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Use of a New Single Multiplex PCR-Based Assay for Direct Simultaneous Characterization of Six *Neisseria meningitidis* Serogroups▽

Christophe Fraisier, Richard Stor, Bernard Tenebray, Yannick Sanson, and Pierre Nicolas*

Unité du Méningocoque, WHO Collaborating Centre, IRBA, Institut de Médecine Tropicale du Service de Santé des Armées, Allée du Médecin Colonel Eugène Jamot, Parc du Pharo, BP 60109, 13262 Marseille Cedex 07, France

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We developed a new *Neisseria meningitidis* multiplex PCR to determine six serogroups, including X-specific primers, and to allow direct W135/Y discrimination. This assay offers a simple and low-cost method for serogrouping *N. meningitidis* from cerebrospinal fluid that could be useful in Africa.

Meningitis is one of the various forms of meningococcal diseases, which may occur as epidemics and severe sepsis, often with a fatal outcome (4). An etiologic diagnosis is confirmed by the isolation of *Neisseria meningitidis* from cerebrospinal fluid (CSF), blood, or other body fluids. Characterization of meningococci by serogroup is compulsory to investigate disease outbreaks and before starting vaccination campaigns. Twelve meningococcal serogroups identified by the capsular polysaccharide antigens are distinguished and chemically defined. Patient strains are nearly always encapsulated, but only six of these groups (A, B, C, W135, Y, and more recently X) (3) have significant pathogenic potential, causing more than 90% of the invasive disease worldwide (12).

Vaccines are available against strains of *N. meningitidis* belonging to serogroups A, C, W135, and Y but not against strains of serogroups B or X. Hence, serogrouping is really necessary to better apply preventive measures (14). Recent outbreaks due to serogroup X have been described in Niger and Ghana (3, 5, 8); thus, the detection of serogroup X becomes essential in African countries and a low-cost assay needs to be developed.

Standard PCR-based assays are routinely used for the detection and identification of serogroups A, B, C, W135, and Y. Simultaneous identification of serogroups A, B, C, W135, and Y has been described in one previous report (14) but a subsequent PCR was necessary to distinguish between W135 and Y. Recently, a separate standard multiplex PCR has also been described for the detection of serogroups 29E, X, and Z (1). Alternatively, a real-time quantitative PCR analysis has also been developed for simultaneous serogroup determination (10), but such an assay might be too expensive for African laboratories.

Here we report for the first time a standard multiplex PCR-based assay for rapid simultaneous identification of the six serogroups A, B, C, W135, Y, and X, with direct discrimination between W135 and Y. We used oligonucleotides in *orf-2* of a gene cassette required for the biosynthesis of the capsule of serogroup A (13) in the *synD* and *synE* genes encoding the polysialyltransferase responsible for the polymerization of polymers of sialic acid containing polysaccharides in strains belonging to serogroups B and C, respectively, and in the *synF* and *synG* genes, encoding proteins that catalyze the linkage of sialic acid with other sugars in the polysaccharide chains of serogroups Y and W135, respectively (6, 10); these primers were those described by Taha (14). The sizes of the expected amplicons are 450 bp (B), 400 bp (A), 250 bp (C), and 120 bp (W135). For direct identification of serogroup Y, the reverse primer in the *synF* gene was identical to that previously described (14), but a new forward primer in the *synF* gene was designed to amplify a Y-specific 75-bp product, distinct from the 120-bp W135-specific amplicon. The 3′ end of the *crtA* gene, which encodes an outer membrane protein involved in capsule transport, is highly conserved among meningococci irrespective of serogroups, but the 5′ end is very variable (7). Both forward and reverse primers specific to serogroup X were then designed within this variable region, giving rise to a serogroup X-specific 190-bp amplification product (Table 1).

The aim of this study was to assess this multiplex PCR assay using DNA extracted either directly from CSF samples (n = 13) or from CSF-isolated meningococcal strains (n = 32). Some strains were also obtained from blood (n = 2), expectoration (n = 1), or pharyngeal carriage (n = 1). The biological source of one sample was not provided. A previous serogroup determination had been performed by agglutination tests (Pastorex and/or Difco) or previous PCR analyses as follows: A (n = 9), B (n = 8), C (n = 4), X (n = 11), W135 (n = 12), and Y (n = 7). Strains belonging to other bacterial species responsible for meningitis, *Haemophilus influenzae* (n = 5) or *Streptococcus pneumoniae* (n = 5), were used as negative controls. *Neisseria meningitidis* samples are listed in Table 2, also indicating the origin (country, source) and the genotype determined by MLST analysis (sequence type [ST]). Strains belonging to different STs within one serogroup were chosen when data were available.

Bacterial DNA was isolated using the High Pure PCR template kit (Roche), by following the manufacturer's instructions, directly from CSF samples or from colonies isolated on blood-agar plates. For serogrouping, the amplification reaction was

* Corresponding author. Mailing address: Unité du Méningocoque, IMTSSA, Allée du Médecin Colonel Eugène Jamot, Parc du Pharo, BP 60109, 13262 Marseille Cedex 07, France. Phone: 33-4-91-150-115. Fax: 33-4-91-59-44-77. E-mail: meningo@imtssa.fr.

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)	Gene amplified (serogroup)	Amplicon length (bp)	Source or reference
98-28	GCAATAGGTGTATATATTCTTCC	*orf-2* (A)	400	14
98-29	CGTAATAGTTTCGTATGCCTTCTT	*orf-2* (A)	400	14
98-19	GGATCATTTCAGTGTTTTCCACCA	*synB* (B)	450	14
98-20	GCATGCTGGAGGAATAAGCATTAA	*synB* (B)	450	14
98-17	TCAAATGAGTTTGCGAATAGAAGGT	*synE* (C)	250	14
98-18	CAATCACGATTTGCCCAATTGAC	*synE* (C)	250	14
98-32	CAGAAAGTGAGGGATTTCCATA	*synG* (W135)	120	14
98-33	CACAACCATTTTCATTATAGTTACTGT	*synG* (W135)	120	14
98-36	ACGATATCCCTATCCTTGCCTA	*synF* (Y)	75	This study
98-35	CTGAAGCGTTTTCATTATAATTGCTAA	*synF* (Y)	75	14
XF	AATGCAAATTCAATTGGTTG	*ctrA* (X)	190	This study
XR	CTTGGGCCTTATACAAAGAC	*ctrA* (X)	190	This study

performed on 50 ng DNA using primers specific to groups A, B, C, W135, Y, and X (Table 2). The reaction was performed in a 25-μl final volume comprising 1 U GoldStar Red DNA polymerase (Eurogentec), 1× polymerase buffer, 300 nM of each primer, 200 nM of each deoxynucleoside triphosphate (Roche), and 2.5 mM $MgCl_2$. The PCR cycles were as follows: denaturation at 94°C for 5 min; amplification at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s for 35 cycles; and 72°C for 10 min. Amplification products were visualized on a 4% agarose gel stained with ethidium bromide so that those with small sizes could be easily separated.

The multiplex PCR described here perfectly determined the serogroup of *N. meningitidis* in all the samples analyzed (Fig. 1). No amplification could be observed with the other bacterial species used in this study. Each sample showed one single specific amplification product, which makes the results of our PCR easy to read, compared to the recent multiplex PCR reported by Bennett et al. (2) showing multiple bands.

We particularly demonstrate that this multiplex PCR allows (i) the direct distinct discrimination between serogroups W135 and Y, using the new forward Y-specific primer in the *synF* gene, and (ii) the specific identification of serogroup X strains with new primers in the 5′ end of the *ctrA* gene.

Although this PCR included oligonucleotides specific to serogroups B and C, the goal of this study was particularly to develop a rapid, simple, and low-cost standard multiplex PCR that could be easily used in African countries for the determination of the main serogroups encountered in the meningitis belt. Thus, this PCR was particularly designed to directly distinguish between W135 and Y serogroups so that it would be more convenient. Comparison of the nucleotide sequences of the *synG* and *synF* genes of W135 and Y strains, respectively (GenBank accession numbers AY234196 and AY234201), shows that the only variable region is located within the 120-bp sequence corresponding to the W135/Y amplicon obtained with the primers described by Taha (14). Since these specific primers show only about a 30% difference, we looked within this 120-bp region to find a possible oligonucleotide that could be used as another W135- or Y-specific primer, giving rise to an amplicon of a smaller size. We then designed the new Y-specific 5′ primer, 98-36, leading to the amplification of a 75-bp product, in combination with the 3′ Y-specific primer, 98-35. Figure 1 shows that W135- and Y-specific amplicons can be easily discriminated on a 4% agarose gel. The nucleotide sequence of this new forward Y-specific primer showed only 26% difference from the corresponding W135 sequence; it had been first tested in single PCR analyses associated with either the reverse Y primer, 98-35, or the reverse W135 primer, 98-33, and was shown to be effectively Y specific (not shown).

Moreover, due to the recent emergence of meningococcus X outbreaks (3, 5, 8), we also focused on the use of a multiplex PCR assay that could be able to identify serogroup X strains in biological samples. As already mentioned above, the aim was to develop this multiplex PCR for African countries, meaning that it needs to be simple, rapid, and low cost. It was then necessary to also include X-specific primers. Primers previously described (1) were shown to work at 62.5°C, generating a 525-bp amplicon, but could also amplify a 650-bp product for some serogroup A strains, albeit different from the X-specific product and from the 400-bp A-specific amplicon obtained with the *orf-2* primers (Table 1). Since our PCR was based on an annealing temperature of 55°C, we designed new forward and reverse X-specific primers using the Primer3 v0.4.0 program (http://frodo.wi.mit.edu/) so that they amplify a product with a distinguishable size at 55°C. The two oligonucleotides, XF and XR, were chosen within the 5′ end of the *ctrA* gene (GenBank accession number AY289931), which was reported to be very variable and group specific (7), and generated a 190-bp amplicon. We demonstrate here that these two oligonucleotides amplified a serogroup X-specific product (Fig. 1) and that primers can be chosen within the 5′ variable *crtA* region for serogroup determination.

As shown in Table 2, the identification of the serogroup with our PCR is in agreement with the previous serogroup determination obtained by agglutination assays. Interestingly, the multiplex PCR allowed the serogroup determination of two samples, 2008-183 and 2007-443, which presented a polyagglutination status. Thus, it confirms that serogrouping by PCR could be helpful when agglutination tests fail or when culture is negative or not available. Indeed, we show that the multiplex PCR worked perfectly on DNA extracted directly from the CSF samples ($n = 13$) included in our study (Table 2), particularly those from Togo collected in 2008 (serogroups X and W135). These samples were indeed also part of another recent study in which serogroup X *Neisseria meningitidis* (ST 181) was reported to cause substantial disease in central Togo (on the border of the meningitis belt) during 2007 and 2008 (11), suggesting that the earlier serogroup X *N. meningitidis* out-

TABLE 2. Samples tested in this study and PCR results[d]

Sample	Serogroup[a]	Sample source	Culture[b]	Country of origin	Sequence type	Multiplex PCR[c]
2008-183	PA	Carriage	Yes	Niger	ND	A
2008-185	A	CSF	Yes	Burkina Faso	2859	A
2008-196	A	CSF	Yes	Burkina Faso	2859	A
2008-197	A	CSF	Yes	Burkina Faso	2859	A
2008-225	A	CSF	Yes	Burkina Faso	2859	A
2008-226	A	CSF	Yes	Burkina Faso	2859	A
2008-227	A	CSF	Yes	Burkina Faso	2859	A
2007-155	A	CSF	No	Niger	7	A
2007-158	A	CSF	No	Niger	7	A
VN73	B	CSF	Yes	Vietnam	ND	B
2008-048	B	CSF	Yes	Vietnam	6985	B
2008-049	B	CSF	Yes	Vietnam	6985	B
2008-308	B	CSF + blood	Yes	France	33	B
2008-309	B	NA	Yes	Vietnam	1576	B
2008-315	B	Blood	Yes	France	162	B
2007-053	B	CSF	Yes	France	269	B
2007-055	B	CSF	Yes	France	1403	B
2008-219	C	CSF	Yes	France	6969	C
2007-047	C	CSF + blood	Yes	France	11	C
2007-054	C	Blood	Yes	France	6347	C
2004-178	C	CSF	Yes	France	3747	C
2008-223	X	CSF	Yes	Burkina Faso	181	X
2008-267	X	CSF	No	Togo	181	X
2008-269	X	CSF	No	Togo	181	X
2008-270	X	CSF	No	Togo	181	X
2008-271	X	CSF	No	Togo	181	X
2007-114	X	CSF	No	Niger	181	X
2007-151	X	CSF	No	Niger	5789	X
2007-340	X	CSF	Yes	Burkina Faso	181	X
2006-078	X	CSF	Yes	Niger	181	X
2006-087	X	CSF	Yes	Niger	5789	X
2008-109	W135	CSF	Yes	Benin	2881	W135
2008-218	W135	CSF	Yes	Burkina Faso	2881	W135
2008-278	W135	CSF	No	Togo	ND	W135
2008-279	W135	CSF	No	Togo	ND	W135
2008-280	W135	CSF	No	Togo	ND	W135
2007-105	W135	CSF	No	Chad	11	W135
2007-107	W135	CSF	Yes	Benin	2881	W135
2007-127	W135	CSF	No	Niger	2881	W135
2007-166	W135	CSF	Yes	Chad	2881	W135
2007-448	W135	CSF	Yes	Togo	2881	W135
2007-449	W135	CSF	Yes	Togo	2881	W135
2007-457	W135	CSF	Yes	Togo	2881	W135
2004-266	W135	CSF	Yes	Burkina Faso	ND	W135
2008-235	Y	CSF	Yes	Burkina Faso	4375	Y
2007-111	Y	Blood	Yes	France	4171	Y
2007-188	Y	CSF	Yes	Burkina Faso	2880	Y
2007-443	PA	Expectoration	Yes	France	167	Y
2006-092	Y	CSF	Yes	Niger	4375	Y
2004-035	Y	CSF	Yes	Benin	767	Y
2004-263	Y	CSF	Yes	Burkina Faso	ND	Y

[a] Serogroup determined by agglutination tests or previous PCR analysis.
[b] DNA extracted with (yes) or without (no) culture.
[c] Serogroup determined by multiplex PCR (this study).
[d] PA, polyagglutination; NA, not available; ND, not determined.

breaks in Niger (3) were not localized events but had expanded to surrounding areas. The recent report on the high prevalence of serogroup X carriage among children in northern Ghana also strongly suggests the need for surveillance of this potentially epidemic variant (8). Additionally, serogroup X *N. meningitidis* has also recently been reported as being involved in an outbreak of meningococcal disease in western Kenya, outside the meningitidis belt, highlighting the presence of serogroup X in East Africa (9). The emergence of serogroup X meningococcal clones in the various countries that make up the African meningitidis belt might lead to a potential epidemic in place of or in addition to serogroup A epidemics, as it was seen with the expansion of serogroup W135 (15). Thus, the identification of serogroup X strains, in addition to the other serogroups, is of particular importance in African countries, pointing out the need for efficient diagnosis tools to detect the causative agent of acute bacterial meningitis in order to adapt the most appropriate treatment or preventive strategies. The multiplex PCR

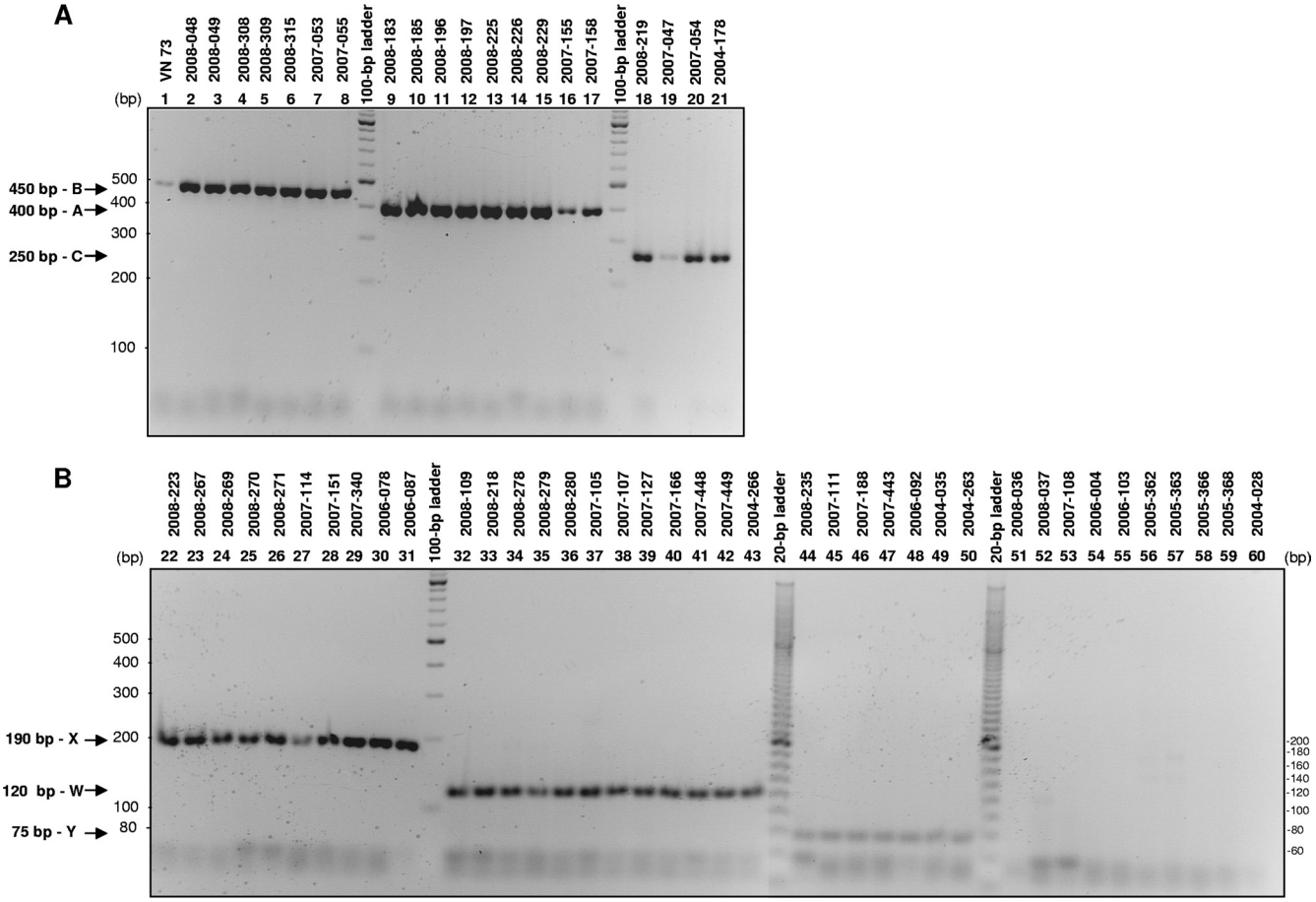

FIG. 1. Multiplex PCR amplification of the *siaD*/*synB* and *siaD*/*synE* (serogroups B and C), *synG* and *synF* (serogroups W135 and Y), *orf-2* (serogroup A), and *ctrA* (serogroup X) genes from samples belonging to serogroup A (lanes 1 to 8), B (lanes 9 to 17), C (lanes 18 to 21) (A) and X (lanes 22 to 31) and W135 (lanes 32 to 43) and Y (lanes 44 to 50) (B). DNA from other species, *Haemophilus influenzae* (lanes 51, 52, 54, 55, 57) and *Streptococcus pneumoniae* (lanes 53, 56, 58, 59, and 60), were used as negative controls. PCR products were separated on a 4% agarose gel. Molecular sizes are indicated.

reported here could be such an helpful tool for laboratories in Africa, allowing low-cost, rapid, simple, and efficient prediction of the six main serogroups; if necessary, this PCR might be possibly more adapted to Africa by using only primers specific to serogroups A, W135, X, and Y, which are the four serotypes encountered most frequently in this part of the world.

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