA* product due to amplification readthrough by primers *ctrA* 1F and *ctrA* UR was also observed in these serogroups. A second *siaD* gene product (104 bp) was amplified with serogroup C isolates due to the *siaD* BR primer also binding to the serogroup C-specific *siaD* gene and generating a second serogroup C-specific product with the *siaD* CF primer. Only two products were obtained with DNA from serogroup A and X isolates (*porA* and *orf-2* [A]/*ctrA* [X]) (Fig. 1A).

N. meningitidis isolates that yielded the *porA* product but not a serogroup-specific product or the *ctrA* (serogroups B, C, H, W135, and Y) product were addressed in the second multiplex assay. Similar to the case for the first multiplex assay, the specific products generated in the second multiplex assay were also distinguishable by size: *ctrA* (A, B, C, 29E, H, W135, X, Y, and Z), 110 bp; *ctrA* (29E), 667 bp; NG (*cnl-1*-like), 179 bp and 432 bp; and NG (*cnl-3*), 377 bp and 432 bp (Fig. 1b, Table 1). Furthermore, only two products were amplified with DNA from any isolate of serogroup 29E (Fig. 1B), while with NG isolates up to four products were amplified but with the presence of a 179-bp band distinguishing NG *cnl-1*-like isolates from NG *cnl-3* isolates (Table 1; Fig. 1B). Isolates of all other serogroups yielded a single product, *ctrA* (A, B, C, 29E, H, W135, X, Y, and Z) (Fig. 1B). The first assay correctly identified each of the 20 serogroup B, C, W135, and Y isolates along with the 10 serogroup X and both serogroup A isolates, and the second assay correctly predicted the 10 serogroup 29E isolates and the 7 *cnl-1*-like and 3 *cnl-3* NG isolates. Therefore, isolates of serogroups A, B, C, W135, X, and Y were identified after the first multiplex assay, and serogroup 29E and true NG isolates, i.e., those harboring the *cnl-3* and *cnl-1*-like sequences, were identified after the second multiplex assay. Isolates that yielded products from either or both of the *ctrA* gene primer pairs but for which no serogroup was identified were subjected to individual PCR assays with serogroup H- and

Z-specific primers (Table 1), using methodology described previously (1, 27) to identify isolates of these serogroups.

This study demonstrated the applicability and high efficacy of these two multiplex assays followed by the individual assays specific for serogroups H and Z in correctly identifying the serogroup and capsular status of all meningococcal isolates tested, and it demonstrated 100% correlation with the results obtained using traditional serotyping and individual PCR assays. These multiplex assays, performing a maximum of three PCRs for any isolate, allow the relatively inexpensive identification (compared to serogroup determination either by individual PCR assays or by DNA sequencing [19]) of nine serogroups of meningococci, including the five serogroups most often associated with invasive disease. In addition, the assays detect the capsule gene (but cannot predict capsule expression), and hence they are suitable for examining isolates that fail to serogroup using conventional slide agglutination and as a consequence may be incorrectly termed NG. The addition of primers specific for the *cnl-3* and *cnl-1*-like sequences that have replaced the capsular biosynthesis and transport loci in several strains of meningococci allows the detection of true NG isolates lacking the genes necessary for capsule biosynthesis and expression. This is particularly important in view of the recent case reports of meningococcal disease caused by meningococcal strains harboring the *cnl* sequence (16, 34). These assays are PCR based and have the potential to allow serogroup identification in the absence of a viable organism (this was not explored in the present study).

These assays allow the accurate, specific, and sensitive identification of meningococcal serogroups within a short time frame. Such rapid resolution of the serogroup and capsular status of meningococcal isolates will facilitate precise and appropriate clinical and public health responses to cases of meningococcal disease and, in addition, will be useful tools in carriage studies.

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