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NOTES

Simultaneous Approach for Nonculture PCR-Based Identification and Serogroup Prediction of *Neisseria meningitidis*

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A nonculture PCR-based method to characterize *Neisseria meningitidis* was used to test 225 clinical specimens. PCR correctly identified and predicted the serogroups of *N. meningitidis* of culture-proven meningococcal diseases and confirmed this diagnosis in 35% of suspected samples. This approach could be useful when culture fails to isolate *N. meningitidis*.

Meningococcal infections (septicemia and meningitis) are usually suspected on the basis of clinical symptoms such as fever, vomiting, neck stiffness, and skin rash (purpura). An etiologic diagnosis is confirmed by the isolation of *Neisseria meningitidis* from cerebrospinal fluid (CSF), blood, or other body fluids. However, this diagnosis is hindered by the failure to isolate bacteria following early treatment (3). Prophylactic measures (vaccination and/or chemoprophylaxis) should be undertaken among contacts of the patient to prevent secondary cases. Vaccines are available against strains of *N. meningitidis* belonging to serogroups A, C, Y, and W135 but not against strains of serogroup B. Hence, serogrouping is necessary to better apply preventive measures. The aim of this prospective study was to establish and to assess a nonculture approach for identification and characterization of *N. meningitidis*.

Biological samples ($n = 225$) were obtained from 150 different patients who were admitted to several hospitals ($n = 25$) in France due to suspected meningococcal infection (Table 1). The hospitals enrolled in this study all have pediatric departments. Patient clinical histories included two or more of the following symptoms and signs: fever, meningism, purpura, and arthralgia. Patients were classified into five categories on the basis of laboratory findings. In patient category 1 ($n = 33$), culture was positive for *N. meningitidis* in CSF, blood, or other body fluids (culture-proven meningococcal infection). *N. meningitidis* was identified and systematically verified using the Api-NH system (Biomérieux). Serogrouping for all strains isolated was determined using group-specific immune sera (Sanofi Diagnostics Pasteur). In patient category 2 ($n = 20$), culture was negative but direct smear and/or antigen detection for *N. meningitidis* was positive (suspected meningococcal disease). Antigen detection was performed using the Pastorex latex agglutination kit (Sanofi Diagnostics Pasteur). In patient category 3 ($n = 147$), culture was negative. Direct smear and antigen detection were negative or not done. However, the CSF was abnormal, with a high cell count (>100 cells/μl of CSF), decreased glucose content, or increased protein (possible meningococcal disease). In patient category 4 ($n = 10$), culture was positive for other bacterial species (other known bacterial meningitis). The diagnoses included pneumococcal meningitis ($n = 1$), staphylococcal meningitis ($n = 4$), *Listeria monocytogenes* meningitis ($n = 2$), *N. sicca* meningitis ($n = 1$), *Bacteroides* meningitis ($n = 1$), and *Mycobacterium tuberculosis* meningitis ($n = 1$). In patient category 5 ($n = 15$), culture, direct smear, and antigen detection were negative, with a low leukocyte count (<100 cells/μl) and normal glucose and protein in the CSF (clinically diagnosed viral meningitis).

To identify *N. meningitidis*, regardless of its serogroup, a PCR screen was designed that amplified a conserved regulatory gene in *N. meningitidis*, *crgA*, which was recently identified in this laboratory. This gene is involved in the regulation of adhesion of *N. meningitidis* to target cells (A. Deghmane, S. Petit, A. Topilko, Y. Pereira, D. Giorgini, M. Larribe, and M.-K. Taha, unpublished data). For serogroup prediction (A, B, C, Y, and W135), a multiplex PCR was performed simultaneously with oligonucleotides in the *siaD* gene (serogroups B, C, Y, and W135) (4) and in *orf-2* of a gene cassette required for the biosynthesis of the capsule of serogroup A (13) (Table 2). The sizes of the expected amplicons from this multiplex PCR are 450 bp (serogroup B), 400 bp (serogroup A), 250 bp (serogroup C), and 120 bp (serogroups Y and W135) (Fig. 1). A PCR for each serogroup was further realized to confirm the result and to discriminate serogroups W135 and Y, for which the amplicon size was 120 bp (Table 2). Oligonucleotides used for the prediction of serogroups were first tested on a collection of meningococcal strains of known serogroups (15 strains of each serogroup were tested). These oligonucleotides were specific for the corresponding serogroup, and no cross-reactivity was observed between meningococcal strains of different serogroups or with strains belonging to other bacterial species (7 strains of *N. gonorrhoeae*, 2 strains of *L. monocytogenes*, 2 strains of *Streptococcus pneumoniae*, and 1 strain of *N. lactamica*) (data not shown). Samples were subjected to one freeze-thaw cycle, heated at 100°C for 3 min, and then centrifuged for 5 min at 10,000 × *g*. In each assay, the final 50-μl reaction mixture contained 15 μl of each sample, 60 mM Tris-HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate, the corresponding oligonucleotides (Table 2) at 0.3 μM, and 1 U of *Taq* polymerase (Promega). The PCR assays were performed in a DNA thermal cycler (Techne) with the following parameters: a first cycle of

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TABLE 1. Categories of patients, samples tested in this study, and PCR results

Category (no. of samples)	No. of patients	Sample source(s) (no. of samples)[a]	Culture		Direct smear			Antigen detection			PCR		No. of samples (patients)	
			−	+	−	+	ND[b]	−	+	ND	−	+	Positive by PCR	Negative by PCR
1 (33)	27	CSF (22)		22	9	8	5	4	8	10	1	21	32 (26)	1 (1)
		Serum (7)		7			7	1	2	4		7		
		PF (3)		3	1	1	1	1	2			3		
		AF (1)		1			1			1		1		
2 (20)	20	CSF (13)	13		1	12			13		1	12	17 (17)	3 (3)
		Serum (7)	7				7		7		2	5		
3 (147)	78	CSF (78)	78		31		47	17		61	48	30	51 (35)	96 (43)
		Serum (69)	69		20		49	15		54	48	21		
4 (10)	10	CSF (10)		10[c]			10			10	10			10 (10)
5 (15)	15	CSF (15)	15		15			15			14	1	1 (1)	14 (14)
Total	150	225	182	33[d], 10[c]	77	21	127	53	32	140	124	101	101 (79)	124 (71)

[a] Samples tested were CSF (n = 138), serum (n = 83), pericardial fluid (PF; n = 3), and articular fluid (AF; n = 1).
[b] ND, not determined.
[c] These samples were positive by culture for other bacterial species.
[d] These samples were positive by culture for *N. meningitidis*.

denaturation at 94°C for 3 min, annealing at 55°C for 30 s, and polymerization at 72°C for 20 s. The subsequent 35 cycles were as follows: 92°C for 40 s, annealing at 55°C for 30 s, and polymerization at 72°C for 20 s. A final cycle of polymerization at 72°C for 10 min was then performed. Amplicons were analyzed by electrophoresis on a standard 2% agarose gel. In order to test whether PCR was feasible for a given sample, a separate PCR was performed after the addition of 3 μl of a suspension of *N. meningitidis* with a known concentration.

PCR correctly identified and predicted the serogroups of *N. meningitidis* in 32 of 33 samples that were also positive for this bacterium by culture (Table 1). The failure of PCR in one culture-proven case (CSF but no serum was available) could be due to the presence of a substance inhibitory for PCR and/or to the small amount of meningococci in that specific CSF sample. Indeed, PCR was less efficient with this specimen when external meningococci were added (data not shown). Moreover, this specimen was from a 2-year-old child who died of meningococcal septicemia. Only 1 CFU was isolated from this sample (200 μl). To circumvent such a problem, both CSF and serum samples should be tested by PCR.

Specimens of category 2 that were positive by both direct observation and antigen detection were also positive by PCR (Table 1). Serogroup prediction by PCR matched that obtained by antigen assays. Three specimens were only positive by antigen detection for serogroup B and were negative by PCR (Table 1). It is noteworthy that cross-reactions between *N. meningitidis* and several other bacterial species were reported in antigen detection assays, in particular with serogroup B (7).

All samples obtained from other cases of nonmeningococcal bacterial meningitis (n = 10) were negative by PCR for the presence of meningococcal DNA. Samples from patients in category 5 (clinically diagnosed viral meningitis) were all neg-

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence	Gene amplified (serogroup)	Amplicon length (bp)
98-6	5′-gctggcgccgctggcaacaaaattc-3′	*crgA*	230
98-10	5′-cttctgcagattgcggcgtgccgt-3′		
98-28	5′-cgcaataggtgtatatattcttcc-3′	*orf-2* (A)	400
98-29	5′-cgtaatagtttcgtatgccttctt-3′		
98-19	5′-ggatcatttcagtgttttccacca-3′	*siaD* (B)	450
98-20	5′-gcatgctggaggaataagcattaa-3′		
98-17	5′-tcaaatgagtttgcgaatagaaggt-3′	*siaD* (C)	250
98-18	5′-caatcacgatttgcccaattgac-3′		
98-32	5′-cagaaagtgagggatttccata-3′	*siaD* (W135)	120
98-33	5′-cacaaccattttcattatagttactgt-3′		
98-34	5′-ctcaaagcgaaggctttggtta-3′	*siaD* (Y)	120
98-35	5′-ctgaagcgttttcattataattgctaa-3′		

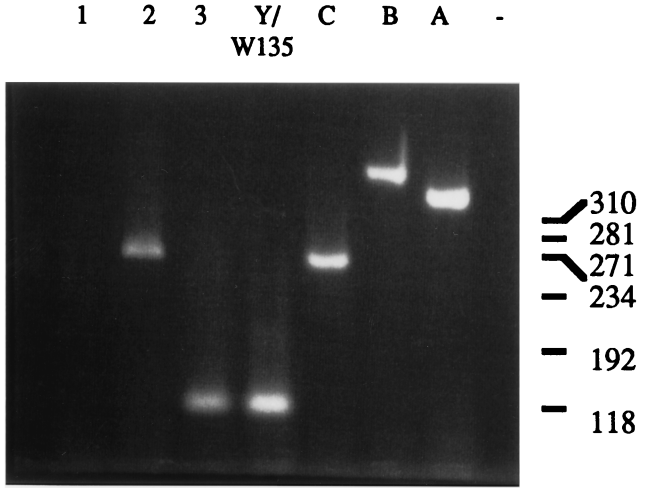

FIG. 1. PCR amplification of the *siaD* (serogroups B, C, and Y and W135) and *orf-2* (serogroup A) genes from strains belonging to serogroups A (strain LNP10824), B (strain LNP10846), C (strain LNP13331), and Y and W135 (strains LNP13145 and LNP13230, respectively) and from three clinical specimens (lanes 1, 2, and 3). The strains used were previously described (6). Lane − is the negative control (no bacteria). Electrophoresis was done on a 2% agarose gel. Size markers (bacteriophage ϕX174 digested by *Hae*III) are indicated in base pairs at the right.

TABLE 3. Two-way table for calculation of efficiency values[a]

PCR result	No. of samples that were culture or direct smear and/or antigen detection	
	Positive	Negative
Positive	49 (*a*)	1 (*b*)
Negative	4 (*c*)	24 (*d*)

[a] Sensitivity = $a/(a + c) = 0.93$, specificity = $d/(b + d) = 0.96$, positive predictive value = $a/(a + b) = 0.98$, negative predictive value = $d/(c + d) = 0.86$.

ative by PCR, except for one case (Table 1). The false-positive sample was CSF from a 5-year-old child who was hospitalized and treated for meningococcal meningitis. However, viral meningitis (category 5) was later kept as a final diagnosis by clinicians, as CSF protein and glucose were normal. The leukocyte count in the CSF was 28 cells/μl (63% polymorphonuclear cells). Several cases of culture-proven meningococcal disease reported in this study had similar CSF findings.

Sensitivity (93%), specificity (96%), positive predictive value (98%), and negative predictive value (86%) were calculated using a two-way table (Table 3) (14). Positive cases (culture or direct smear and/or antigen detection) corresponded to categories 1 ($n = 33$) and 2 ($n = 20$). Negative cases, which represent a rather small number, corresponded to categories 4 ($n = 10$) and 5 ($n = 15$) (Tables 1 and 3).

For possible meningococcal diseases (category 3), 51 (35%) specimens were confirmed by PCR. These cases were undiagnosed by currently used methods. This percentage is comparable to that (31%) obtained by Newcombe et al. using PCR in suspected cases of meningococcal disease (12).

Other methods for nonculture diagnosis of *N. meningitidis* have been recently reported. They are based on the amplification of the 16S rRNA gene, IS*1106*, or the *porB* gene (8, 10, 12, 15). However, these methods usually identify the presence of *N. meningitidis* without prediction of the serogroup or only predict two serogroups (1). To the best of my knowledge, no method is available for the prediction of serogroup A, which is the serogroup encountered in the major epidemics in Africa. Moreover, false-positive diagnosis of meningococcal infection by the IS*1106* PCR has been reported (2). The amplification of two different meningococcal genes, as reported here, should limit such a false-positive response. The identification and the prediction of serogroups by this method can be performed rapidly. Subsequently, it could be combined with our previously described nonculture *N. meningitidis* typing method, which is based on analysis of the polymorphism of a meningococcal gene (*pilA*) (5). The distribution of *pilA* alleles among different strains was previously shown to correlate well with the typing obtained with the reference typing method, multilocus enzyme electrophoresis (6, 9, 11). When this technique (the polymorphism of *pilA*) was applied to samples which were positive by PCR, different alleles of *pilA* were observed, indicating different genetic lineages among the strains incriminated. This result is in agreement with an endemic situation of meningococcal disease in France. PCR assays should not be developed as an alternative to culture. The development of specific and sensitive PCR methods represents an efficient approach in the surveillance of meningococcal disease when culture fails to isolate *N. meningitidis*.

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