A novel porA-based real-time PCR for detection of meningococcal carriage

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Real-time PCR based on the capsule transfer gene (ctrA) is a significant aid in the diagnosis of meningococcal infection but fails to detect a high proportion (60%) of non-groupable strains associated with nasopharyngeal carriage. This study aimed to design a novel real-time (TaqMan) PCR that would detect more strains of meningococci and be suitable for large-scale carriage studies. Primer and probe sequences were based on the meningococcal porA gene and designed specifically to exclude the highly related porA pseudogene in Neisseria gonorrhoeae. The specificity of the assay was confirmed by testing strains of N. gonorrhoeae known to contain the porA pseudogene together with commensal strains of Neisseria lactamica and Neisseria sicca. None of these was detected in the assay. Neisseria meningitidis strains representing a wide range of serogroups together with non-groupable strains isolated from the nasopharynx were tested by ctrA assay and the novel porA-based TaqMan PCR. All carriage strains were detected by the porA-based assay including four that gave weak or no reaction with the ctrA assay. Comparison of ctrA and porA assays on 71 throat swabs obtained from university students showed that the porA assay detected meningococcal DNA in all samples that were ctrA positive plus three that were ctrA negative but culture positive. This novel porA-based TaqMan assay provides a highly specific method for detecting meningococcal DNA that is more sensitive than the ctrA assay for detecting meningococcal carriage and is particularly suitable for carriage studies where non-groupable strains and other Neisseria are present.

INTRODUCTION

Meningitis and septicaemia caused by Neisseria meningitidis are serious causes of morbidity and mortality. Effective vaccines based on the polysaccharide capsule have reduced the burden of disease in the UK caused by serogroup C strains of meningococci (Communicable Disease Surveillance Centre, 2001). However, disease caused by serogroup B organisms still persists and vaccines against these based on the outer-membrane protein are being developed (Van der Voort \textit{et al.}, 1997). Rapid and sensitive detection of meningococcal carriage is an important aid to human population studies, either during outbreaks or as an integral part of vaccine efficacy trials. The detection of nasopharyngeal carriage of meningococci has traditionally relied on selective culture from throat swabs. In a recent study, it was shown that detection of carriage could be improved by inclusion of real-time (TaqMan) PCR based on the capsule transfer gene, \textit{ctrA} (Jordens \textit{et al.}, 2002). However, the majority of the carriage strains were non-groupable and 60% of these could not be detected or were only poorly detected with the \textit{ctrA} PCR, showing that prompt culture was still essential. The aim of the current study was to design a real-time PCR that would detect a greater proportion of the non-groupable strains associated with carriage and that could be used in surveys of large populations.

METHODS

DNA. Supernatant fluid obtained from boiled suspensions of bacterial strains after overnight culture on clear typing media (Diaz & Heckels, 1982) was used for PCR. The specificity of the assay was determined with extracts from laboratory cultures of the following: Haemophilus influenzae ATCC 49766, Haemophilus parainfluenzae NCTC 4101, Streptococcus anginosus NCTC 8037, Streptococcus pneumoniae ATCC 49619, Neisseria gonorrhoeae strain P9 (Diaz & Heckels, 1982) and three fresh clinical isolates (provided by the Public Health Laboratory, Southampton, UK), Neisseria lactamica NCTC 10617 and Neisseria sicca NCTC 4591. The detection of a wide range of serogroups was determined with extracts from cultures of laboratory strains with serogroups A, B, C, X (three strains), Y, Z, W135 and 29E, and from two non-groupable strains.

The detection of a wide range of serotypes associated with carriage was determined with extracts from 18 strains of meningococci (15 non-groupable and three group W135) isolated from the nasopharynx of
products were detected by ethidium bromide staining of agarose gels.

Amplification by PCR. The presence of a porA pseudogene in the N. gonorrhoeae strains was determined by conventional PCR amplification with primers specific for the VR1 region. The primers were 5'-CCGCACCTGCCTTGGG-3' and 5'-CCAAACAGCTTCCAGC

Amplification by TaqMan PCR. Reaction mixtures contained 1 mM primers, 200 μM each dCTP, dGTP, dATP and dTTP (Promega), 1× Optibuffer, 2 mM MgCl2 and 2 U (0.5 μl) Bio-X-Act polymerase (Bioline). Amplification was carried out using a Perkin Elmer 9600 cycler with 35 cycles of 40 s at 96°C (Bioline). Amplification was carried out using a Perkin Elmer 9600 cycler with 35 cycles of 40 s at 96°C, 40 s at 50°C and 30 s at 72°C. Products were detected by ethidium bromide staining of agarose gels.

Amplification of porA by TaqMan PCR. Reaction mixtures contained 1× universal mix (Perkin Elmer), 0.3 nM each primer (Crucachem), 200 nM probe (Crucachem) and 5 μl DNA extract in a total volume of 25 μl. Amplification parameters consisted of 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The threshold cycle (the cycle at which sample fluorescence exceeds a threshold value, indicating a positive result and which is proportional to the number of gene copies present) was recorded for each sample. Experiments included separate positive- and negative-control DNA samples (N. meningitidis and N. gonorrhoeae, respectively) and at least three water (no template DNA) controls. Dilutions (10-fold) of meningococcal DNA covering the range 10^{-3} to 10^{7} copies ml^{-1} were also included.

ctrA TaqMan assay. All samples (except the three clinical isolates of N. gonorrhoeae and the non-Neisseria species) were tested in the modified ctrA TaqMan assay, as described previously (Corless et al., 2001; Jordens et al., 2002), either as part of the previous study (throat swab samples) or specifically for this study.

RESULTS

Primer and probe design

The proposed assay needed to be species specific and to detect a wide range of meningococcal strains. For specificity, it was essential to avoid amplification and detection of similar sequences in related species. porA was chosen as the target gene as DNA sequence data for this was available for a large number of meningococcal strains and the only closely related gene reported in other species is a porA pseudogene detected in N. gonorrhoeae (Feavers & Maiden, 1998). To detect the maximum number of meningococcal strains, highly variable regions within porA, for example VR1 and VR2 (McGuinness et al., 1990), were excluded as primer and probe target sequences. To locate species-specific DNA sequences, a consensus porA sequence from N. meningitidis was aligned with a consensus porA pseudogene sequence from N. gonorrhoeae (made from all six gonococcal porA pseudogene sequences deposited in GenBank) together with related sequences representing other Neisseria porin genes, namely consensus porB2 and porB3 from N. meningitidis, consensus porB1a and porB1b from N. gonorrhoeae, and the related

Given the constraints above, the guidelines for designing TaqMan primers and probes provided by the manufacturer (Perkin Elmer Biosystems) were followed as closely as possible. For ease of use, reaction conditions were kept consistent with those used for other TaqMan assays (Corless et al., 2001; Jordens et al., 2002). Primer and probe sequences were designed manually and Primer Express software (Applied Biosystems) was used to calculate Tm and to detect any possible internal loops or primer dimers. The sequences designed were as follows: forward primer, 5'-GTCTCGGT

Amplification of DNA from cultures

PCR amplification with primers specific for conserved sequences flanking the VR1 region of the meningococcal porA gene yielded products of the expected size (~250 bp) from all 12 laboratory control strains of N. meningitidis, 18 carriage strains of N. meningitidis and also from three of the four N. gonorrhoeae isolates, indicating the presence of porA, or porA pseudogene, in these strains. In addition N. lactamica and N. sicca produced weak bands corresponding to products of different sizes.

The porA TaqMan assay was negative for H. influenzae, H. parainfluenzae, S. anginosus and S. pneumoniae, and also for the four N. gonorrhoeae, N. lactamica and N. sicca strains tested, despite the presence of porA-related sequences in the latter strains. The assay detected all 12 laboratory control strains of N. meningitidis, including two non-groupable strains, one of which was not detected with the ctrA assay. All 18 N. meningitidis carriage strains were detected with the porA assay, whereas three were not detected with the ctrA assay and one was detected only very weakly. Ethidium bromide staining of porA TaqMan amplification products on agarose gels showed products of the expected size from a sample of six control strains (serogroups B, C, X, Y, W135 and 29E) and three non-groupable carriage strains, but no
products were detected from the four *N. gonorrhoeae* tested, confirming that the pseudogene was not amplified with the *porA* primers.

**Amplification of DNA from throat swabs**

A total of 71 throat swab extracts were tested in the *porA* assay designed in this study. Of the 14 samples previously shown to contain meningococci by culture or PCR, four were not detected by the *ctrA* assay (Table 1). The four samples that were *ctrA*-negative all contained non-groupable meningococci when cultured. The *porA* assay detected three of these *ctrA*-negative samples, while one culture-positive sample was not detected by either assay. The remaining 57 samples were negative by all methods.

**DISCUSSION**

The novel *porA* TaqMan assay described in this study provides a simple, rapid method for the detection of meningococcal DNA from throat swabs. The assay was more sensitive than the *ctrA* TaqMan assay and at least as sensitive as traditional culture for the detection of meningococcal carriage in the population tested. Hence, this *porA*-based TaqMan assay should be suitable for large-scale carriage studies, and has advantages over the *ctrA* TaqMan assay for these samples as it detects more non-groupable *N. meningitidis* specifically associated with nasopharyngeal carriage.

TaqMan assays are convenient for large studies as swab washings can be stored at −80 °C and processed at a later date for testing in a 96-well format. On a single instrument, a total of about 200 samples can be tested by one person in a day, making the screening of large numbers of samples, especially those taken at sites not near the testing laboratory, much simpler than culture.

The swab sample that was not detected by either TaqMan assay yielded only a few colonies by culture. As swabs were used to inoculate culture media first, it is likely that the number of bacteria remaining was insufficient for detection when diluted for PCR. Hence, the sensitivity is likely to be even better when a TaqMan assay is used alone. The level of detection of both TaqMan assays was comparable (about 50 organisms per PCR reaction or 10^4 organisms per ml supernate). The culture-negative results obtained for two TaqMan-positive samples probably reflect sampling differences in these low-count samples. For optimal detection of meningococci from throat swabs, prompt culture and *porA* TaqMan PCR should be undertaken, as samples with few organisms may be missed using either assay alone.

Initially it was hoped that a sequence containing regions encoding VR1 and/or VR2 of meningococcal *porA* could be amplified so that products could be characterized by sequencing after amplification. The presence of the *porA* pseudogene in some strains of *N. gonorrhoeae* with 91 % similarity to the gene in *N. meningitidis* prevented this. Clarke et al. (2001) have described a method for *porA*-based sequence typing of meningococci that they suggest can be used directly on clinical samples. Although this may be suitable for clinical samples in which there is high suspicion of meningococcal disease, it is unlikely to be suitable for carriage studies where other neisserial species with related *porA* genes are present, since the primers used amplify the complete *porA* gene (Maiden et al., 1991) and have been shown to amplify the gonococcal *porA* pseudogene (Feavers & Maiden, 1998).

TaqMan type assays are ideal for distinguishing between highly related sequences, such as the meningococcal *porA* and gonococcal *porA* pseudogenes, as they use a highly specific probe that can discriminate between just a few bases. The lack of detection of *N. gonorrhoeae* strain P9, which has previously been shown to contain the *porA* pseudogene (J. E. Heckels & K. Jolley, unpublished data), and the two clinical isolates that were *porA* positive by conventional PCR, together with the lack of reaction with the other *Neisseria* species and respiratory organisms tested, shows that the *porA* TaqMan assay described here is species specific.

Feavers & Maiden (1998) undertook extensive sequence analysis of *porA* pseudogenes from *N. gonorrhoeae* and confirmed by phylogenetic analysis that the only closely related gene to this was *porA* in *N. meningitidis*. The probe in this study targeted the sequence showing the most variation between meningococcal and gonococcal *porA*; five of the 27 bases differed. Translation of the probe sequence showed it to map to loop 7 of the porin protein (RGKKGENT) (Van der Ley et al., 1991), a region that is highly conserved in *N. meningitidis*, with only occasional strains having a single-base change. The *porA* assay detected two such strains known to have a single mismatch (data not shown). The six *porA* pseudogene sequences in GenBank were also identical.

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**Table 1. Detection of *N. meningitidis* in throat swabs from carriers by culture, *ctrA* TaqMan assay and *porA* TaqMan assay**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Culture*</th>
<th><em>ctrA</em></th>
<th><em>porA</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>−</td>
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</tr>
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</tr>
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<td>82</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total positive</td>
<td>12</td>
<td>10</td>
<td>13</td>
</tr>
</tbody>
</table>

*Results from a previous study (Jordens et al., 2002).*

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in this region, while N. lactamica porin, N. sicca porin, gonococcal porB1a and porB1b, and meningococcal porB2 and porB3 differed from porA at a total of between 7 and 16 bases. The high degree of conservation of the target sequence within N. meningitidis indicates that it is a stable target, and the lack of similarity with related porins shows that specificity is unlikely to be compromised by simple genetic changes in related species. Strains of N. meningitidis lacking porA have been isolated from patients only rarely (Van der Ende et al., 1999), so are unlikely to compromise the assay.

The porA TaqMan assay designed and tested in this study provides a highly specific method for detecting meningococcal DNA that is particularly suitable for carriage studies where non-groupable meningococci, other Neisseria species and respiratory organisms are present.

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REFERENCES


