

5' Exonuclease Assay for Detection of Serogroup Y *Neisseria meningitidis*

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The incidence of serogroup Y meningococcal disease has increased recently in the United States. Here, we describe the development of a 5' exonuclease assay for the detection of serogroup Y *Neisseria meningitidis* and demonstrate the usefulness of this assay for resolving serogroup identification of strains that are resistant to conventional serogrouping and for the nonculture identification of serogroup Y meningococcal disease.

Serogroup classification of *Neisseria meningitidis* strains is often useful for investigating outbreaks of meningococcal disease, for case contact management, and for monitoring meningococcal vaccine effectiveness. Thirteen serogroups of *N. meningitidis* have been described based on the antigenic properties of the capsular polysaccharide. Worldwide, meningococcal infections are most often associated with serogroups A, B, C, W135, and Y. While asymptomatic nasopharynx carriage of *N. meningitidis* is observed in 5 to 10% of a given population, transmission of the infection to susceptible individuals can lead to an acute bacterial illness that presents clinically as fever, nausea, vomiting, nuchal rigidity, and/or petechial rash. Invasive disease, characterized by meningococcemia or meningococcal meningitis, has a case fatality rate approaching 15%. Recently, an increase in the prevalence of meningococcal infections caused by serogroup Y *N. meningitidis* has been observed within the United States (5, 6). California, like other regions of the United States, experienced a rise in serogroup Y meningococcal infections reaching a high of 29% of the isolates submitted to the state's reference laboratory in 1998. In addition to increasing numbers of sporadic cases, the number of outbreaks attributed to serogroup Y meningococcal infection has also increased in the United States and other parts of the world (6). The reason for this increase in serogroup Y meningococcal infections remains unclear, but possible explanations include the emergence of a virulent clone or a decrease in herd immunity to this serogroup.

Traditionally, serogroup determination has been accomplished by a simple bacterial agglutination test using serogroup-specific antisera. Unfortunately, serologic classification can occasionally prove difficult because of specificity problems associated with grouping antisera, down regulation of *N. meningitidis* capsule expression, or the propensity of certain strains to autoagglutinate (8). In addition, the detection of *N. meningitidis* by culture isolation suffers from poor sensitivity, due in part to early antimicrobial intervention prior to specimen col-

lection (2). To enhance the detection and the reliability of serogroup determination, Guiver et al. (4) recently developed a real-time PCR assay for the molecular detection and serogroup determination of *N. meningitidis* from clinical specimens. The 5' exonuclease (or Taqman) assay developed by these investigators identified *N. meningitidis* by amplification of a capsular transfer gene (*ctrA*) and in separate amplification reactions tested for serogroup B and serogroup C strains by targeting serogroup-specific gene sequences in a capsular biosynthesis gene called *siaD*. The *siaD* gene encodes a polysialyltransferase that functions in the polymerization of sialic acids into the polysaccharide capsule of the bacteria. Sequence differences between the *siaD* genes of serogroup B and serogroup C strains translate into functional differences in the polysialyltransferases produced by these serogroups (3). The functional differences in the polysialyltransferases lead to structural differences in the polysaccharide capsules and to antigenic differences between the two serogroups. These sequence differences in the *siaD* genes were exploited to design primer and probe sets for the detection of serogroup B and serogroup C nucleic acid signatures in clinical specimens. Similarly, regions of sequence divergence can be found in the otherwise highly homologous *siaD* genes of serogroups W135 and Y (3). To build upon this platform and extend serogroup coverage, we describe the development of a real-time, 5' exonuclease assay for the detection of serogroup Y *N. meningitidis*, its usefulness in the resolution of some nongroupable *N. meningitidis* isolates, and its application to the nonculture identification of infections caused by serogroup Y strains.

To develop a 5' exonuclease assay for the detection of serogroup Y *N. meningitidis* strains, a region of the *siaD* gene that encompasses the most divergent region of sequence between serogroup Y (GenBank accession no. Y13969.2) and serogroup W135 (GenBank accession no. Y13970.1) was targeted. A primer set capable of amplifying both serogroups was chosen with the PrimerSelect program of DNASTAR (Lasergene, Madison, Wis.). The forward primer sequence, 5'-TCC GAGCAGGAAATTTATGAGAA, shares perfect identity with the serogroup Y sequence but possesses a single nucleotide difference (G-for-A substitution at nucleotide 21 of the

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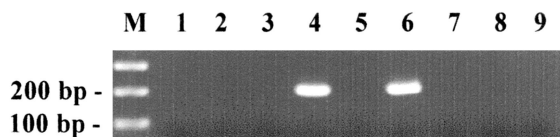


FIG. 1. Amplification of a 197-bp fragment of the *siaD* gene from various serogroups of *N. meningitidis*. PCR samples were electrophoresed on a 2% agarose gel, and the DNA was detected by ethidium bromide staining. *N. meningitidis* strains tested for amplification included those from the following serogroups: A (lane 1), B (lane 2), C (lane 3), W135 (lane 4), X (lane 5), Y (lane 6), Z (lane 7), and Z' (lane 8). No DNA template was added to the PCR shown in lane 9. The lane containing a 100-bp DNA ladder is designated M.

primer) from the serogroup W135 sequence. The reverse primer sequence, 5'-GCCATTCCAGAAATATCACCAG, was identical to the *siaD* sequence for both serogroup Y and serogroup W135. The ability of this primer set to amplify the *siaD* target from different serogroups of *N. meningitidis* was tested by PCR. All *N. meningitidis* strains used in this study were isolated from sterile sites, and the serogroup designation was previously determined in our laboratory by bacterial agglutination with commercially available, serogroup-specific sera (Difco Laboratories Inc., Detroit, Mich.; Murex Biotech Ltd., Dartford, England). PCRs were set up with the following components: 0.5 μ M (each) primer, 2.5 mM MgCl₂, 1 mM GeneAmp deoxynucleoside triphosphate blend (Applied Biosystems, Foster City, Calif.), 1 U of FastStart *Taq* DNA polymerase (Roche Applied Sciences, Indianapolis, Ind.), 0.5 U of uracil-DNA glycosylase (UNG) (heat labile; Roche Applied Sciences), and 2 ng of purified *N. meningitidis* DNA. The amplification parameters included an initial 10-min incubation at 20°C for UNG activity followed by a 10-min incubation at 95°C to inactivate UNG and activate the *Taq* polymerase. This was then followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s. An extension step of 7 min at 72°C was included as the final step of the amplification reaction. The amplification of a 197-bp product from each *N. meningitidis* serogroup was evaluated by agarose gel electrophoresis and ethidium bromide staining (Fig. 1). An amplification product of the appropriate size was detected for serogroups Y and W135, whereas no amplification products were observed for representative strains of serogroups A, B, C, X, Z, and Z'. Despite the single nucleotide mismatch between the forward primer and the W135 *siaD* sequence, no difference in assay sensitivity was observed when serial dilutions of serogroup Y and W135 template were tested (data not shown).

To discriminate serogroup Y strains from serogroup W135 strains, a dual-labeled hybridization probe was designed based on sequence differences within the 197-bp amplicon region described above. A 32-nucleotide region of the serogroup Y *siaD* gene sequence was selected for construction of the probe. Within this region, seven nucleotide differences can be found between the serogroup Y and W135 *siaD* sequences. A probe representing this 32-nucleotide region of the serogroup Y *siaD* sequence, 5'-ATAGGCAAGGATAGGGATATCGTACACC ATAC, was synthesized with a 5', 6-carboxyfluorescein modification (6-FAM reporter) and a 3', 6-carboxytetramethylrhodamine label (TAMRA quencher) by Operon Technologies

(Alameda, Calif.), and a 5' exonuclease assay was developed for rapid-cycling, real-time PCR. As designed, the probe's quencher molecule prevents emission of fluorescence from the reporter molecule. With the 5' exonuclease assay, the probe hybridizes to the complementary target sequence of the amplicon and is cleaved by the 5'-to-3' exonuclease activity of *Taq* polymerase during the primer extension step of the PCR. The exonuclease activity of *Taq* polymerase on the probe disassociates the reporter molecule from close association with the quencher molecule. Free reporter molecules can then emit fluorescence, and the amount of fluorescence is directly related to the amount of amplicon present in the reaction tube. Fluorescence is continually monitored during the course of the PCR by a rapid-cycling, real-time PCR instrument. Components of the 5' exonuclease assay for the detection of serogroup Y *N. meningitidis* were optimized and included 0.5 μ M (each) primer (as described above); 0.25 μ M dual-labeled, serogroup Y hybridization probe; 5 mM MgCl₂; 1 mM GeneAmp deoxynucleoside triphosphate blend; 1 \times FastStart DNA master hybridization probes (Roche Applied Sciences); and 0.5 U of UNG. Real-time PCR was performed with the Light-Cycler (Roche Applied Sciences) with the following amplification parameters: 2 min at 50°C, 10 min at 95°C, and 30 cycles of 95°C for 10 s and 60°C for 30 s. During amplification, the samples are monitored at each cycle for fluorescence at 530 nm, and a positive result is determined as the cycle at which the fluorescent signal increases exponentially and exceeds the background fluorescence of a control containing no template.

The specificity of the serogroup Y 5' exonuclease assay was assessed with a panel of *N. meningitidis* isolates representing eight clinically relevant serogroups. For DNA template preparation, a 1- μ l loopful of solid growth was resuspended in 100 μ l of Tris-EDTA buffer, boiled for 10 min, and microcentrifuged at full speed for 3 min to pellet the cell debris. Five microliters of the supernatant was used as template in the assay as configured above. Compared to conventional serologic grouping, the assay correctly identified 47 of 47 isolates as serogroup Y, whereas none of the 51 non-serogroup Y, *N. meningitidis* isolates were positive in the assay. Representatives from other serogroups included 8 strains of serogroup A; 9 strains of serogroup B; 22 strains of serogroup C; 9 strains of serogroup W135; and 1 strain each of serogroups X, Z, and Z'. Therefore, the specificity of the serogroup Y, 5' exonuclease assay for grouping *N. meningitidis* isolates was 100%. Whereas the primers and probe provide specificity for discriminating serogroup Y strains from serogroups A, B, C, X, Z, and Z', it should be emphasized that the assay's ability to discriminate between serogroup Y and W135 strains is entirely dependent upon the specificity of the probe, since the primer set will amplify both serogroups.

Over the last 10 years, greater than 90% of the *N. meningitidis* isolates received by the Microbial Diseases Laboratory (the microbiology reference laboratory for the state of California) for conventional serogrouping were identified as serogroup B, C, or Y. The relative percentage of each serogroup determined over this period was 48.5% for serogroup B; 23.8% for serogroup C; 18.2% for serogroup Y; 1.3% for serogroup W135; and 1.2% for serogroups A, X, Z, and Z'. Serogroup identification could not be determined for 7% of the submitted isolates. To evaluate whether molecular techniques might re-

TABLE 1. Utility of the *siaD* 5' exonuclease assays for grouping strains of *N. meningitidis* that are resistant to serologic grouping

Isolate	Source	<i>siaD</i> PCR result			Designation
		B	C	Y	
95A10220	Blood	-	-	-	Unknown
96A7452	CSF	-	-	+	Y
97A2159	Blood	-	+	-	C
98A1721	CSF	-	-	+	Y
98A1723	Blood	-	-	+	Y
98A6877	Blood	+	-	-	B
98A12181	CSF	+	-	-	B
99A6553	Blood	-	-	+	Y
00A0225	Blood	-	-	+	Y
00A1520	CSF	+	-	-	B
00A2205	Blood	-	-	+	Y
00A2764	CSF	+	-	-	B
00A4849	Blood	+	-	-	B
00A8920	CSF	+	-	-	B
00A9121	Blood	+	-	-	B
00A9349	Blood	+	-	-	B
00A9496	Blood	+	-	-	B
01A0664	CSF	-	-	-	Unknown
01A0839	Blood	-	-	-	Unknown

solve problems encountered with serogroup identification, we tested the utility of the 5' exonuclease assays for serogroups B, C, and Y against a panel of 19 isolates for which serogrouping was unsuccessful by serologic testing. The 5' exonuclease assays for detecting the *siaD* genes of serogroups B and C have been previously described by Guiver et al. (4). While the primer and probe sequences remained the same, other reaction components and amplification parameters for the serogroup B and serogroup C 5' exonuclease assays were modified to match those described above for the serogroup Y 5' exonuclease assay. The test results for the molecular serogrouping of the 19 isolates are shown in Table 1. No confounding results were observed. The 5' exonuclease assays unambiguously assigned nine of the isolates to serogroup B, six to serogroup Y, and one to serogroup C. Three isolates could not be serogrouped by these assays. Presumably, these isolates represent serogroups other than B, C, or Y.

In addition to resolving serologic grouping of *N. meningitidis* isolates, the 5' exonuclease assays can also facilitate nonculture detection and serogrouping of *N. meningitidis* infections. Guiver et al. (4) have applied the use of 5' exonuclease assays for the identification of *N. meningitidis* (*ctrA* assay) and for serogroup determination (*siaD* assays for serogroups B and C) directly from clinical specimens such as blood, serum, and cerebrospinal fluid (CSF). We have added our serogroup Y 5' exonuclease assay to this panel of assays for direct detection and serogrouping of *N. meningitidis* in clinical specimens. For increased assay sensitivity, the number of amplification cycles was increased to 45 for testing clinical specimens. With these amplification parameters, the limit of detection for each assay was 10 fg of purified *N. meningitidis* DNA (data not shown). In our laboratory, clinical specimens are tested for *N. meningitidis* DNA by the *ctrA* assay. Specimens positive by the *ctrA* assay are subsequently tested for serogroup determination by the three *siaD* assays (serogroups B, C, and Y). The utility of the serogroup Y 5' exonuclease assay for nonculture serogroup determination is illustrated by the data for selected meningococcal cases shown in Table 2.

TABLE 2. Direct detection of serogroup Y *N. meningitidis* in clinical specimens by the *ctrA* and *siaD* 5' exonuclease assays

Case	Source	Result by:				
		Culture	PCR assay			
			<i>ctrA</i>	B	C	Y
1	CSF	-	+	-	-	+
2	CSF	-	+	-	-	+
3	CSF	+	+	-	-	+

All three cases presented as classic meningococcal sepsis. Cases 1 and 2 were epidemiologically linked. *N. meningitidis* was isolated postmortem from the case 3 patient, but no culture isolates were available for the first two cases—most likely due to antimicrobial intervention prior to specimen collection. The isolate from the case 3 patient, however, was not groupable by serologic methods. To find evidence of meningococcal infection for each of these cases, total DNA from an 0.5-ml sample of CSF was purified with DNeasy spin columns (Qiagen Inc., Valencia, Calif.) and tested by the 5' exonuclease assays. The CSF specimens from all three patients tested positive by the *ctrA* assay. Subsequent testing revealed that the specimens were negative for serogroups B and C but were positive in the serogroup Y assay. The isolate from the case 3 patient was also tested by the 5' exonuclease assays and confirmed as serogroup Y.

The increased prevalence of meningococcal illness caused by serogroup Y strains and the sporadic quality control problems associated with commercially prepared group Y antisera have prompted our laboratory to implement a simple and rapid alternative to conventional serogrouping. Building upon the 5' exonuclease assays for serogroup B and C determination described by Guiver et al. (4), we have developed a compatible assay for serogroup Y *N. meningitidis* identification. The 5' exonuclease assay offers several advantages over other detection formats including (i) real-time detection of amplicon accumulation for rapid reporting of test results, (ii) product verification by a specific hybridization probe, and (iii) reduced risk of amplicon carryover contamination by utilization of a closed-tube detection system. These features represent significant advantages over previously described molecular approaches for identification of serogroup Y *N. meningitidis* (1, 7).

As designed, the primers described here will amplify both serogroup Y and W135 strains. The design and addition of a W135-specific hybridization probe with a distinct reporter fluorophore would allow the detection and differentiation of serogroup Y and W135 strains to be performed in a single reaction tube. While the addition of a W135-specific hybridization probe may be appropriate in areas where this serogroup is prevalent, the paucity of this serogroup in California would preclude the usefulness of such an assay in our geographic region. Used together, however, the serogroup B, C, and Y 5' exonuclease assays will provide serogroup identification for greater than 90% of the isolates reported in California. As illustrated in this report, these assays can help resolve inconclusive serogrouping results and thereby may also prove useful as a secondary assay after conventional serologic approaches. Finally, the capacity of these rapid PCR assays to detect nu-

cleic acids from nonviable organisms may significantly improve laboratory confirmation and serogroup determination for meningococcal disease, particularly in cases of culture-negative, clinically suspected meningococcal illness.

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