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

Hongfei Zhu, Quan Wang, Liuqing Wen, Jianguo Xu, Zhujun Shao, Mingliang Chen, Peter R. Reeves, Boyang Cao, and Lei Wang

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 ~4 × 10^9 genomes) or 3 × 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.

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N. meningitidis 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% CO2 in air for 24 h.

Genomic DNA extraction. Genomic DNA was extracted with a DNA extraction kit (QiAamp DNA minikit; Qiagen, Hilden, Germany).

Capsular gene complex amplification, sequencing, and analysis. Primers wl-14395 (5'-CGGCAATCTTC1CGCCACACCA-3') and wl-22861 (5'-CGACGGGAGAAAATCAGCGATGC-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'-CGGCAATGC1GCACCATGACT-3') and wl-27419 (5'-CGGCAAGACC1ACTGGCGATGTG-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 72°C for 30 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 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 using 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.

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 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 2.5 mM MgCl2, 0.4 mM deoxy-

**TABLE 1** Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain no. (source of strain)</th>
<th>Total no. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neisseria meningitidis (n = 15) serogroup</td>
<td></td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>29010 (CMCC), 29019 (CMCC)</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>29011 (CMCC), 29061 (CMCC)</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>29012 (CMCC)</td>
</tr>
<tr>
<td><strong>29E</strong></td>
<td>29034 (CMCC)</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>29031 (CMCC)</td>
</tr>
<tr>
<td><strong>I</strong></td>
<td>29044 (CMCC)</td>
</tr>
<tr>
<td><strong>K</strong></td>
<td>29047 (CMCC)</td>
</tr>
<tr>
<td><strong>L</strong></td>
<td>43828 (ATCC)</td>
</tr>
<tr>
<td><strong>W135</strong></td>
<td>29037 (CMCC), 29057 (CMCC)</td>
</tr>
<tr>
<td><strong>X</strong></td>
<td>M8210 (ICDC)</td>
</tr>
<tr>
<td><strong>Y</strong></td>
<td>29038 (CMCC)</td>
</tr>
<tr>
<td><strong>Z</strong></td>
<td>33582 (ATCC)</td>
</tr>
</tbody>
</table>

N. meningitidis clinical isolate (n = 143)

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>23 (ICDC), 2 (SCDC)</td>
</tr>
<tr>
<td>Blood</td>
<td>4 (ICDC)</td>
</tr>
<tr>
<td>TS</td>
<td>61 (ICDC), 6 (SCDC)</td>
</tr>
<tr>
<td>CP</td>
<td>30 (SCDC)</td>
</tr>
<tr>
<td>BP</td>
<td>4 (SCDC)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (ICDC), 4 (SCDC)</td>
</tr>
</tbody>
</table>

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

**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 University, China; CSF, cerebrospinal fluid; CP, chocolate agar plate; BP, blood agar plate; TS, throat swab.
nucleoside triphosphate (dNTP), 0.1 U 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 these two serogroups. For serogroups W135 and Y, a minor difference between the primer pairs could amplify the expected PCR products in the corresponding serogroup(s) but not in other serogroups. Primers based on specific genes could amplify the expected PCR products in the two expected PCR products in 

**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. gonorhoeae* (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*, *wmnB*, *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 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 PCR efficiencies. The primer concentration that
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, and 988 bp for I/K). The DNA of strains that belonged to serogroups Z, L, W135, X, H, and W135/Y produced the expected PCR products of distinct sizes (470 bp for Z, 988 bp for L, 777 bp for X, 1,454 bp for H, 893 bp for W135/Y, and 257 and 158 bp for W135). In addition, the 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.

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.

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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 reference strains, clinical isolates from different locations and years, and genogrouping of all N. meningitidis serogroups will be more applicable and can be developed in future studies.

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