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# Development of a Multiplex PCR Assay for Detection and Genogrouping of *Neisseria meningitidis*

**Hongfei Zhu,[a,b] Quan Wang,[a,b,c] Liuqing Wen,[a,c] Jianguo Xu,[d] Zhujun Shao,[d] Min Chen,[e] Mingliang Chen,[e] Peter R. Reeves,[f] Boyang Cao,[a,g] and Lei Wang[a,g,h]**

TEDA School of Biological Sciences and Biotechnology, Nankai University, TEDA, Tianjin, China[a]; Key Laboratory of Molecular Microbiology and Technology, Ministry of Education, Tianjin, China[b]; The Engineering and Research Center for Microbial Functional Genomics and Detection Technology, Ministry of Education, Tianjin, China[c]; National Institute for Communicable Disease Control and Prevention and State Key Laboratory for Infectious Disease Prevention and Control, Chinese Center for Disease Control and Prevention, Beijing, China[d]; Center for Disease Control and Prevention, Shanghai, China[e]; School of Molecular and Microbial Biosciences (G08), University of Sydney, Sydney, Australia[f]; Tianjin Key Laboratory of Microbial Functional Genomics, Tianjin, China[g]; and Tianjin Research Center for Functional Genomics and Biochip, Tianjin, China[h]

***Neisseria meningitidis*** **is a leading pathogen of epidemic bacterial meningitis and fulminant sepsis worldwide. Twelve different *N. meningitidis* serogroups have been identified to date based on antigenic differences in the capsular polysaccharide. However, more than 90% of human cases of *N. meningitidis* meningitis are the result of infection with just five serogroups, A, B, C, W135, and Y. Efficient methods of detection and genogrouping of *N. meningitidis* isolates are needed, therefore, in order to monitor prevalent serogroups as a means of disease control and prevention. The capsular gene complex regions have been sequenced from only seven out of the 12 serogroups. In this study, the capsular gene complexes of the remaining five serogroups were sequenced and analyzed. Primers were designed that were specific for *N. meningitidis* species and for the 12 individual serogroups, and a multiplex PCR assay using these specific primers was developed for *N. meningitidis* detection and genogrouping. The assay was tested using 15 reference strains covering all 12 serogroups, 143 clinical isolates, and 21 strains from closely related species or from species that cause meningitis. The assay could detect *N. meningitidis* serogroups and was shown to be specific, with a detection sensitivity of 1 ng of genomic DNA (equivalent to $\sim 4 \times 10^5$ genomes) or $3 \times 10^5$ CFU/ml in noncultured mock cerebrospinal fluid (CSF) specimens. This study, therefore, describes for the first time the development of a molecular protocol for the detection of all *N. meningitidis* serogroups. This multiplex PCR-based assay may have use for the clinical diagnosis and epidemiological surveillance of *N. meningitidis*.**

*Neisseria meningitidis* is an encapsulated Gram-negative bacterium and the leading pathogenic cause of epidemic bacterial meningitis and fulminant sepsis worldwide. Infections with *N. meningitidis* are a significant cause of mortality and morbidity in young children and adolescents (16, 23). The bacterial capsular polysaccharide (CPS) is the most important virulence factor for *N. meningitidis* (14, 20, 26, 28, 32). Twelve different serogroups, A, B, C, 29E, H, I, K, L, W135, X, Y, and Z, have been identified based on antigenic variation in the CPSs (6, 22). More than 90% of human cases of *N. meningitidis* meningitis are the result of infection with serogroups A, B, C, W135, and Y (21). Rare meningitis cases caused by serogroup K and Z infections have also been reported over the last few decades (11, 25). Detection and genogrouping of *N. meningitidis* isolates are important to monitor changes in the population of prevalent circulating serogroups for the purposes of disease control and prevention, vaccination strategy, and contact management (3, 12, 30).

Several genes, which include *ctrA*, *porA*, *crgA*, and 16S rRNA, have been widely used as the gene targets for *N. meningitidis* detection by PCR-based assays (7, 16, 33). In particular, genes such as *sacB* and *siaD* have been used to genogroup the most prevalent A, B, C, W135, and Y serogroups (3, 4, 10). However, to our knowledge, no molecular method that distinguishes all 12 *N. meningitidis* serogroups has ever been developed. The conventional serogrouping method that uses specific antisera is still in routine practice in many diagnostic laboratories; however, it is labor-intensive, and antisera for all serogroups are very hard to obtain. Therefore, developing a rapid, accurate, easy to use, and inexpensive method for the detection and genogrouping of *N. meningitidis* is in urgent need.

Variations in genes clustered in the chromosomal capsular gene complex are responsible for CPS differences in the *N. meningitidis* serogroups. A typical *N. meningitidis* capsular gene complex consists of regions A, B, C, D, and E. Regions A and C are located between the *galE* and *tex* genes on the chromosome and are transcribed independently (31). Region B is upstream of region A or downstream of region C (35). Genes in region A are responsible for the synthesis of the polysaccharide. Genes in region B, which include *lipA* and *lipB*, are involved in translocation and surface expression of capsule polymers. Region C contains four genes (*ctrA*, *-B*, *-C*, and *-D*) that are necessary for the transport of the capsule to the membrane (35). Region D is comprised of a cassette of genes, which include *rmlA*, *-B*, and *-C* and *galE*, and is not involved in capsule expression but is responsible for menin-

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Address correspondence to Lei Wang, wanglei@nankai.edu.cn, or Boyang Cao, boyangcao@nankai.edu.cn.
H. Zhu and Q. Wang contributed equally to this article.
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gococcal lipooligosaccharide (LOS) biosynthesis (13). Region E contains only one gene, *tex*, that has been proposed to regulate CPS synthesis (19). The gene sequences in region A of the complex are different in individual serogroups, and those in regions B, C, D, and E are highly conserved between serogroups (32).

Out of the 12 serogroups, only the seven capsular gene complexes from serogroups A, B, C, 29E, W135, X, and Y have been characterized previously (8, 27). In this study, the capsular gene complexes of the five uncharacterized serogroups (H, I, K, L, Z) were sequenced, which made it possible to develop primers to differentiate genes specific for all 12 *N. meningitidis* serogroups. A multiplex PCR-based assay for *N. meningitidis* detection and genogrouping was developed based on "screening out" specific genes and primers for each *N. meningitidis* serogroup. This assay was tested on 179 bacterial isolates and proved to be specific, accurate, and sensitive.

## MATERIALS AND METHODS

**Bacterial strains.** The 179 strains used in this study are listed in Table 1. They include 15 reference strains and 143 clinical strains of the 12 targeted *N. meningitidis* serogroups (A, B, C, 29E, H, I, K, L, W135, X, Y, and Z) and 15 strains of other *Neisseria* species, 5 strains of *Streptococcus pneumoniae*, and one strain of *Haemophilus influenzae*. *N. meningitidis* strains were grown on chocolate agar plates at 37°C in the presence of 5% $CO_2$ in air for 24 h.

**Genomic DNA extraction.** Genomic DNA was extracted with a DNA extraction kit (QIAamp DNA minikits; Qiagen, Hilden, Germany).

**Capsular gene complex amplification, sequencing, and analysis.** Primers wl-14395 (5′-CGCCATTTCTTCCGCCAACACCA-3′) and wl-22861 (5′-CCAGCCCGAAAGTAGCCGATGC-3′) were designed based on the sequences of *galE* and *gltS* genes, respectively, and were used to amplify the capsular gene complexes of serogroups D, H, and K. The primers wl-14396 (5′-GCGTTTGCGTGCAGCATCGACT-3′) and wl-27419 (5′-GCCGAGAACCTTAACTGGCATATTGTGAA-3′) were designed based on *tex* and *lipB* gene sequences, respectively, and were used to amplify the capsular gene complexes of serogroups I, L, and Z. Long, high-fidelity PCR was carried out under the following conditions: denaturation step at 95°C for 15 s, annealing step at 62°C for 30 s, and extension step at 68°C for 10 min for 32 cycles. The PCR products were gel purified on UNIQ-10 columns (Sangon, Shanghai, China), and the sample DNA was sheared at speed code 2 (20 cycles) to the desired fragment length of 2 to 3 kb by HydroShear fragmentation (GeneMachines, CA). The fragments were then cloned into the pUC18 vector after end-blunting reaction with $T_4$ polymerase and Klenow fragments (New England BioLabs, MA) to produce shotgun banks.

The samples were sequenced on an ABI 3730 automated DNA sequencer (Applied Biosystems, CA), and sequencing data were assembled by use of the Staden package software (29). The Artemis program (www.sanger.ac.uk) was used to identify open reading frames (ORFs) and annotations (24). BLAST and PSI-BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to search several databases (1), which included GenBank (www.ncbi.nlm.nih.gov/GenBank), the Clusters of Orthologous Groups (COG; www.ncbi.nlm.nih.gov/COG/), and Pfam (pfam.sanger.ac.uk) protein motif databases (2, 34).

**Primer design.** All the primers used in this study are listed in Table 2. The genes *porA* and *ctrA* were used as the gene targets for *N. meningitidis* species-specific primers. Sequences of *N. meningitidis porA* and *ctrA* genes were obtained from the GenBank database and aligned using ClustalX software (www.clustal.org). The universal primers were designed based on the most conserved regions of these two genes. Serogroup-specific primers were designed based on the *sacD* gene sequence for serogroup A, the *siaD* gene sequence for serogroups B and C, the *synG/synF* gene sequence for W135/Y, the *cap29EH* gene sequence for serogroup 29E, the *wnmB* gene sequence for serogroups I/K, the *capZC* gene sequence for serogroup Z, the *lcbB* gene sequence for serogroup L, the *xcbA* gene sequence for serogroup X, and the *wnmA* gene sequence for serogroup H. In addition, the primers used to differentiate serogroups I and K were based on the *lipA* gene sequence (sense primer) and *galE* gene sequence (antisense primer) of serogroup I. The primers that were used to differentiate serogroups W135 and Y were based on *synF and synG* gene sequences. At least two pairs of primers were designed for screening each serogroup.

**Specificity of primers.** Primers based on *porA* and *ctrA* gene sequences were used to amplify the DNA templates of *N. meningitidis* reference strains from the 12 serogroups, from *Neisseria flavescens*, *Neisseria subflava*, *Neisseria mucosa*, *Neisseria lactamica*, *Neisseria sicca*, *Neisseria gonorrhoeae* strains, and from *S. pneumoniae* and *H. influenzae*. The PCRs were carried out in a reaction mixture that contained 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 2.5 mM $MgCl_2$, 0.4 mM deoxy-

**TABLE 1** Bacterial strains used in this study[a]

| Serogroup, sample type, or species | Strain no.[b] (source of strain) | Total no. of strains |
|---|---|---|
| *Neisseria meningitidis* reference strain ($n = 15$) serogroup | | |
| A | 29010 (CMCC), 29019 (CMCC) | 2 |
| B | 29011 (CMCC), 29061 (CMCC) | 2 |
| C | 29012 (CMCC) | 1 |
| 29E | 29034 (CMCC) | 1 |
| H | 29031 (CMCC) | 1 |
| I | 29044 (CMCC) | 1 |
| K | 29047 (CMCC) | 1 |
| L | 43828 (ATCC) | 1 |
| W135 | 29037 (CMCC), 29057 (CMCC) | 2 |
| X | M8210 (ICDC) | 1 |
| Y | 29038 (CMCC) | 1 |
| Z | 35562 (ATCC) | 1 |
| *N. meningitidis* clinical isolate ($n = 143$) sample type | | |
| CSF | 23 (ICDC), 2 (SCDC) | 25 |
| Blood | 4 (ICDC) | 4 |
| TS | 61 (ICDC), 6 (SCDC) | 67 |
| CP | 30 (SCDC) | 30 |
| BP | 4 (SCDC) | 4 |
| Unknown | 9 (ICDC), 4 (SCDC) | 13 |
| Strains from other species ($n = 21$) | | |
| *Neisseria lactamica* | 3719 (CCM), 29114 (CMCC) | 2 |
| *Neisseria flavescens* | 2827 (CCM) | 1 |
| *Neisseria subflava* | 3482 (CCM), 4392 (CCM), 4400 (CCM), 29109 (CMCC), 29110 (CMCC), 29111 (CMCC), 29121 (CMCC) | 7 |
| *Neisseria mucosa* | 3483 (CCM) | 1 |
| *Neisseria sicca* | 4404 (CCM), 4405 (CCM) | 2 |
| *Neisseria gonorrhoeae* | No. 1 (DDTGH), no. 2 (DDTGH) | 2 |
| *Streptococcus pneumoniae* 6A | G1839 (NU) | 1 |
| *Streptococcus pneumoniae* 1 | G1863 (NU) | 1 |
| *Streptococcus pneumoniae* 19A | G1921 (NU) | 1 |
| *Streptococcus pneumoniae* 23F | G1934 (NU) | 1 |
| *Streptococcus pneumoniae* 6B | G1940 (NU) | 1 |
| *Haemophilus influenzae* | 58528 (CMCC) | 1 |

[a] CMCC, China's Medical Culture Center; ATCC, American Type Culture Collection; ICDC, Chinese Center for Disease Control and Prevention, Beijing, China; SCDC, Shanghai Center for Disease Control and Prevention; CCM, Czech Collection of Microorganisms; DDTGH, Dermatological Department of Tianjin General Hospital; NU, Nankai Univeristy, China; CSF, cerebrospinal fluid; CP, chocolate agar plate; BP, blood agar plate; TS, throat swab.

[b] For *N. meningitidis* clinical isolate sample types, the number of strains is given instead of strain number.

**TABLE 2** Primers used in this study

| Primer pair | Targeted gene | GenBank accession no. | Amplicon size (bp) | Targeted serogroup | Sequence of primer pairs (5′→3′) |
|---|---|---|---|---|---|
| wl-35663/38969 | *ctrA* | GU391296 | 257 | All | GTCGCGGTGATGTGGTTA/AATCTCTGCCTCACTGCCAT |
| wl-40218/40170 | *porA* | AY319969 | 158 | All | CTCATAGCCGCCCGTCA/GCGGTTTTGCCGGGAACTAT |
| wl-37416/37417 | *sacD* | NC_003116 | 470 | A | TTTTATTCTTAGATGTTGACGTTTT/ATGCCAGAAATGTTT AGGAGTT |
| wl-37410/37411 | *siaD* | NC_003112 | 555 | B | TTTTTAGCATATTCAGGAAAGG/TTCAATGTGGTTGACAAC ATCT |
| wl-37469/37470 | *siaD* | NC_008767 | 381 | C | TGTGCTAATCCCGCCTGA/AGAAAGCCGGGAATCGTT |
| wl-39429/39430 | *cap29EH* | AJ576117 | 694 | 29E | TTGGCGGTTGAAACCTTAC/GCGTATCATGCTCCATTACCA |
| wl-39433/39434 | *wnmB* | HQ437687 | 893 | I/K | AATGTGTGTGCAGGAGCTTG/ATGCTTCGGTTGCCTGTC |
| wl-37709/37710 | *capZC* | HQ437689 | 656 | Z | TATTGGCCTGAGCACCG/AGGCGTACCGTCTGTAACTG |
| wl-37406/37407 | *lcbB* | HQ437688 | 988 | L | TTTGAATGTACCCTCTCCTCTG/TAACAGTCCTGATATCAC TCCGTA |
| wl-39431/39432 | *xcbA* | AY289931 | 777 | X | CTCTCGTCTACCAAATGGCTAT/GTGAGGGTGACATCCGCTA |
| wl-36794/36795 | *wnmA* | HQ437685 | 1454 | H | TGATCTACCCAAGGCACATAC/CAATCGGCTTATTGAGGGT |
| wl-39435/39436 | *synG* | EU038215 | 520 | W/Y | AAGGTGAATCTTCCGAGCAG/CCATTGGAAATTTTCGCTT |
| wl-32526/45168 | *galE/lipA* | HQ437687 | 5030 | I | TATTGGTTCGCATACTGTCATT/GTCATAGACGATGGAAT AGGGA |
| wl-37473/37475 | *synG* | EU038216 | 476 | W135 | ACGGTATCTGATGAAATGCTG/TCATATACAACGATTGG AATATC |

nucleoside triphosphate (dNTP), 1 U r*Taq* DNA polymerase, 0.1 μM each primer, and 50 to 100 ng of DNA template in a final volume of 20 μl. The PCR program used was as follows: a denaturation step at 94°C for 5 min and then 31 cycles of a denaturation step at 94°C for 30 s, an annealing step of 56°C for 45 s, and an extension step of 72°C for 1 min, with a final extension at 72°C for 5 min.

**Development of the multiplex PCR-based assay.** This assay consisted of three multiplex PCRs. PCR 1 was used to detect serogroups A, B, C, 29E, and I/K, and PCR 2 was used to detect serogroups Z, L, X, H, and W135/Y. Reaction 3 was used to differentiate serogroups I and K and W135 and Y, which could not be distinguished by PCRs 1 and 2 (see Fig. S1 in the supplemental material). The PCR mixture for reactions 1, 2, and 3 contained the same reagents except for the primers, and the details are described in the supplemental material. The PCR program for reactions 1 and 2 was as follows: a denaturation step at 94°C for 5 min and then 24 cycles of a denaturation step at 94°C for 30 s, an annealing step at 59°C for 45 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR program for reaction 3 was the same as that for reactions 1 and 2 except that the annealing step was 54°C for 45 s and the extension step was 72°C for 3 min.

**Nucleotide sequence accession numbers.** The DNA sequences of the capsular gene complexes of *N. meningitidis* serogroups H, I, K, L, and Z have been deposited in the GenBank database under the accession numbers HQ437685, HQ437686, HQ437687, HQ437688, and HQ437689, respectively.

## RESULTS

**Capsular gene complexes of six *N. meningitidis* serogroups.** The capsular gene complexes of the remaining five serogroups, H, I, K, L, and Z, were sequenced in this study. DNA sequences of 23,488, 24,676, 27,709, 18,453, and 21,871 bp in length were obtained from serogroups H, I, K, L, and Z, respectively. These gene complexes contained 19, 19, 22, 14, and 15 ORFs, respectively (see Fig. S2 in the supplemental material). The average GC contents of the sequences were 45.13, 48.04, 45.03, 45.62, and 46.97% in serogroups H, I, K, L, and Z, respectively. The functions of each ORF in these capsular gene complexes were predicted based on sequence homology by search of the listed databases and are summarized in Tables S1 to S5 in the supplemental material.

**Genes specific for *N. meningitidis* detection and genogrouping.** *ctrA* or *porA* genes have been used individually to identify *N. meningitidis* (15, 16). However, we found that our primers based on the *ctrA* sequence produced PCR products from both *N. meningitidis* and *N. lactamica*, and those based on the *porA* sequence gave PCR products from both *N. meningitidis* and *N. gonorrhoeae* (data not shown). Therefore, it was necessary to use a combination of these two genes as the targets to distinguish *N. meningitidis* from other species. Serogroup-specific genes were chosen based on the capsular gene sequences of all 12 *N. meningitidis* serogroups from those genes responsible for different CPS structures. These genes included ones that encode glycosyltransferase (*sacD*, *wnmB*, *lcbB*), polysialyltransferase (*siaD*), polymerase (*synF*, *synG*), phosphotransferase (*capZC*, *wnmA*, *xcbA*), and D-arabinose-5-phosphate isomerase (*cap29EH*). However, the gene sequences responsible for CPS synthesis in serogroups I and K, and those in serogroups W135 and Y, were too similar to be useful for differentiation. Genes *lipA* and *galE*, whose location is different in serogroups I and K, were used to differentiate the two serogroups. For serogroups W135 and Y, a minor difference between *synF* and *synG* sequences was used to differentiate these two serogroups.

Primers based on the *ctrA* and *porA* gene sequences gave both of the two expected PCR products in *N. meningitidis*, but only one or no PCR product in other species. Primers based on serogroup-specific genes could amplify the expected PCR products in the corresponding serogroup(s) but not in other serogroups. Primers that differentiated the serogroups I and K, or serogroups W135 and Y, gave PCR products only for serogroups I and W135, respectively, and no PCR product was detected for serogroups K or Y. The positions of the serogroup-specific primers are shown in Fig. S2 and S3 in the supplemental material.

**Development of a multiplex PCR-based assay.** Three groups of primer sets were made based on the screened specific primers. The primer pair efficiency in each group was determined on the basis of achieving amplicons of the expected sizes at a range of different primer concentrations. The primer concentration that

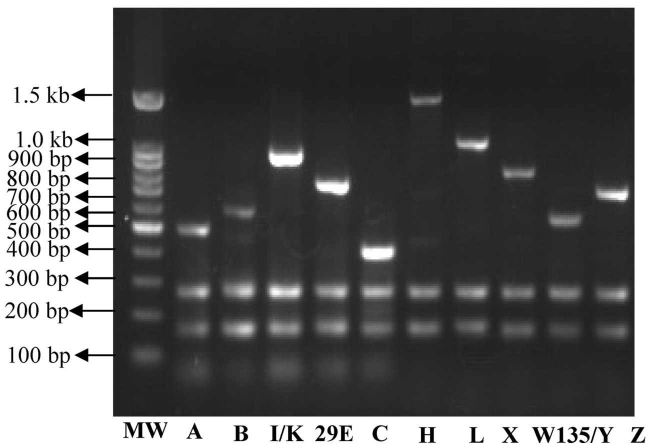


**FIG 1** Agarose gel electrophoresis of the PCR products obtained from multiplex PCRs 1 and 2 in the assay. Lane MW, 100-bp DNA ladder marker; lane A, serogroup A; lane B, serogroup B; lane I/K, serogroup I/K; lane 29E, serogroup 29E; lane C, serogroup C; lane H, serogroup H; lane L, serogroup L; lane X, serogroup X; lane W135/Y, serogroup W135/Y; lane Z, serogroup Z.

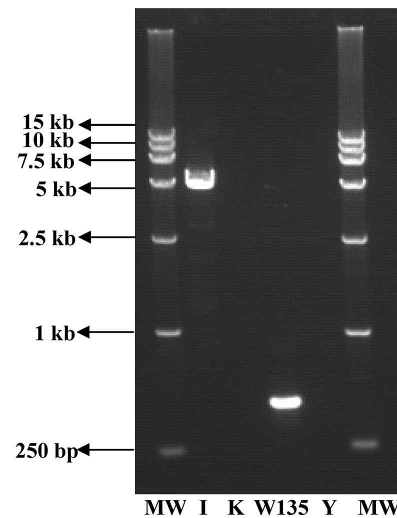


**FIG 2** Agarose gel electrophoresis of the PCR products obtained from multiplex PCR 3. Lanes MW, DNA marker DL15,000; lane I, serogroup I; lane K, serogroup K; lane W135, serogroup W135; lane Y, serogroup Y.

resulted in high-signal products was used as described in Materials and Methods. At the optimized primer concentration ratio, the DNA of strains that belonged to serogroups A, B, C, 29E, and I/K produced the expected PCR products of distinct sizes (470 bp for serogroup A, 555 bp for B, 381 bp for C, 694 bp for 29E, 893 bp for I/K, and 257 and 158 bp for *ctrA* and *porA* of all the tested serogroups) in the group 1 multiplex PCRs (Fig. 1). The DNA from serogroups Z, L, X, H, and W135/Y produced the expected PCR products (656 bp for Z, 988 bp for L, 777 bp for X, 1,454 bp for H, 520 bp for W135/Y, and 257 and 158 bp for *ctrA* and *porA* of all the tested serogroups) in the group 2 multiplex PCRs (Fig. 1). Serogroups I and W135 gave expected PCR products of 5,030 bp and 476 bp, respectively, in the group 3 multiplex PCRs (Fig. 2).

**Specificity of the multiplex PCR-based assay.** The developed multiplex PCR-based assay was tested with the 15 reference strains that belonged to the 12 *N. meningitidis* serogroups, with 97 clinical strains that belonged to A, B, C, W135, 29E, X, or Y or nongroupable isolates from different locations and years; with 15 strains of closely related species such as *N. flavescens*, *N. subflava*, *N. mucosa*, *N. lactamica*, *N. sicca*, and *N. gonorrhoeae*; and with six strains from species also reported to cause meningitis that included five *S. pneumoniae* isolates (belonging to serotypes 1, 23F, 6A, 6B, and 19A, reported to be more virulent) and one *H. influenzae* isolate (Table 1; see also Table S6 in the supplemental material). All the reference strains were detected with 100% specificity. Among the 97 clinical isolates, 90 of known serotype were detected and confirmed by the specific antiserum serogrouping. Five out of the seven strains that were classified as nongroupable by antisera could be classified as belonging to a specific serogroup by the multiplex PCR assay (see Table S6). For strains that belonged to other *Neisseria* species, only one or no PCR products that corresponded to segments of *ctrA* or *porA* genes could be detected. There was no PCR product obtained for *S. pneumoniae* and *H. influenzae* strains. These results indicate that the multiplex PCR-based assay is specific for individual *N. meningitidis* serogroups.

**Double-blinded test to verify the multiplex PCR assay.** A double-blinded test was performed in order to verify the stability and specificity of the assay. Forty-six clinical isolates were used for the test (see Table S6 in the supplemental material). The isolates were also characterized by specific antisera at the Center for Disease Control and Prevention, Shanghai, China. The detection results of the PCR assay were consistent with the results by antiserum serogrouping except for the isolate NM033 (see Table S6). The reason for the different detection results for isolate NM033 using antisera and the multiplex PCR assay are unknown, and the strain needs to be further analyzed.

**Detection sensitivity of the multiplex PCR assay.** The detection sensitivities for genomic DNA and isolates in noncultured mock CSF specimens were both determined. Serial dilutions of genomic DNA (10 ng, 1 ng, 100 pg, 10 pg, and 1 pg per ml) of *N. meningitidis* serogroups A, B, C, 29E, and Z reference strains were used to test the sensitivity of the multiplex assay. As little as 1 ng of DNA (equivalent to $\sim 4 \times 10^5$ genomes) could be detected by the assay. Pure cultures of strain NM055 (serogroup C, isolated from a patient) were mixed with 1 ml CSF specimens from healthy people with the concentrations of $10^7$, $10^6$, $10^5$, $10^4$, to $10^3$ CFU/ml, respectively, and tested with the multiplex assay. The lower limit of detection was determined to be $3 \times 10^5$ CFU/ml. All the reference strains of other serogroups could also be detected by this assay at the level of $\sim 4 \times 10^5$ genomes or $3 \times 10^5$ CFU/ml. Therefore, $\sim 4 \times 10^5$ genomes or $3 \times 10^5$ CFU/ml strains in the mock CSF specimen were the lower limits for the detection using this assay.

## DISCUSSION

Molecular protocols for the detection of *N. meningitidis* have been reported previously. However, serogrouping of *N. meningitidis* is dependent mainly on methods that use specific antisera. The capsular gene complexes from seven out of the 12 *N. meningitidis* serogroups have been identified previously. The complexes of the remaining five serogroups were sequenced in this study, and these sequences enabled the design of primers for further genogrouping. As far as we know, this study is the first to describe a molecular protocol for the detection and genogrouping of all 12 *N. meningitidis* serogroups.

In the multiplex PCR-based assay developed in this study, primers for *ctrA* and *porA* genes were combined to be used for *N. meningitidis* identification. Genes that encode glycosyltransferase, polysialyltransferase, polymerase, phosphotransferase, and D-arabinose-5-phosphate isomerase in the capsular gene complexes were used as the serogroup-specific genes, as these genes were responsible for differences in the CPSs between the serogroups. For serogroups W135 and Y, it was reported that the amino acid 310 in *synF* and *synG* was responsible for the different capsular forms of the two serogroups (5). A primer pair based on minor divergence between *synF* and *synG* sequences was used to differentiate serogroups W135 and Y. The antisense primer covered three consistently different positions (L324V, A325V, N327D) between the two genes, and the fragment amplified contained six of the nine consistently different positions (including G310P) of the two genes.

A total of 179 strains, which included the *N. meningitidis* reference strains, clinical isolates from different locations and years, those of closely related species, and those of species that can be detected in blood or CSF causing meningitis, were used to characterize the specificity of the assay. The results corresponded well with the findings by the traditional serology-based method. Some isolates previously classified as nongroupable were also able to be genogrouped by our assay. The total time to perform this assay was only 2 to 4 h, following bacterial isolation, and a PCR block is the only major equipment necessary. The accurate and efficient detection of a pathogenic microorganism is crucial for the prevention and effective treatment of disease, especially in the case of pathogen outbreaks. Therefore, this multiplex PCR-based assay has the advantage for applications in clinical diagnosis and epidemiological surveillance.

Although the detection sensitivity ($4 \times 10^5$ genomes or $3 \times 10^5$ CFU/ml) is close to the level of *N. meningitidis* in most clinical CSF specimens ($10^5$ CFU/ml) (17), a better sensitivity will be helpful for the assay to be applied. It was reported that real-time PCR assays for *N. meningitidis* had a sensitivity less than 100 genomes (250 fq) (9, 18); therefore, real-time PCR assays for the detection and genogrouping of all *N. meningitidis* serogroups will be more applicable and can be developed in future studies.

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