Detection and genotyping of meningococci using a nested PCR approach

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An effective vaccine against *Neisseria meningitidis* serogroup B is required. Outer-membrane protein vaccines have been developed, which may provide protection against common circulating serotypes and serosubtypes in some countries. However, limited genosubtyping data are available because most laboratories use mAbs directed against a limited number of specific serotypes and serosubtypes and laboratories do not genosubtype directly from body fluids due to the lack of a sensitive PCR method. A nested PCR was therefore developed that enables the amplification of the *porA* gene directly from clinical samples and has the required sensitivity for nucleotide sequencing of the three main variable regions, VR1, VR2 and VR3. Data were compared with those from culture-based nucleotide sequencing, and the use of this method increased the availability of genosubtype information by 45 %, thereby indicating the impact that this methodology has on the data provided and the implications for vaccine design.

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INTRODUCTION

Meningococcal disease remains a significant public health problem, and a vaccine against *Neisseria meningitidis* serogroup B is urgently needed (Rosenstein *et al.*, 2001). However, the use of a capsule-based vaccine is unlikely because the meningococcal polysaccharide is identical to a human carbohydrate [α (2 → 8) *N*-acetyl neuraminic acid or polysialic acid] (Finne *et al.*, 1987; Hayrinen *et al.*, 1995). Vaccines based on other antigens therefore require development and the outer-membrane proteins (OMPs) of the meningococcus have been investigated (Rosenstein *et al.*, 2001; Al'Aldeen & Cartwright, 1996). Although OMP-based vaccines are not the solution for combating serogroup B meningococcal infection, they may be useful in some circumstances (Al'Aldeen & Cartwright, 1996; Rappuoli, 2001). However, such proteins are highly antigenically variable, and an OMP vaccine cannot protect against all serotypes and serosubtypes of *N. meningitidis*. Therefore, selected OMPs must be used, based on the prevalent serotypes and serosubtypes in a given population. However, to develop such vaccines, high-quality data must be gained to determine such prevalence. To date, the characterization of meningococci has relied on serogrouping, serotyping and serosubtyping using serological methods, thereby limiting the designation of serotypes and serosubtypes because a

panel of mAbs is used. Newer nucleotide sequence methods, based on the detection and sequencing of the *porB* and *porA* genes for serotype (class 2/3 OMP) and serosubtype (class 1 OMP), respectively, have led to a better understanding of the variability of these proteins (Urwin *et al.*, 1998a, b, c; Clarke *et al.*, 2001a; Jelfs *et al.*, 2000; Molling *et al.*, 2000; Feavers *et al.*, 1996; Saunders *et al.*, 1993; Smith *et al.*, 1995). However, most of these methods are only useful when *N. meningitidis* has been isolated from blood or cerebrospinal fluid (CSF) and they therefore rely on the availability of a culture (Jelfs *et al.*, 2000; Feavers *et al.*, 1996). Other methods rely on sufficient numbers of meningococci being present in body fluids (Urwin *et al.*, 1998a; Clarke *et al.*, 2001a; Saunders *et al.*, 1993). Although the PCR has been used with great success for the laboratory confirmation of meningococcal disease (Ni *et al.*, 1992; Guiver *et al.*, 2000; Corless *et al.*, 2001; Diggle *et al.*, 2001a), some methods have lacked the sensitivity required for the detection of DNA and subsequent nucleotide sequence analysis from body fluids. With the success of clinical trials with candidate OMP vaccines, there is a need for better understanding of the epidemiology of *porA* sequence variation. Although there are eight exposed surface loops (I–VIII) within *porA* (van der Ley *et al.*, 1991), to date, most variation has been observed within loops I, IV and V, otherwise known as variable regions (VRs) 1, 2 and 3 (Molling *et al.*, 2000; van der Ley *et al.*, 1991; Maiden *et al.*, 1991; McGuinness *et al.*, 1993; Clarke *et al.*, 2001b). We have therefore developed a nested PCR method for amplification of the *N. meningitidis porA* gene directly from body fluids and sequenced three variable regions of interest, VRs 1, 2 and 3, to

Abbreviations: CSF, cerebrospinal fluid; OMP, outer-membrane protein; SMPRL, Scottish Meningococcus and Pneumococcus Reference Laboratory; VR, variable region.

indicate their sequence variability. We have compared this information with data gained from culture-based sequencing only and also discussed the impact that this methodology has on the data provided and the implications for vaccine design.

METHODS

Patients and specimens. All patients with suspected meningococcal disease in Scotland between January and June 2001 were included in this study. Samples were sent from microbiology diagnostic laboratories throughout Scotland to the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) and consisted of *N. meningitidis* cultures isolated from blood or CSF or samples of body fluid or tissue for meningococcal PCR.

Phenotypic characterization of *N. meningitidis* strains. All strains were isolated on horse-blood agar (Oxoid) at 37 °C in an atmosphere of 5% CO₂. Serogrouping of *N. meningitidis* was performed by latex agglutination, co-agglutination and *siaD* PCR as described previously (Eldridge *et al.*, 1978; Olcen *et al.*, 1975; Borrow *et al.*, 1997, 1998a). Serotyping and serosubtyping were performed as described previously (Frasch *et al.*, 1985; Clarke *et al.*, 2002).

Genotypic characterization of *N. meningitidis* strains. A liquid-handling robot, the RoboAmp-4204 (MWG-Biotech), was used for all PCR and sequencing set-up procedures. This robot possesses four washable tips, two integrated thermocyclers and an integrated vacuum manifold for high-throughput liquid handling, PCR and DNA clean-up, respectively. An automated 96-capillary DNA sequencer, the Molecular Dynamics MegaBACE 1000 (Amersham Pharmacia Biotech), was used for automated DNA sequencing. Programming of the RoboAmp-4204 liquid-handling system was performed according to the manufacturer's instructions. This allowed the automation of most of the procedures required for the DNA amplification of the *porA* gene and subsequent sequence-labelling from meningococcal isolates. Clinical isolates of *N. meningitidis* were cultured on horse-blood agar (Oxoid) and incubated overnight in the presence of 5% CO₂ at 37 °C. A few fresh colonies were suspended in 0.5 ml 18-MΩ distilled water and boiled for 10 min. The suspension was centrifuged at 15 000 r.p.m. for 2 min and the supernatant was used as a source for the detection of meningococcal DNA. All PCR reagents were maintained at 4 °C on the platform. Each PCR was performed in a final volume of 50 µl using 1.1 × Reddymix PCR master mix, containing 1.25 U *Taq* DNA polymerase (ABgene), 75 mM Tris/HCl (pH 8.8 at 25 °C), 20 mM (NH₄)₂SO₄, 1.5 mM

MgCl₂, 0.01% (v/v) Tween 20 and 0.2 mM each of dATP, dCTP, dGTP and TTP. For a 50 µl reaction, 45 µl PCR master mix and 1 µl of each *porA* primer, PorA-F and PorA-R (Table 1), were added to produce a master mix volume of 47 µl. These pre-prepared master mixes were placed on the RoboAmp-4200 refrigerated reagent rack and the DNA samples were placed on the sample area. Within a refrigerated 96-well microtitre plate, 47 µl master mix was added automatically to appropriate wells using a washable tip along with 3 µl DNA preparation, making a final 50 µl reaction mixture. After each stage of the set-up, the washable tip was washed automatically with 2 ml 18-MΩ distilled water. The microtitre plate was placed automatically into the integrated MWG-Biotech Primus 96 thermocycler. The PCR conditions were described previously (Clarke *et al.*, 2001b) and were 25 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min followed by one cycle of 72 °C for 2 min. After the PCR, the microtitre plate was removed automatically from the thermocycler to a refrigerated block.

Meningococcal PCR from body fluids. Meningococcal PCR requests were received from patients with suspected meningococcal disease. DNA was extracted from whole blood or serum using the Nucleospin Blood kit (ABgene). CSF samples were boiled for 5 min and centrifuged at 15 000 r.p.m. for 2 min and the supernatant was used in the PCR. DNA samples were then processed for a routine *IS1106* PCR assay (Ni *et al.*, 1992; Newcombe *et al.*, 1996) followed by the *ctrA* PCR assay (Diggle *et al.*, 2001a) as described previously but performed on another liquid-handling robot, the RoboAmp-4200 PE, which possesses a non-cross-contamination (NCC) system. The reactions were set up as for the *porA* PCR but respectively with *IS1106* and *ctrA* primers (Table 1). For the *IS1106* PCR, the thermocycler conditions were one cycle each of 37 °C for 10 min and 95 °C for 10 min, followed by 32 cycles of 95 °C for 25 s, 61 °C for 40 s and 72 °C for 1 min. The *ctrA* PCR conditions were one cycle each of 50 °C for 5 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. After the PCR, the *IS1106* PCRs were removed automatically from the thermocycler to a refrigerated block whilst the *ctrA* PCRs were moved automatically to an integrated fluorescence reader. For the *IS1106* PCR, analysis of DNA products was performed by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized on a transilluminator. For the *ctrA* PCR, fluorescence emissions were analysed automatically as described previously (Diggle *et al.*, 2001a).

PCR, nested PCR and DNA sequencing of *porA* from body fluids. DNA extracted from body fluids was subjected to separate standard PCRs and nested PCRs. The standard PCR was performed as for meningococcal cultures (for primers see Table 1). The nested PCR was performed initially with a first-round PCR using primers designed using

Table 1. Primers used for PCR amplification and sequencing of *porA*

Primer	Gene target	Primer sequence (5'→3')	Reference
Amplification primers			
IS1106-1	<i>IS1106</i>	ATTATTCAGACCGCCGGCAG	Newcombe <i>et al.</i> (1996)
IS1106-8	<i>IS1106</i>	TGCCGTCCTGCAACTGATGT	Newcombe <i>et al.</i> (1996)
NMPD1F	<i>porA</i>	CGTAAGCAGATTGGCAGTCAGATTGC	This study
NMPD1R	<i>porA</i>	GAAGACATATCGGGTGTTTGCCCGAT	This study
PorA-F	<i>porA</i>	ATGCGAAAAAACTTACCGCCCTC	Suker <i>et al.</i> (1994)
PorA-R	<i>porA</i>	AATGAAGGCAAGCCGTCAAAAACA	Maiden <i>et al.</i> (1992)
CtrAF	<i>ctrA</i>	TTGTGTGGAAGTTTAATTGTAGGATGC	Guiver <i>et al.</i> (2000)
CtrAR	<i>ctrA</i>	TCAGATTGTTGCCCTAAAGAGACA	Guiver <i>et al.</i> (2000)
Sequencing primers			
porA-SF (103)	<i>porA</i> VR1	AACGGATACGTCTTGCTC	Suker <i>et al.</i> (1994)
porA-SR (8U)	<i>porA</i> VR2 and VR3	TCCGTACGCTACGATTCTCC	Maiden <i>et al.</i> (1992)

the GeneFisher software (<http://bibiserv.techfak.uni-bielefeld.de/genefisher>). The PCR was set up in a final volume of 50 µl using 1 × Reddymix PCR master mix as before but using 1 µl of each of primers NMPD1F and NMPD1R (Table 1). The PCR conditions were 25 cycles of 95 °C for 1 min, 62 °C for 1 min and 72 °C for 2 min followed by one cycle of 72 °C for 2 min. After the PCR, the NCC plate was removed automatically from the thermocycler to a refrigerated block. The second-round PCR was then performed as for the genotypic characterization of *N. meningitidis* strains.

PCR product purification. A 40 µl aliquot of each PCR product was subsequently transferred into a 384-well Millipore MultiScreen 384-PCR filter plate. The plate was transferred automatically onto the integrated vacuum manifold and a vacuum of 450 mbar (approx. 30 cm Hg) was applied for 20 min. The PCR products were then resuspended in 30 µl 18-MΩ distilled water by multi-pipetting a 15 µl volume 20 times. This process removed unused primers and dNTPs.

PCR sequence-labelling. Aliquots of 11 µl of each purified PCR product were transferred to two wells of a 96-well microtitre plate (one well for the forward direction and one well for the reverse direction). To these wells, 8 µl DYEnamic ET premix (Amersham Pharmacia Biotech) was added, followed by 1 µl of the necessary primer at a concentration of 5 pmol to give a final volume of 20 µl. The sequencing cycle was 30 cycles of 92 °C for 20 s, 50 °C for 15 s and 60 °C for 1 min. Appropriate washing of the washable tip occurred with 18-MΩ distilled water throughout the procedure. The plate was placed automatically into the integrated thermocycler. Afterwards, the plate was removed automatically from the thermocycler and placed onto a refrigerated block.

Sequence product purification. All sequence products were adjusted to 20 µl with 0.3 mM EDTA and were transferred into a Millipore MultiScreen 384-SEQ plate and placed on the vacuum manifold. A pressure of 850 mbar was applied for 20 min or until the plate was dry. The product was then washed in 25 µl 0.3 mM EDTA. Pressure was applied again at 850 mbar for 5 min or until the plate was dry. Each sequence product was then resuspended in sterile distilled water with repeat pipetting. At this point, the samples were ready for injection on the MegaBACE DNA sequencer (Molecular Dynamics, Amersham Pharmacia Biotech) as outlined in the manufacturer's instructions.

Sequence interpretation of *porA* gene fragments. The sequence data were read automatically from the MegaBACE sequencer using the integrated image analysis and data collection software. Each gene sequence was downloaded onto a locally compiled database using DiscoverIR (Licor Biosciences UK). After sequence comparisons and appropriate editing, the nucleotide sequences from the *porA* VRs 1 and 2 were translated into amino acid sequences using the program TRANSLATE (<http://ca.expasy.org/tools/dna.html>) and submitted to the *Neisseria meningitidis* PorA Variable Region database (<http://neisseria.org/nm/typing/pora/>) and were based on the scheme of Suker *et al.* (1994). New sequences located in VR1 or VR2 were assigned numbers through this database. Amino acid sequences for PorA VR3 were assigned names according to the same scheme as Suker *et al.* (1994), as performed in previous studies (Molling *et al.*, 2000, 2001; Clarke *et al.*, 2001b).

RESULTS

Phenotypic characterization of *N. meningitidis* strains

Specimens were sent from a total of 555 patients with suspected meningococcal disease in Scotland between January and June 2001. Cultures isolated from blood or CSF were received from patients with meningococcal disease.

There were 35 isolates of *N. meningitidis* from 31 different patients received by the SMPRL for confirmation and typing (Table 2). Twenty-five were from blood and ten were from CSF. They were identified as serogroups B (18), C (11) and W135 (4), with two being non-groupable. Serotype information was not available for 15 isolates but serosubtype information was available for all isolates.

Genotypic characterization of *N. meningitidis* strains

porA gene sequencing was performed on all 35 *N. meningitidis* strains to gain nucleotide sequence information for VRs 1, 2 and 3 (Table 2). All strains were successfully sequenced and provided 21 different *porA* types, the most common being 18-1, 3, 38 and 22, 14, 36.

Meningococcal PCR from body fluids

There were 538 requests for meningococcal PCR between January and June 2001. These were serum (314), plasma (118), CSF (102), endotracheal (ET) aspirate (2) and adrenal tissue (2). Of these, 20 samples from 17 different patients were positive by the IS1106 PCR method; these were from CSF (12), plasma (5), serum (1), ET aspirate (1) and adrenal tissue (1) (Table 3). Using the *ctrA* PCR method, only 13 of these were positive.

PCR, nested PCR and DNA sequencing of *porA* from body fluids

All 20 IS1106 PCR-positive samples were subjected to both the standard *porA* and nested *porA* PCR methods. Using the standard *porA* PCR, only six were positive; however, when the nested *porA* PCR was used, 15 samples from 14 different patients were positive and the DNA was used as a template for nucleotide sequencing. Information relating to the nucleotide sequence of VRs 1, 2 and 3 was gained from all 15 nested *porA*-positive samples (Table 3). Of the seven standard *porA* PCR positives, three were negative by the nested *porA* PCR method and there was insufficient DNA for nucleotide sequencing. Nucleotide sequence data gained from the samples reflected those of the culture isolates. For example, *porA* VR sequences such as 5-1, 10-8, 36b and 18-1, 3, 38 were found, which are respectively common in serogroup C and W135 strains.

DISCUSSION

Surveillance of bacterial infections is important and leads to a better understanding of the epidemiology of disease. Molecular methods have further improved our knowledge of the population biology of some bacteria, and this has been exemplified by the use of multilocus enzyme electrophoresis and multilocus sequence typing (Diggle *et al.*, 2001b). The development of vaccines relies on the availability of such data so that the design represents strains circulating in the community. The use of sequence-based systems for bacterial typing has been highlighted previously (Clarke *et al.*, 2001b,

Table 2. Phenotypic characterization and *porA* gene sequencing results for *N. meningitidis* isolates

Abbreviations: NG, non-groupable; NT, non-typable with mAbs; NST, non-subtypable with mAbs.

Isolate	Isolate type	Phenotypic characterization			<i>porA</i> VR sequence types		
		Serogroup	Serotype	Serosubtype	VR1	VR2	VR3
01-1773	Blood	W135	NT	P1.15	19-1	16	36
01-2667	Blood	C	2a	P1.5	5-1	10-8	36-1
01-1647	Blood	B	NT	P1.12, 13	19	13	35a
01-1624	Blood	B	NT	P1.9	22	9	35a
01-1138	CSF	B	NT	P1.9	22	9	35a
01-1678	CSF	W135	NT	P1.3, 6	18-1	3	38
01-1597	Blood	W135	NT	P1.3	18-1	3	38
01-2362	CSF	B	NT	P1.16	7	4	37
01-1801	CSF	B	15	P1.7, 16	7	16	35
01-1711	Blood	W135	NT	P1.3	18-1	3	38
01-2767	CSF	C	2a	P1.2, 5	5	2	36b
01-1575	Blood	C	2a	NST	5	2	36
01-3328	Blood	B	NT	P1.14	22	14	36
01-1784	Blood	B	NT	P1.3, 6	18-1	3	38
01-1506	Blood	B	1	P1.13	19	13	36
01-2210	Blood	B	NT	P1.9	22	9	35a
01-2398	Blood	B	NT	P1.16	19	16	38-1
01-2757	CSF	B	1	P1.14	22	14	36
01-2729	Blood	B	NT	P1.9	20	9	35a
01-1638	Blood	B	4	NST	19-1	15	36
01-2782	Blood	C	2a	P1.5	5-1	10-8	36-1
01-1446	CSF	B	NT	P1.5	5-1	10-4	36
01-1470	Blood	B	NT	P1.5	5-1	10-4	36
01-1152	Blood	C	2a	NST	5	2	38
01-2782	Blood	C	2a	P1.5	5-1	10-1	36
01-2948	Blood	B	14	P1.14	22	14	36
01-1430	Blood	B	4	P1.16	19	16	38-1
01-2979	Blood	C	2a	P1.5	5-1	10-8	36-1
01-2035	Blood	C	2a	P1.2, 5	5	2	36-1
01-3166	CSF	NG	1	P1.14	22	14	36
01-3167	Blood	NG	1	P1.14	22	14	36
01-3326	Blood	C	2a	P1.5	5-1	10-8	36-1
01-2980	CSF	C	2a	P1.15	5-1	10-8	36-1
01-1308	CSF	B	14	P1.6, 3	18-1	3	38
01-3181	Blood	C	2b	P1.2, 5	5	2	36-1

c; Maiden *et al.*, 1998) and the SMPRL currently provides a national genotyping service for the characterization of *N. meningitidis* cultures by multilocus sequence typing and *porA* gene sequencing (Clarke *et al.*, 2001c). Such systems can often provide rapid results when cultures are available and can be useful for public health management (Clarke *et al.*, 2001b; Feavers *et al.*, 1999). The provision of these data also provides long-term surveillance data that can be used for informing vaccine policy.

There is an urgent need for a vaccine against serogroup B meningococci, as this serogroup is now the most common

cause of infection in many countries (Connolly & Noah, 1999). Since the introduction of meningococcal serogroup C conjugate vaccines in the UK, there has been a decrease in the incidence of serogroup C disease, and serogroup B is therefore even more predominant. OMP vaccines may provide a short-term solution to this problem by providing protection in countries where a limited number of serosubtypes exist, although they are unlikely to eliminate endemic disease. However, information is required on the sequence variation of OMPs and studies of meningococcal cultures have been performed that indicate such variation (Molling *et al.*, 2000; Clarke *et al.*, 2001b). In the present study, we have shown that

Table 3. Serogrouping and serosubtyping data from non-culture-confirmed cases of meningococcal disease

Sample	Sample type	Serogroup	PCR				<i>porA</i> VR sequence types		
			IS1106	<i>ctrA</i>	<i>porA</i>	<i>porA</i> nested	VR1	VR2	VR3
01-1062	ET aspirate	C	+	+	—	+	5-1	10-8	36b
01-1415	CSF	NG	+	+	—	+	7-2	4	37
01-1500	Plasma	NG	+	+	—	+	5-1	10-8	36b
01-1501	Adrenal tissue	NG	+	+	—	+	5-1	10-8	36b
01-1505	CSF	NG	+	+	—	+	5-1	10-4	36b
01-1755	Plasma	NG	+	—	—	+	18-1	3	38
01-1970	Serum	NG	+	—	—	+	7-2	4	37
01-2124	CSF	C	+	+	+	+	5-1	10-8	36b
01-2187	CSF	NG	+	+	—	+	7-2	4	37
01-2711	Plasma	NG	+	+	—	+	5-1	10-8	36b
01-2877	CSF	B	+	+	+	+	22	14	36
01-2981	CSF	C	+	+	+	+	5-1	10-8	36b
01-3183	CSF	C	+	+	—	+	5	2	36b
01-3251	Plasma	NG	+	—	—	+	5	2	36b
01-3368	CSF	NG	+	—	—	+	5-2	10-1	36b
01-1290	Plasma	NG	+	+	—	—	NA	NA	NA
01-1852	CSF	NG	+	+	—	—	NA	NA	NA
01-2124	CSF	NG	+	—	+	—	NA	NA	NA
01-2877	CSF	NG	+	—	+	—	NA	NA	NA
01-2981	CSF	NG	+	—	+	—	NA	NA	NA

NA, Not applicable.

a nested PCR approach can be used to provide nucleotide sequence data and therefore serosubtype information relating to the infecting meningococcus. All IS1106 PCR-positive clinical samples were tested by *ctrA*, *porA* and nested *porA* PCRs. Interestingly, seven samples were *ctrA* PCR-negative. All *ctrA* PCR-negative samples were non-groupable, so this could be explained by meningococci not possessing the genes necessary for capsule expression, as reported by Claus *et al.* (2002). Of the seven *ctrA* PCR-negative samples, three were *porA* PCR-positive and four were nested *porA* PCR-positive; although potential false-positives have been reported when using IS1106 as a target (Borrow *et al.*, 1998b), this was clearly not the case in this study. This would result in three positive results being missed in laboratories using the *ctrA* PCR method even though some laboratories use the fluorescence-based *ctrA* PCR assay (Guiver *et al.*, 2000; Diggle *et al.*, 2001a).

Fifteen samples were nested *porA* PCR-positive and were therefore suitable for nucleotide sequencing. Although three non-nested *porA* PCRs were positive, there was insufficient DNA present for nucleotide sequencing. Nucleotide sequence data from the three VRs analysed indicated variation amongst strains causing invasive disease. The nucleotide sequence data gained after nested *porA* PCR were comparable to those gained from nucleotide sequencing directly from isolates.

The results presented here indicate that a nested *porA* PCR method increased the overall serosubtype data gained from meningococci causing disease in a given population. Without such a method, the amount of information is severely reduced. In the present study, the amount of serosubtype information was increased by 45 % when the nested *porA* nucleotide sequencing results were included, even taking into account multiple specimens from the same patient. Therefore, this method increases the amount of information available for public health management of cases and their contacts. It also provides data for vaccine design, which may have an impact on the design and development of OMP vaccines against serogroup B meningococci.

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