Simultaneous single-tube PCR-based assay for the direct identification of the five most common meningococcal serogroups from clinical samples

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Abstract
This study presents a stepdown multiplex PCR assay for the simultaneous detection of the five most common Neisseria meningitidis serogroups (A, B, C, W-135 and Y) in 530 clinical samples obtained from 428 patients (271 blood and 259 cerebrospinal fluid). The sensitivity and the specificity was calculated to 100% [positive predictive value 100% (95%, CI 99.0–100%) and negative predictive value 100% (95% CI 99.0–100%)]. The overall effectiveness permits the rapid, accurate and inexpensive detection of the five most prevalent meningococcal serogroups in clinical samples. It is potentially a valuable tool for diagnosis and epidemiological monitoring of disease due to N. meningitidis.

Introduction
Meningococcal disease receives a high level of medical, public and media attention because of its rapid onset and high level of morbidity and mortality (van Deuren et al., 2000). The rapid progression of symptoms and potentially devastating effect of this disease necessitate early recognition and immediate treatment (Rosenstein et al., 2001).

The developments in meningococcal polysaccharide–protein conjugate vaccines have increased the need for accurate laboratory confirmation to monitor the effect of vaccine implementation and continuing effectiveness (Zimmer & Stephens, 2004). It is important to establish the distribution of meningococcal serogroups circulating in the population, to detect the emergence of serogroups associated with disease, particularly those belonging to hyperinvasive lineages (Stollenwerk et al., 2004; Bennett & Cafferkey, 2006).

Characterisation of isolates by serogroup is an integral part of the management of contacts of patients with meningococcal infection. Currently, 13 serogroups (A, B, C, D, 29E, H, I, K, L, W-135, X, Y and Z) of Neisseria meningitidis are recognized based on the antigenic variation among the different capsular polysaccharides. Serogroups A, B, C, W-135 and Y account for the majority of invasive meningococcal disease (IMD) worldwide (World Health Organization, 1996; Centres for Disease Control and Prevention, 2000; Taha et al., 2000; Maiden & Begg, 2001; Tsolia et al., 2006; Tzanakaki & Manstantonio, 2007).

Several assays based on PCR have been developed for the detection and identification of those common serogroups for routine use in many diagnostic laboratories (Borrow et al., 1997, 1998; Taha, 2000; Diggle et al., 2003; Bennett & Cafferkey, 2006). These are particularly valuable for the confirmation of N. meningitidis cases in which early antibiotic treatment prevents detection by culture (Tzanakaki et al., 2003, 2005). The use of individual assays for the identification of the serogroups requires several consecutive PCR assays, which is demanding on time and resources.

The aim of the present study was to develop and evaluate a single-tube multiplex PCR assay for the simultaneous detection of serogroups A, B, C and W-135/Y of N. meningitidis in clinical samples as a tool for improved
noncultural diagnosis. The sensitivity and specificity of the PCR assay indicate that the method will be a valuable tool for diagnosis and epidemiological studies.

**Materials and methods**

**Sources of specimens**

The multiplex PCR assay was applied to a total of 530 clinical samples which were identified as N. meningitidis either by culture or PCR assay as described previously (Tzanakaki et al., 2005). Of those, 267 blood, 259 cerebrospinal fluid (CSF) and four blood cultures were obtained from 428 patients with IMD admitted to different hospitals throughout Greece between 2003 and June 2007. All meningococcal strains were sent to the Reference Laboratory in charcoal medium for further identification, while clinical samples such as CSF (frozen) and blood (room temperature) samples were sent by courier.

From the above samples, 196 obtained from 136 patients were confirmed by culture and PCR: CSF = 27/136 (19.8%), blood = 49/136 (36.1%) and both CSF and blood = 60/136 (44.1%). There were 334 samples obtained from 292 patients confirmed only by PCR: CSF = 130/292 (44.5%), blood = 116/292 (39.7%), CSF and blood = 42/292 (14.4%) and blood cultures = 4 (4/292, 1.4%). Details of the collection of clinical data and samples have been published previously (Tzanakaki et al., 2003). In addition, for the evaluation of the multiplex PCR assay, 62 meningococcal strains (mainly isolated from carriers) were also included. These were previously serogrouped by slide agglutination with capsular typing sera.

**DNA isolation**

Bacterial colonies were suspended in 200 μL of 10 mM Tris-EDTA buffer (pH 8.0), vortexed, heated at 100 °C for 10 min and centrifuged (Hettich Zentrifugen, Tuttlingen, Germany) at 20,000 g for 12 min. The supernatant was retained and the DNA concentration was estimated spectrophotometrically.

DNA was extracted from whole blood samples with the Nucleospin Blood Quickpure kit (Macherey-Nagel, Duren, Germany) according to the manufacturer’s instructions. The CSF samples were processed as described by Zambardi et al. (1985). In brief, 500 μL of sample was centrifuged at 1700 g for 10 min. Supernatant was discarded to leave 150 μL which was added to 650 μL of sterile double-distilled H2O and 150 μL of Chelex/Tween-80 buffer. The samples were heated at 100 °C for 30 min and centrifuged at 10,280 g for 8 min. A volume of 200 μL of the supernatant was used for the PCR assay.

DNA from blood cultures was extracted as described previously (Shrestha et al., 2002). In brief, 500 μL of blood culture vial was centrifuged at 850 g for 2 min to remove the charcoal medium. The supernatant was centrifuged at 11,500 g for 5 min. The pellet was resuspended in 200 μL lysis buffer and incubated at 100 °C for 10 min. The sample was centrifuged at 850 g for 2 min and 170 μL of the supernatant was retained.

**PCR primers**

For the simultaneous detection of the meningococcal serogroups A, B, C, W-135 and Y, a multiplex PCR was designed.

Specific primers for each of the serogroups A, B and C were used based on those described previously (Tzanakaki et al., 2003) for the amplification of specific genetic loci (Table 1). A common pair of primers was used for the simultaneous detection of serogroups W-135 and Y (Probert et al., 2002). In order to improve sensitivity and specificity for serogroup A, a new pair of primers was designed inside the sacB gene (orf2) (GenBank Accession number AF019760) according to the software FASTPCR (Kalander, 2007). Each primer pair was tested for the presence of secondary structure as described by Zuker (2003). The specificity of the designed primers was tested in silico against the N. meningitidis available complete genomes of serogroup A (Z2491 GenBank Accession number: AL157959, position 20 000 g for 12 min. The supernatant was retained and the DNA concentration was estimated spectrophotometrically.

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**Table 1. Oligonucleotides used for the identification of meningococcal serogroup**

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Primers</th>
<th>Gene amplified</th>
<th>Sequence (5' → 3')</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SA1</td>
<td>sacB (orf-2)</td>
<td>5'-tgttgacatgaaaactcagcag-3'</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>SA2</td>
<td></td>
<td>5'-tcttgatgacttgtggtctcg-3'</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>B1</td>
<td>siaD</td>
<td>5'-ggatctcctgttcttccacca-3'</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td></td>
<td>5'-gcctgctgggaattgccctaa-3'</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>C1</td>
<td>siaD</td>
<td>5'-tcaatagcttgctcaatggat-3'</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td></td>
<td>5'-aatctcaagatgtgcaactgac-3'</td>
<td></td>
</tr>
<tr>
<td>W-135/Y</td>
<td>W/Y1</td>
<td>siaD</td>
<td>5'-tcctgacaggaatttagaraa-3'</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>W/Y2</td>
<td></td>
<td>5'-gctcattccagaaatcaccag-3'</td>
<td></td>
</tr>
</tbody>
</table>
Amplification reactions (25 μL) contained the following: four primer pairs, with final concentration of 0.28, 0.24, 0.12 and 0.2 μM for serogroups A, B, C and W-135/Y respectively; 4.5 mM MgCl₂ (ABgene, Surrey, UK); 0.8 mM dNTPs (ABgene, Surrey, UK); 1.3 x DyNaZyme II Buffer; 1.2 U Taq polymerase (DyNaZyme II Hot Start, Finnzymes, Finland) and 5 μL of DNA sample.

For the PCR amplification protocol, the conditions were: 95°C for 10 min; 10 cycles at 95°C for 50 s; 60°C for 45 s and 68°C for 90 s. The next step consisted of: 25 cycles at 95°C for 50 s; 59°C for 45 s; 68°C for 1.45 min; and final extension at 68°C for 3 min. The PCR was performed in a Robocycler Gradient 96 Cycler (Stratagene, La Jolla, CA). Amplicons were visualized under UV fluorescence following electrophoresis in agarose 2.0% (w/v) gels and staining with ethidium bromide. Positive controls of DNA from reference strains of each serogroup, as well as negative controls, were included in each assay.

Serial dilutions of spectrophotometrically quantified DNA (0.5–0.001 ng per 25 μL) from each serogroup were amplified with the individual and multiplex assay to determine the sensitivity of the assay. Specificity was tested with DNA extracted from clinical specimens to which DNA of other serogroups (X, Z and nongroupable) had been added.

Results

Assessment of the PCR performance

The ability of the PCR to detect limited bacterial load was assayed with respect to two parameters: the ability to amplify each serogroup regardless the presence of DNA from other serogroups; and the ability to detect serial dilutions of DNA samples of known serogroup (0.5–0.001 ng per serogroup per reaction). The amplification of each individual DNA was not affected by the presence of DNA from other serogroups. For the sensitivity limitations, repeated experiments were conducted with serial dilutions of the DNA samples of five serogroups. DNA of five serogroups was detected in 0.001 ng per 25 μL reaction.

The issue of specificity of the new PCR assay was addressed using meningococcal serogroups which were not targets of the multiplex PCR assay. These included serogroups X, Z and nongroupable (NG) (Table 2).

Detection of meningococcal DNA in clinical samples

The technique was applied to simultaneous detection of the five serogroups in clinical samples. The assay was applied in both CSF and blood samples if these were available for the same patient. For 295/334 (88.32%) samples, serogroup was identified by the single PCR assay, while 39 samples were nongroupable (NG). In contrast, the multiplex PCR assay identified 93.7% of the clinical samples (313/334) into serogroups, while 21 clinical samples remained nongroupable (21/334) (Table 2). Of these, 10 nongroupable clinical samples, identified by the single PCR, were found, by the application of the present multiplex PCR, to belong to serogroup A, while seven belonged to serogroup B, and one to serogroup W-135. Figure 1 shows examples of the results obtained when the multiplex PCR assay was carried out.

Sensitivity of the PCR assay

The sensitivity of the multiplex PCR assay was evaluated with data obtained from the 196 N. meningitidis clinical samples.
samples. These were from culture confirmed cases for which the serogroup was determined by a single PCR assay, and the slide agglutination test was performed for their respective isolates (Table 2). The sensitivity was estimated as 100% with positive predictive value (PPV) 100% (95%, CI 99.0–100%) and negative predictive value (NPV) 100% (95% CI 99.0–100%).

Assessment of specificity of the PCR assay
The specificity of the PCR assay was evaluated in relation to 62 meningococcal isolates which were not targets of the multiplex PCR assay, serogroups X, Z and NG. The specificity of the assays was 100% with positive predictive value (PPV) 100% (95%, CI 99.0–100%) and negative predictive value (NPV) 100% (95% CI 99.0–100%) (Table 2).

Discussion
Bacteriological confirmation and serogroup determination of clinically suspected IMD is important for contact management, outbreak recognition and for detailed epidemiological surveillance of these infections.

This study demonstrated that a single-tube multiplex PCR assay correctly identified the serogroup of all meningococcal samples tested. There was 100% correlation with the results obtained using traditional serogrouping and individual PCR assays. In comparison to serogroup determination by individual PCR assays or by DNA sequencing, the proposed protocol provides a relatively inexpensive, rapid and reliable method for identification of the five serogroups most often associated with invasive meningococcal disease (Lewis et al., 2003).

In contrast to other multiplex PCR-based assays (Bennett & Cafferkey, 2006), this method enabled us to identify the serogroup in a large number of clinical samples known to contain smaller quantities of bacterial DNA in the presence of large amounts of human genomic DNA, and inhibitors (e.g. heme in blood) (Abu Al Soud & Radstrom, 2001). This is important since early antibiotic treatment impairs detection by culture; the method could contribute significantly to accurate early recognition and surveillance of meningococcal infections.

The hot start polymerase used in combination with the two annealing step – in contrast to the conventional PCR conditions (data not shown) – reduced the nonspecific amplification and increased the yield of the specific amplicons. As a result, quantities as low as 0.001 ng of the genomic DNA were detected.

The newly designed primer for serogroup A clearly distinguished serogroup A and serogroup B PCR products (initial amplicon sizes 400 and 450 bps for serogroups A and B respectively). Also, it enabled further identification of 10 nongroupable clinical samples as serogroup A.

In conclusion, the high sensitivity and specificity observed suggest that the method is a robust, simple, reliable, easily implemented, rapid and cost-effective way for identification of the most prevalent meningococcal serogroups. The direct application of this assay for assessment of clinical samples could contribute to better monitoring the meningococcal serogroups, especially after the implementation of the monovalent and quadrivalent conjugated vaccines.

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