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Use of Real-Time PCR To Resolve Slide Agglutination Discrepancies in Serogroup Identification of *Neisseria meningitidis*

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***Neisseria meningitidis* is a leading cause of bacterial meningitis and septicemia in children and young adults in the United States. Rapid and reliable identification of *N. meningitidis* serogroups is crucial for judicious and expedient response to cases of meningococcal disease, including decisions about vaccination campaigns. From 1997 to 2002, 1,298 *N. meningitidis* isolates, collected in the United States through the Active Bacterial Core surveillance (ABCs), were tested by slide agglutination serogrouping (SASG) at both the ABCs sites and the Centers for Disease Control and Prevention (CDC). For over 95% of isolates, SASG results were concordant, while discrepant results were reported for 58 isolates. To resolve these discrepancies, we repeated the SASG in a blinded fashion and employed *ctrA* and six serogroup-specific PCR assays (SGS-PCR) to determine the genetic capsule type. Seventy-eight percent of discrepancies were resolved, since results of the SGS-PCR and SASG blinded study agreed with each other and confirmed the SASG result at either state health laboratories or CDC. This study demonstrated the ability of SGS-PCR to efficiently resolve SASG discrepancies and identified the main cause of the discrepancies as overreporting of these isolates as nongroupable. It also reemphasized the importance of adherence to quality assurance procedures when performing SASG and prompted prospective monitoring for SASG discrepancies involving isolates collected through ABCs in the United States.**

Neisseria meningitidis is an important cause of morbidity and mortality worldwide and a leading cause of bacterial meningitis and septicemia in children and young adults in the United States. Over the past several decades, rates of meningococcal disease in the United States have remained relatively stable at 0.8 to 1.3 per 100,000 (18), but changes in the epidemiology of meningococcal disease that have important implications for vaccination and other prevention strategies have occurred. New meningococcal conjugate vaccines, which are expected to be licensed based on immunogenicity studies, should be available in the United States in the next 2 years; identification and characterization of *N. meningitidis* will be crucial for recommendations and evaluations of these vaccines.

Rapid and reliable identification of *N. meningitidis* serogroups remains an important responsibility of the U.S. public health laboratories. In the late 1980s and early 1990s, most meningococcal disease was due to either MenB or MenC; during that time period, MenY accounted for only 2% of reported cases (18). While MenB and MenC still cause most outbreaks and sporadic meningococcal disease, the proportion of disease caused by MenY has risen from 2% during the period from 1989 to 1991 (9) to 25% in 2002 (ABCs data [unpublished]), and outbreaks due to MenY have also been reported (18, 25; C. Woods, N. Rosenstein, and B. A. Perkins, Abstr. 38th Annu. Meet. Infect. Dis. Soc. Am., abstr. 99, 1998). Even though outbreak-associated cases represent only 2 to 3% of the total U.S. disease burden, they cause tremendous public health concern.

A total of 1,298 *N. meningitidis* isolates that were collected through the ABCs between 1997 and 2002 were tested by SASG at both the SHL and the CDC. While the majority of these isolates (95.5%; $n = 1,240$) had CR in SASG, DR were reported for 58 isolates. The goal of this study was to determine whether comparison of the capsule expression of these isolates with their genetic capsule type could resolve these discrepancies.

A number of PCR approaches have been developed over the past several years to detect targets within the *ctrA* gene (capsule transport), as well as the genes required for serogroup-specific capsule biosynthesis of *N. meningitidis* (1, 2, 6, 8, 11, 13, 17, 22). In this study, we attempted to resolve the DR by using (i) real-time SGS-PCR targeting the capsule biosynthesis genes *sacB* (MenA), *siaD* (MenB or MenC), *synG* (MenW135), *xcbB* (MenX), and *synF* (MenY) (5, 20, 21) to detect the genetic capsule type and (ii) SASG in a controlled blinded fashion to determine capsule expression.

MATERIALS AND METHODS

In the present study, we analyzed a total of 447 bacterial isolates: 132 *N. meningitidis* isolates collected between 1997 and 2002 through ABCs, and 315

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TABLE 1. *N. meningitidis* reference strains

Strain	Serogroup	GenBank accession no.		Use[a] in:		
		ctrA	*sacB*, *siaD*, *synF*, *synG*, or *xcbB*	SASG (controls)	SGS-PCR	
					LLD	Controls
M7060	A	AY281028	AY281049	+	+	+
M4775	A	AF315847	AY234202		+	
M2677	A	AF315845	AY234204			
M1124	A	AF315844	AY234203			
M3562	A	AF315846	AY234205			
M5178	B	AY281029	AY281043	+	+	+
M1019	B	AF315849	AY234192		+	
M4211	B	AF315851	AY234190			
M3315	B	AF315850	AY234191			
M5177	B	AF315848	AY234193			
M3045	C	AY281030	AY281044	+	+	+
M980	C	AF315853	AY281045		+	
M209	C	AF315854	AY281046			
M3515	C	AF315855	AY281047			
M4642	C	AF315852	AY281048			
M7034	W	AY281031	AY281050	+	+	+
M6477	W	AF315856	AY234196		+	
M2190	W	AF315857	AY234194			
M3029	W	AF315859	AY234195			
M1013	W	AF315858	AY234197			
M2578	Y	AY281032	AY281051	+	+	+
M1000	Y	AF315861	AY234198		+	
M3027	Y	AF315862	AY234199			
M4440	Y	AF315863	AY234200			
M5005	Y	AF315860	AY234201			
M9601	X	AY281034	AY281039	+	+	+
M9592	X	AY281033	AY281038		+	
M9554	X	AY281035	AY281040			
M8210	X	AY281036	AY281041			
M2526	X	AY281037	AY281042			

[a] +, strain was used for this component of the study.

isolates (*N. meningitidis* and others) used for validation of the *ctrA* and SGS-PCR assays.

Abbreviations. The following abbreviations are used in this paper: ABCs, Active Bacterial Core surveillance; CDC, Centers for Disease Control and Prevention; CI, confidence interval; CR, concordant slide agglutination results; CR-NG, nongroupable concordant slide agglutination results; CR-SG, serogroupable concordant slide agglutination results; Ct, cycle threshold; DR, discrepant slide agglutination results; FDA, U.S. Food and Drug Administration; LCA, latent class analysis; LLD, lower limit of detection; MenA, *N. meningitidis* serogroup A; MenB, *N. meningitidis* serogroup B; MenC, *N. meningitidis* serogroup C; MenW135, *N. meningitidis* serogroup W135; MenX, *N. meningitidis* serogroup X; MenY, *N. meningitidis* serogroup Y; MenZ, *N. meningitidis* serogroup Z; Men29E, *N. meningitidis* serogroup 29E (Z′); NG, nongroupable slide agglutination result; SASG, slide agglutination serogrouping; SGS-PCR, serogroup-specific PCR; SHL, state health laboratories.

PCR validation. (i) Bacterial strains. A total of 315 bacterial isolates (282 *N. meningitidis* isolates and 33 isolates representing other species) were used to validate the *ctrA* assay and the SGS-PCR assays for *sacB* (MenA), *siaD* (MenB or MenC), *synG* (MenW135), *xcbB* (MenX), and *synF* (MenY) (5, 20, 21). For all isolates, cells were harvested from overnight growth on tryptic soy agar II plus 5% sheep blood plates (BBL, Cockeysville, Md.), suspended in 1.0 ml of 10 mM Tris buffer (pH 8.0), and heat killed by boiling for 10 min.

(a) *N. meningitidis* reference strains (n = 30). Thirty randomly chosen *N. meningitidis* isolates (5 from each serogroup, i.e., A, B, C, W135, X, and Y) were selected for sequencing of the *ctrA* gene and the six serogroup-specific genes (Table 1). Two sets of 6 isolates representing each serogroup, selected from the 30 reference isolates, were used for determining the LLD of all PCR assays (*ctrA* assay and six SGS-PCR assays). One of these sets was also used as positive controls for all PCR assays (Table 1).

(b) *N. meningitidis* strains used for evaluation of specificity of real-time SGS-PCR (n = 282). A total of 282 *N. meningitidis* isolates, including the 30 reference strains described above, were used for determining the specificity of the *ctrA* assay and the six SGS-PCR assays: 47 MenA isolates, 43 MenB isolates, 49 MenC isolates, 46 MenW135 isolates, 58 MenY isolates, 22 MenX isolates, and 17 other *N. meningitidis* strains (8 Men29E, 4 MenZ, and 5 NG). They were selected to represent the diversity of serogroups and previously defined hypervirulent clonal groups (subgroup III, ET-5 complex, and ET-37 complex) collected through ABCs from 1993 through 2001 (n = 111) and worldwide from 1963 through 2002 (n = 171) and also because of their association with well-defined and epidemiologically investigated outbreaks or sporadic cases of meningococcal disease (3, 14, 16, 24).

(c) Negative controls (n = 33). Thirty-three strains which are either close relatives of *N. meningitidis*, could be found in cerebrospinal fluid, or are commonly misidentified as *N. meningitidis* were used as negative controls. They included *Staphylococcus aureus*; *Haemophilus influenzae* types a, b, c, d, e, and f and nontypeable; *H. influenzae* biogroup aegyptius; *Haemophilus aphrophilus*; *Haemophilus parainfluenzae*; *Haemophilus haemolyticus*; *Neisseria cinerea*; *Neisseria gonorrhoeae*; *Neisseria sicca*; *Neisseria subflava*; *Neisseria lactamica*; *Moraxella catarrhalis*; *Streptococcus* groups A, B, C, D, and G; *Streptococcus pneumoniae* types 19f (two strains), 18c, 23f, 14, and 6b; *Escherichia coli* K-1; *Corynebacterium diphtheriae*; and *Mycobacterium tuberculosis* (two strains).

(ii) Sequencing of *ctrA* and serogroup-specific genes. (a) Determination of *ctrA* consensus sequence. The *ctrA* genes of 30 *N. meningitidis* reference strains (5 from each serogroup, i.e., A, B, C, W135, X, and Y) (Table 1) were amplified by standard PCR with primers designed from the *N. meningitidis* capsular transport gene sequence (GenBank accession number M57677) (Table 2) or, in the case of MenX, with previously published primers (19) as follows. PCR mixtures (100 μl) contained 5 U of Expand DNA polymerase (Roche Diagnostics, Indianapolis, Ind.); 2 μl of bacterial whole-cell suspension; 10 mM Tris-HCl (pH 8.0); 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM dATP, dCTP, dGTP, and dTTP; and a 0.4 μM concentration of each primer. The PCR mixtures were first incubated for 5 min at 95°C, and then 35 cycles were performed as follows: 15 s at 94°C, 15 s at the appropriate annealing temperature (Table 2), and 90 s at 72°C. The reaction mixtures were then incubated at 72°C for 5 min. PCR products of the appropriate sizes were visualized on a 1.2% E-gel (Invitrogen Corp., Carlsbad, Calif.) after electrophoresis for 20 min at 70 V. PCR products were purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's protocol. The amplified product for *ctrA* was then sequenced by using the primer set shown in Table 2. Sequencing was performed with the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Sequencing products were purified by using Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.) and were resolved on an Applied Biosystems model 3100 automated DNA sequencing system. The 30 *ctrA* sequences were aligned by using the GCG package, version 10.1 (Genetics Computer Group, Madison, Wis.), and the most conserved region of the resulting consensus sequence was identified and used for real-time PCR primer and probe design.

(b) Determination of serogroup-specific consensus sequences. As for *ctrA*, consensus sequences for capsule genes were obtained based on five reference strains for each serogroup (Table 1). Primers used for amplification of *sacB* (MenA), *siaD* (MenB or MenC), *synG* (MenW135), *xcbB* (MenX), or *synF* (MenY) are shown in Table 2 and were designed from the sequences in GenBank with accession numbers AF019760, M95053, U75650, Y13969, and Y13970, respectively. Primers specific for *xcbB* were designed from the capsule-specific gene sequence (the *xcbB* sequence was provided by David S. Stephens, Emory University [personal communication]) (Table 2). Sequencing was performed as described above for *ctrA*. Primers and serogroup-specific amplified DNA product sizes are shown in Table 2.

(iii) Real-time SGS-PCR assays. (a) Primer and probe design. Real-time PCR assays were designed to target *ctrA*, *sacB* (MenA), *siaD* (MenB or MenC), *synG* (MenW135), *xcbB* (MenX), or *synF* (MenY). The consensus region for each gene was searched for appropriate primers and probes by using Primer Express software (Applied Biosystems) (Table 2). Primers and probes were synthesized at the CDC Biotechnology Core Facility (Atlanta, Ga.). Primers were optimized by testing in the range of 0.3 to 0.9 μM (final concentration), and fluorescence-labeled probes were optimized by testing in the range of 100 to 400 nM (Table 2). Reactions were carried out with the ABI Prism 7700 or 7000 sequence detector (Applied Biosystems). Each reaction mixture contained 2 μl of whole-cell suspension, 2 μl of each primer, 2 μl of probe, and 12.5 μl of 2× TaqMan master mix (Applied Biosystems). PCR-certified Apex water (Mo Bio Laboratories, Inc., Encinitas, Calif.) was added to bring the reaction volume to 25 μl. PCR mixtures were first incubated for 10 min at 50°C, and then, 40 cycles of 1 min at 95°C and 1 min at 60°C were performed. The ABI 7700 and 7000 instruments read each sample every few seconds and computed a mean baseline

reading for early PCR cycles. A positive result, as reported by its Ct value, was indicated by the cycle at which fluorescence exceeded the mean baseline by 10 standard deviations. Ct values of >35 were considered negative. A positive control of *N. meningitidis* whole-cell suspension was included on every run, as were multiple no-template controls.

(b) LLD of real-time PCR assays. Two strains from each serogroup (A, B, C, W135, X, and Y) were selected for LLD testing (Table 1). Bacteria were collected from a single-colony subculture by swiping a loop across a 3-cm^2 area of dense growth, suspended in 5 ml of prewarmed Mueller-Hinton broth (BBL), and incubated for 6 h at 37°C. A 100-ml volume of prewarmed Mueller-Hinton broth (BBL) was then inoculated with 0.5 ml of the 5-ml culture, and bacteria were allowed to grow overnight at 37°C with shaking at 200 rpm. A 30-ml sample of the overnight culture was processed to extract genomic DNA by using the Qiagen genomic DNA purification kit according to the manufacturer's protocol. The final DNA pellet was resuspended in 1 ml of PCR-certified Apex water (Mo Bio Laboratories, Inc.) with incubation at 55°C for 1 to 2 h. DNA concentration and purity were determined with the MBA 2000 DNA calculator (Perkin-Elmer, Boston, Mass.). Using genomic DNA for each serogroup, adjusted to the same starting concentration (100 ng/μl), 10-fold serial dilutions (10^{-1} to 10^{-9}) were made in PCR-grade water. Real-time PCR was performed as described above with the optimized primer and probe concentrations shown in Table 2. The LLD for the *ctrA* assay and each serogroup-specific assay was determined to be the dilution that yielded a Ct value less than or equal to the cutoff of 35.

Evaluation of ABCs isolates (n = 132). (i) Selection of ABCs isolates. Active laboratory- and population-based surveillance for invasive disease caused by *N. meningitidis* is part of the ongoing multistate ABCs project coordinated by CDC as part of the Emerging Infections Program (18). Between 1997 and 2002, CDC collaborated with investigators in state and local health departments and universities in seven to nine geographically dispersed areas of the United States. Participating areas include all or part of the following states: California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New York, Oregon, and Tennessee. Because surveillance was not conducted continuously in all nine surveillance areas, the aggregate population under surveillance varied from 28.9 million in 1997 to an estimated 35.4 million in 2002. Census data for 2000 were used to estimate the annual surveillance population because census data for 2001 and 2002 were not available. A case of meningococcal disease was defined as the isolation of *N. meningitidis* from a normally sterile site, such as blood or cerebrospinal fluid, in a resident of the surveillance area. All available isolates were sent to CDC for further study. From 1 January 1997 to 31 December 2002, 1,783 *N. meningitidis* isolates were cultured from patients with meningococcal disease at all nine surveillance sites. Of those 1,783 isolates, 1,298 were collected and tested by SASG (15), at both SHL and CDC. The majority of these isolates (95.5%; n = 1,240) had CR results between SHL and CDC. Only 58 isolates (4.5%) had DR between SHL and CDC. To investigate reasons for these DR, 132 isolates that constituted three groups were selected for analysis in this study: (i) the 58 DR isolates, (ii) all 12 isolates that were reported as NG by both SHL and CDC (CR-NG) and were selected because of the high proportion of DR isolates reported as NG by at least one site, and (iii) 62 CR-SG isolates that provided a statistical representation of all 1,240 CR isolates to reduce the bias that might be associated with conducting LCA with a set of only DR isolates. These 62 isolates were selected randomly within serogroups B (14 of 461), C (19 of 350), W135 (13 of 17), and Y (16 of 393).

(ii) PCR analysis of ABCs isolates. The 132 ABCs isolates were analyzed by PCR assays for *ctrA*, MenA, MenB, MenC, MenW135, MenX, and MenY. A MenB mixed-base probe (Table 2), which included a single base change from the original probe, was used to confirm the serogroup identification of two MenB isolates.

(iii) SAGS of ABCs isolates. (a) SASG at SHL. SASG was conducted at SHL with commercially available antisera produced by either Difco Laboratories (Detroit, Mich.) or Murex (Remel, Lenexa, Kans.).

(b) SASG at CDC. Following SASG testing at SHL, meningococcal isolates are routinely forwarded to CDC for serogroup confirmation, storage, and further molecular testing. For the isolates used in this study, SASG was repeated at CDC with one of four different diagnostic antiserum sets: Difco antisera, FDA-produced antisera (Bureau of Biologics, Rockville, Md.), CDC-produced antisera, or U.S. Navy-produced antiserum for MenC only (Naval Biological Lab, U.S. Navy, Berkeley, Calif.). A result of 0, +/−, 1+, or 2+ was designated negative and was identified by no visible agglutination or minimal agglutination, with the suspension remaining cloudy and smooth. A result of 3+ or 4+ was designated positive and was identified by visible clumping with clearing of the suspension (Fig. 1). An isolate was identified as NG if no agglutination occurred with any of the antisera and saline (no reaction), when there was cross-reactivity with more than one antiserum (cross-reaction), or when there was agglutination in only saline or in saline with one or more antisera (autoagglutination).

(c) Quality assessment of diagnostic antiserum sets used in the SASG blinded study. For quality assessment, one well-characterized reference strain was selected to represent each of the serogroups A, B, C, W135, X, and Y (Table 1). All strains were tested with four sets of antisera (Difco, Murex, FDA, and CDC). Results were read and interpreted as described above. Antisera were considered satisfactory if they gave 3+ or 4+ agglutination (Fig. 1) with homologous antigens and if they did not react with strains of other serogroups. All *N. meningitidis* reference strains except the MenB strain (CDC M5178) were positive in SASG with all available homologous antisera. Difco MenB antiserum was not considered satisfactory but was used in the blinded study because it was the only lot available at the time.

(d) SASG blinded study design. An SASG blinded study was conducted with the 132 *N. meningitidis* ABCs isolates (see above). All isolates were maintained in sterile defibrinated sheep blood at −70°C and were recovered from freezer stocks by overnight incubation on tryptic soy agar II plus 5% sheep blood plates (BBL) at 37°C in a 5% CO_2-enhanced atmosphere for 18 to 24 h. Cell suspensions were made in 300 μl of 0.5% formalinized physiologic saline and briefly vortexed. Four sets of antisera (Difco, Murex, FDA, and CDC) were used by a single laboratory worker to test all isolates according to standard laboratory protocols (15). Each isolate was coded in a blinded fashion and randomly tested.

For the SASG blinded study, the Difco set contained antisera for serogroups A, B, C, W135, X, and Y and the Murex set contained antisera for serogroups A, B, C, and W135. The CDC-produced set contained antisera specifically reactive with serogroups B, C, W135, X, and Y, and the FDA-produced set contained antisera for serogroups A, B, C, and Y. When the SASG was performed, the order in which the four sets of antisera were used was random.

Statistical analysis. LCA was used to determine the sensitivity and specificity of all antisera used in the blinded SASG study. The serogroup of each isolate was predicted, and the sensitivity and specificity of all tests included in the model were estimated. LCA is a mathematical method that uses a statistical model to relate unobserved (latent) conditions to multiple test results. LCA models the probability of each combination of results conditionally on the latent class ("true" serogroup) (12). SASG DR between SHL and CDC were considered resolved when SGS-PCR agreed with the result of the SASG blinded study.

Nucleotide sequence accession numbers. The 60 gene sequences (30 for *ctrA* and 30 for serogroup-specific genes) determined in this study have been deposited in GenBank under the accession numbers listed in Table 1.

RESULTS

PCR validation. (i) Sensitivity and specificity of *ctrA* PCR and SGS-PCR assays. All 289 *N. meningitidis* strains used in the evaluation of sensitivity of the real-time PCR assays were positive in the species-specific assay targeting the *ctrA* gene. Two hundred seventy-two *N. meningitidis* strains of serogroups A, B, C, W135, X, and Y were also positive in the appropriate SGS-PCR assays (100% sensitivity for each assay). The remaining 17 *N. meningitidis* strains (8 Men29E, 4 MenZ, and 5 NG), for which serogroup-specific PCR assays were not available, were negative in the MenA, MenB, MenC, MenW135, MenX, and MenY assays (100% specificity). Strains of other species of the genus *Neisseria* were consistently negative in the *ctrA* assay. Furthermore, DNAs from 33 strains representing other bacterial species gave negative results in the *ctrA* assay (100% specificity).

(ii) LLD of *ctrA* PCR and SGS-PCR assays. The LLD of the real-time PCR assays for *ctrA* and SGS-PCR assays for MenA, MenB, and MenX were found to be in the range of 20 to 200 fg of genomic DNA (equivalent to 8 to 80 genomes, based on a 2.3-Mb genome). LLDs for SGS-PCR assays for MenC, MenW135, and MenY were in the range of 200 fg to 2 pg (80 to 800 genomes). We observed a 10-fold difference in LLD between the two reference strains of MenC and MenW135.

Analysis of *N. meningitidis* ABCs isolates. (i) Real-time PCR assays for *ctrA* and serogroup-specific capsule genes. All 132

TABLE 2. Primers and probes for gene sequencing and real-time PCR

Gene and group	Designation	5′-3′ nucleotide sequence	*ctrA*, *sacB*, *siaD*, *synF*, *synG*, or *xcbB*				Real-time PCR		
			Amplification	Annealing temp (°C)	Amplified product size (bp)	Sequencing	SGS-PCR	Amplified product size (bp)	Final primer or probe concn (nM)
ctrA	L1289	GTCTCTTTAGGGCAACAATCTGA	+[a]	55	1,154	+			
	U157	AACTTTTCTTTTTCGGCTTTTTA	+			+			
	MenXF	ATGCGGTGGCTGCGGTAGGT	+	65	491	+			
	MenXR	CCGGCGAGAACACAAACGACAAG	+			+			
	F623	CCCACCGCACCCATAGACGTA				+			
	R793	GTGGCTGCGGTAGGTGGTTCA				+			
	F1097	AGGCCACCACCAAACAATACT				+			
	F753	TGTGTTCCGCTATACGCCATT					+	114	300
	R846	GCCATATTCACACGATATACC					+		900
	Pb820	AACCTTGAGCAATCCATTTATCCTGACGTTCT					+		100
sacB (MenA)	F21335	TAAGTGATTCAGGCGGCATAC	+	56	2,041	+			
	R23376	CGGCAGCCATTCAACTCAAAT	+			+			
	F1856	TAACAATCAAAACGCAATAGGTGTAT				+			
	F2966	AGTAACGGGCTATCTCTATCATCATT				+			
	R1854	ACCTATTGCGTTTTGATTGTTAAG				+			
	R2841	TGAGGGGAGTGATGTAGTTTAGT				+			
	R2557	GAAGGGCACTTTGTGGCATAATTT				+			
	F2531	AAAATTCAATGGGTATATCACGAAGA					+	92	300
	R2624	ATATGGTGCAAGCTGGTTTCAATAG					+		900
	Pb2591	CTAAAAGTAGGAAGGGCACTTTGTGGCATAAT					+		100
slaD (MenB)	F2949	GGAGACTTCAGCGTCAACGAA	+	60	1,647	+			
	R4572	TTGTCGGCGGAATAGTAATAATGTT	+			+			
	F3854	TTTGTTAGTCAACGCTACCCCATTTC				+			
	R3854	GAAATGGGGTAGCGTTGACTAACAAA				+			
	F3872	CCCATTTCAGATGATTTGT					+	162	900
	R4013	AGCCGAGGGTTTATTTCTAC					+		900
	Pb3974	ATGGGCAACAACTATGTAATGTCTTTATT					+		100
	Pb3974(C/T)[b]	ATGGG(C/T)AACAACTATGTAATGTCTTTATT					+		100
siaD (MenC)	F109	TTTATTATTTCTACATTTGCCCAACT	+	60	990	+			
	R1077	CAATTAAAGCGGTGTTCTCTTGT	+			+			
	F648	TTCAGGCGGGATTAGCACAAG				+			
	R648	CTTGTGCTAATCCCGCCTGAA				+			
	F478	CTTTCCCTGAGTATGCGAAAAAA					+	77	900
	R551	TGCTAATCCCGCCTGAATG					+		300
	Pb495	TTTCAATGCTAATGAATACCACCGTTTTTTTGC					+		100
synF/*G* (MenW/Y)	F85	ATCAGAACCTTATTTCAACTATTACT	+	50	2,228	+			
	R2288	GTCTGCGGATACTCCATTACAATACT	+			+			
	F827[b]	TCTATTGTTGGCAG(C/T)ATTCAG				+			
	F1267	ATATAATCACAATCGCTTCTT				+			
	F1797	TACTGATTGCTTTACCCAACA				+			
	F2117	ACCGGAAAATGGGAAAAACAC				+			
	R827[b]	CTGAAT(A/G)CTGCCAACAATAGA				+			
	R1267	AAGAAGCGATTGTGATTATAT				+			
	R1797	TGTTGGGTAAAGCAATCAGTA				+			
	R2117	GTGTTTTTCCCATTTTCCGGT				+			
MenW	F1068	GTGAGGGATTTCCATATATATTTA					+	147	900
	R1214	TTGCCATTCCAGAAATATCA					+		900
	Pb1139	TATGGAGCGAATGATTACAGTAACTATAA					+		100
MenY	F1019	GAGCAGGAAATTTATGAGAATACAGA					+	140	300
	R1158	CTAAAATCATTCGCTCCATAT					+		900
	Pb1098	GTATGGTGTACGATATCCCTATCCTTGCCTATAAT					+		100
xcbB (MenX)	F127	TCGTACACTGAATTAGGGGTTA	+	51	83	+			
	R940	CGCGAGCTAGATTAGAACT	+			+			
	F7	CCCTCACATCACAGCCTAGTGG				+			
	R937	CGCGAGCTAGATTAGAACTTGT				+			
	F173	TGTCCCCAACCGTTTATTGG					+	66	900
	R237	TGCTGCTATCATAGCCGCC					+		900
	Pb196	TGTTTGCCCACATGAATGGCGG					+		100

[a] +, strain was used for this component of the study.
[b] Mixed-base primer or probe.

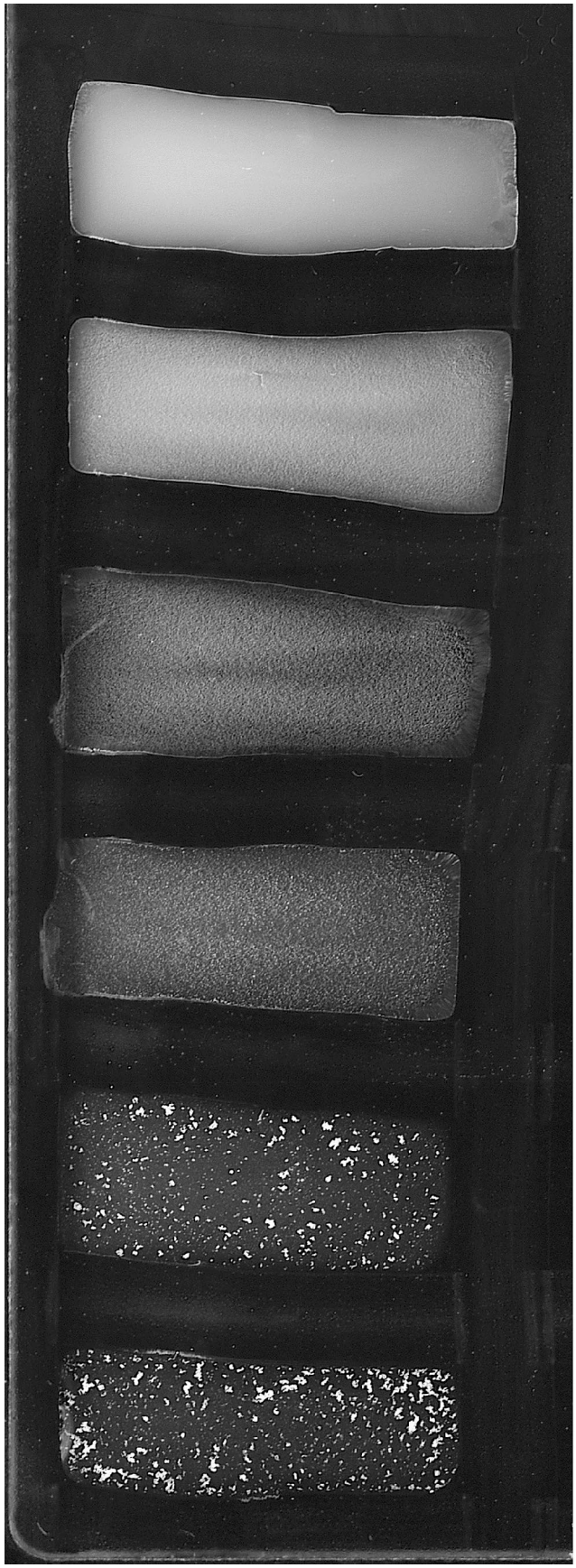

FIG. 1. Interpretation of SASG. A result of 0, +/−, 1+, or 2+ was designated negative and identified by minimal agglutination or by no visible agglutination, with the suspension remaining cloudy and smooth. A result of 3+ or 4+ was designated positive and was identified by visible clumping with clearing of the suspension.

SASG results between SHL and CDC	SASG			SGS - PCR	No. of isolates tested	
	SHL	CDC	Blinded study			
DR	B	NG	B	B	7	58
	C	NG	C	C	14	
	Y	NG	Y	Y	4	
	NG	B	B	B	6	
	NG	C	C	C	1	
	NG	Y	Y	Y	4	
	B	C	C	C	1	
	C	B	C	C	2	
	C	Y	Y	Y	1	
	W	Y	W	W	2	
	Y	C	C	C	2	
	Z	Y	Y	Y	1	
	B	NG	NG	B	1	
	Y	NG	NG	Y	3	
	NG	Y	NG	Y	1	
	C	B	NG	C	1	
	Y	W	W	Y	4	
	NG	B	NG	Y	1	
	B	NG	NG	Y	1	
	W	NG	NG	Y	1	
CR-NG	NG	NG	C	C	1	12
	NG	NG	NG	C	1	
	NG	NG	NG	Y	8	
	NG	NG	NG	NG	2	
CR-SG	B	B	B	B	14	62
	B	B	NG	Y	1	
	C	C	C	C	19	
	W	W	W	W	13	
	Y	Y	Y	Y	15	
Total						132

FIG. 2. Overall comparison of SASG and SGS-PCR for 132 *N. meningitidis* ABCs isolates. SASG results in the blinded study were predicted by LCA. Green or blue, SASG result at either SHL or CDC agreed with blinded study and SGS-PCR or with only SGS-PCR, respectively; gray, neither result agreed with SGS-PCR; orange, both results agreed with blinded study and SGS-PCR.

ABCs isolates were *ctrA* positive. For all 58 DR isolates, SGS-PCR detected 14 MenB, 21 MenC, 2 MenW135, and 21 MenY isolates. SGS-PCR detected the serogroup-specific capsule genes for 10 of the 12 CR-NG isolates, (2 MenC and 8 MenY); the remaining 2 isolates were negative. For all 62 CR-SG isolates, SGS-PCR detected 14 MenB, 19 MenC, 13 MenW135, and 16 MenY.

(ii) SASG blinded study. All 132 ABCs isolates were also tested by SASG in a blinded fashion with four sets of antisera produced by Difco, Murex, FDA, and CDC. For each isolate, the serogroup was predicted according to the LCA previously described. Of the 58 DR isolates, 49 were identified as follows: 13 MenB, 20 MenC, 6 MenW135, and 10 MenY (Fig. 2). The remaining nine isolates were NG. Of the 12 CR-NG isolates,

TABLE 3. Sensitivity and specificity of antisera for serogroup identification of 132 *N. meningitidis* ABCs isolates used in the SASG blinded study

Antiserum	Sensitivity		Specificity	
	%	95% CI	%	95% CI
MenB				
CDC	100	99–100	100	95–100
Difco[a]	0	0	0	0
FDA	93	73–100	95	91–99
Murex	26	9–43	100	99–100
MenC				
CDC	68	53–82	100	99–100
Difco	100	99–100	100	99–100
FDA	95	88–100	100	99–100
Murex	100	99–100	100	99–100
Men Y[b]				
CDC	100	99–100	100	99–100
Difco	62	43–80	100	99–100
FDA	100	99–100	88	82–94
Men W135[c]				
CDC	100	99–100	100	99–100
Difco	85	69–100	100	99–100
Murex	100	99–100	100	99–100

[a] Difco MenB antiserum did not agglutinate any of the isolates in this study.
[b] Murex MenY antiserum was not available.
[c] FDA MenW135 antiserum was not available.

11 were still identified as NG in the blinded study and 1 was identified as MenC (Fig. 2). The 62 CR-SG isolates were identified as 14 MenB, 19 MenC, 13 MenW135, 15 MenY, and 1 NG (Fig. 2).

The sensitivity and specificity for all antisera used in the SASG blinded study were determined by LCA (Table 3). The sensitivity for CDC- and FDA-produced antisera ranged from 93 to 100% for the available serogroups, except in the case of CDC MenC antiserum (68% sensitivity; 95% CI, 53 to 82%). The sensitivity for the commercially available Difco antisera ranged from 0 to 100% for serogroups B, C, Y, and W135. The sensitivity for Murex MenB antiserum was 26%, and that for Murex MenC and MenW135 antisera was 100%. All individual antisera in the four sets were 100% specific, except in the cases of FDA MenB (95% specificity; 95% CI, 91 to 99%) and FDA MenY (88% specificity; 95% CI, 81 to 94%) antisera.

(iii) Overall comparison of SASG and SGS-PCR. As presented in Fig. 2, serogroup identifications of 45 of 58 DR isolates (78%) were considered resolved because the results of the SASG blinded study and SGS-PCR agreed with each other and confirmed the SASG result at either SHL or CDC. For the remaining 13 DR isolates (22%), the blinded study and SGS-PCR results disagreed with each other; therefore, the SASG result discrepancy between SHL and CDC could not be resolved. Six of these 13 DR isolates were NG by the SASG blinded study, but the SGS-PCR result agreed with the serogroup result at either SHL or CDC. Another 4 of the 13 isolates were identified as MenW135 by the SASG blinded study but as MenY by SGS-PCR. The remaining 3 of the 13 isolates were identified as NG by the SASG blinded study but as MenY by SGS-PCR; for these 3 isolates, the SGS-PCR result did not agree with the SASG result obtained by SHL, CDC, or the blinded study.

Of the 12 CR-NG isolates, 2 were NG by both SASG blinded study and SGS-PCR, while the remaining 10 were positive by SGS-PCR (Fig. 2). The serogroup identifications previously determined at SHL and CDC for the 62 CR-SG isolates were confirmed by both the SASG blinded study and SGS-PCR, with a single exception (Fig. 2). One isolate was serogrouped at SHL and CDC as MenB but was identified as NG by the SASG blinded study and as MenY by SGS-PCR.

DISCUSSION

This study demonstrated the applicability and high efficacy of SGS-PCR in resolving DR in serogroup identification of meningococcal ABCs isolates and also identified the main cause of the DR as the overreporting of isolates as NG. SGS-PCR was able to identify specific capsule types for all 58 DR isolates. Since SGS-PCR detects the capsule gene but cannot predict capsule expression or SASG outcome under laboratory conditions, we considered a DR resolved only if the SGS-PCR result agreed with the result of the SASG blinded study (78%).

Even though the number of discrepancies was small, it nevertheless was important to resolve them, as judicious and expedient responses by public health departments to possible cases of meningococcal disease depend upon rapid and accurate serogroup identification of *N. meningitidis* isolates. Epidemiologists and other public health officials rely upon serogroup identification to make decisions about vaccination and antimicrobial prophylaxis campaigns to prevent the further spread of disease (4). If the discrepant results are occurring due to poor-quality diagnostic antisera or inappropriate quality control procedures, it is important to work with the antiserum manufacturers on production of products of higher quality as well as to improve laboratory quality control procedures and protocols.

For the present study and for future use with clinical samples, we developed and validated real-time PCR assays for *ctrA* and serogroup-specific capsule genes for MenA, MenB, MenC, MenW135, MenX, and MenY. The assays presented in this study improve the sensitivity and specificity of existing assays, ensure that the new targets were conserved among a diverse subset of strains in our extensive collection, and expand the diagnostic spectrum of real-time PCR by including novel assays for MenW135 and MenX.

Among the 58 DR isolates examined in this study, serogroup results of SGS-PCR and the SASG blinded study agreed with each other and with the SASG results at either SHL or CDC for 45 isolates (78%). DR for these isolates were therefore considered resolved. It is apparent that for these 45 isolates, their identification as NG at either SHL or CDC was not due to lack of capsule expression but rather to a technical problem with SASG. Human subjectivity in result interpretation, human error, or poor-quality antiserum may have been a factor when the isolates were originally tested at SHL and CDC, as we previously demonstrated was the case for *H. influenzae* serotyping (10). For example, certain isolates cross-react with some antisera, as has been observed for MenB-MenC and MenW135-MenY. Consequently, any time that agglutination is observed with more than one serogroup-specific antiserum, the isolate is

reported as NG. The low sensitivity of certain Difco and Murex reagents used in this study was apparently due to inferior manufacturer lots, as retesting with newer lots of Difco MenB and MenY antisera produced 100% sensitivity with the 58 DR isolates (data not shown). The fact that Difco and Murex are the only two commercially available serogrouping products in the United States and are used at SHL suggests that poor reagent quality or lot-to-lot variability may have contributed to the overidentification of MenB and MenY as NG. This is in agreement with previous studies that reported variable sensitivity and specificity of commercially available serogrouping reagents (23). Overidentification of MenB, MenC, and MenY as NG at CDC was likely due to poor performance of antisera from other sources (FDA and U.S. Navy) and to human subjectivity in reading and interpreting SASG results.

As for any other diagnostic approach, several factors are crucial for obtaining reproducible and reliable results. For serogrouping of *N. meningitidis* isolates, we continue to support SHL use of SASG, with the following specific recommendations: (i) implementation and consistent use of quality assurance procedures and (ii) use of well-characterized control strains for testing of all new lots of diagnostic antisera. These recommendations reiterate those set forth by the Clinical Laboratory Improvement Amendment of 1988 (regulations part 493, section 1261), which specifically require the use of positive and negative reaction controls every time a new reagent batch is prepared in-house, when a new shipment and/or lot number of commercially available reagent is opened, and every 6 months thereafter.

According to the criteria established for this study, 13 of the 58 DR isolates (22%) remained unresolved. For 10 of the 13 isolates, SGS-PCR did not agree with the blinded study result but did agree with either SHL or CDC. Further sequencing analysis of the capsule biosynthesis genes is under way in an effort to determine the genetic capsule types of these isolates.

Of the 12 CR-NG isolates, 10 were positive in SGS-PCR, but 9 of these 10 were consistently NG in SASG (NG at SHL, CDC, and by the blinded study). We postulate that for these nine isolates, the absence of capsule is most likely due to a specific genetic event such as one of those described in two recent publications on *N. meningitidis* carriage isolates (7, 19). These investigations showed that phase variation such as slipped-strand mispairing, presence of an insertion element in a capsule gene, or deletion of part of the capsule region was responsible for the lack of capsule expression in these NG carriage isolates. In our study, the nine CR-NG isolates were positive by SGS-PCR; however, positive SGS-PCR results do not necessarily indicate the presence of the entire gene or prove that the gene is functional and expressed.

The high efficacy of SGS-PCR in resolving DR has prompted the initiation of a prospective study of all *N. meningitidis* isolates collected through ABCs. As of 1 July 2003, in addition to standard serogroup identification procedures, all isolates are tested by SGS-PCR at CDC. This allows for continuous monitoring of DR results and further elucidation of underlying genetic and procedural causes.

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