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Amplification of the meningococcal *porB* gene for non-culture serotype characterization

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SUMMARY

Since 1992, the proportion of culture-confirmed meningococcal infections compared with numbers of notified cases of meningococcal disease has decreased in England and Wales. As most meningococcal strain characterization methods depend on a clinical isolate, this has resulted in a loss of epidemiological information. To address this problem, and to aid non-culture diagnosis, a semi-nested PCR protocol for the amplification of the meningococcal *porB* gene from clinical specimens was developed. This gene encodes the meningococcal serotyping antigen; strain typing data was provided by hybridization of allele-specific oligonucleotide probes to the digoxigenin-labelled *porB* amplicon in a 'PCR ELISA'. This assay was specific for meningococcal DNA and sensitivities of 0·81 for cerebrospinal fluid (CSF), 0·57 for serum, and 0·62 for whole blood taken from patients with proven meningococcal infection were obtained.

INTRODUCTION

Confirmation of infection by *Neisseria meningitidis* and strain characterization are necessary for case ascertainment and surveillance of meningococcal disease. Accurate epidemiological data are of particular importance before and during the implementation of new meningococcal vaccines in order to determine vaccine efficacy and to monitor any antigenic change in the organism. In England and Wales since 1992 there has been a growing discrepancy between the numbers of cases of meningococcal disease notified to the Office of National Statistics (ONS) and the numbers of culture-confirmed cases of infection reported to the Communicable Diseases Surveillance Centre (CDSC) [1]. This decline is thought to be due largely to the increasing practice of pre-admission antibiotic treatment of suspected cases of meningococcal infection and a reluctance of clinicians to perform lumbar punctures [2, 3].

Historically, cases of suspected meningococcal disease were confirmed by culture methods, microscopic identification of Gram-negative diplococci in CSF, or antigen detection by latex agglutination [4]. More recently, the polymerase chain reaction (PCR) has been used for the non-culture diagnosis of meningococcal infection by amplification of meningococcal DNA in clinical specimens [5–7] – a technique which is not affected by prior antibiotic treatment [6]. Improved specificity and sensitivity have been achieved by hybridization of amplicons with internal oligonucleotide probes in a colorimetric hybridization assay (PCR ELISA) [8]. Some assays also provide epidemiological information, identifying the sero-

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group (*siaD* PCR ELISA) [9] and serosubtype (*porA* PCR) [10, 11] of the infecting meningococcus.

Serotypes, which are defined by the reaction of antibodies with variants of the class 2 and class 3 (PorB) outer membrane proteins (OMPs), are commonly used for epidemiological studies of the meningococcus [12, 13]. Antibodies to these proteins are opsonizing [14] and PorB proteins are included in a number of outer membrane vesicle (OMV) meningococcal vaccines [15–17]. Structural models if the PorB protein predict eight surface exposed loops (I–VIII) interspersed with conserved membrane-spanning regions [18, 19]. Nucleotide sequence analyses of *porB* alleles from meningococci expressing different serotypes have identified serotype-specific sequences in the regions of *porB* predicted to encode the surface loops of the PorB protein [20, 21]. DNA-based typing methods have been developed that target these serotype-specific sequences with oligonucleotide probes [20].

In this report we describe a typing assay based on the amplification of meningococcal *porB* genes from clinical specimens using a semi-nested PCR, followed by hybridization with a panel of biotinylated type-specific oligonucleotide probes in a colorimetric solution hybridization assay (PCR ELISA). The panel of probes developed identified the *porB* alleles most frequently associated with invasive meningococci in England and Wales. This provided a method for non-culture confirmation of meningococcal disease combined with serotype identification of the infecting organism.

METHODS

Bacterial strains and clinical specimens

N. meningitidis isolates were from a collection of strains referred to the Meningococcal Reference Unit (MRU) at Manchester Public Health Laboratory, UK, for immunological characterization. Other bacterial species (*N. canis*, $n = 1$; *N. cavaie*, $n = 1$; *N. cinerea*, $n = 1$; *N. cuniculo*, $n = 1$; *N. denitrificans*, $n = 1$; *N. elongata*, $n = 2$; *N. gonorrhoeae*, $n = 3$; *N. lactamica*, $n = 5$; *N. mucosa*, $n = 2$; *N. ovis*, $n = 1$; *N. pharyngis*, $n = 1$; *N. subflava*, $n = 3$; *N. sicca*, $n = 1$; *N. flavescens*, $n = 2$ and *Moraxella catarrhalis*, $n = 7$) were obtained from the MRU collection or from the UK National Collection of Type Cultures (NCTC). Strains were propagated overnight on heated blood agar plates, using an atmosphere of 5% CO_2 for *Neisseria* species.

Serum and CSF samples from patients with proven and suspected meningococcal disease were from those submitted to the MRU. Samples from proven cases of meningococcal disease were those where a clinical diagnosis by the referring physician was confirmed by at least one of the following from CSF or blood samples: (i) isolation of meningococci; (ii) microscopic detection of Gram-negative diplococci; (iii) a positive latex agglutination reaction for meningococcal capsule. Samples from patients with clinical symptoms of meningococcal disease (fever and haemorrhagic rash), but without laboratory confirmation, were defined as coming from cases of suspected meningococcal disease. CSF samples from patients with meningitis of other aetiologies were obtained from the Karim Centre for Meningitis Research, London, UK. These included *Escherichia coli* ($n = 3$), group B streptococci ($n = 2$), *Haemophilus influenzae* ($n = 2$) and *Streptococcus pneumoniae* ($n = 2$). The specimens in this work were not blinded.

Sample preparation

Bacterial suspensions and clinical specimens were treated prior to PCR as follows: approximately 50 μl of each sample, plus 50 μl of PCR reaction buffer (Gibco–BRL) for serum samples, was boiled for 15 min and then chilled on ice for 15 min. The samples were centrifuged at 12000 ***g*** for 10 min and the supernatant fraction collected. Meningococcal DNA was extracted from 2·5 ml EDTA blood samples by a semi-automated extraction procedure (Extragen; VH Bio Ltd, UK) using the manufacturer's protocol.

PCR Primers and oligonucleotide probes

PCR primers complementary to conserved sequences in the coding region of *porB* were designed using the Primerselect package (DNASTAR, Inc., Madison, USA). For first round amplification, primer 23F was located in the part of the gene encoding the signal peptide and primer 3R was located in conserved region 8 of *porB* [21]. For second round amplification, a primer 2F, which was internal to 23F but still in the signal peptide domain, was used with primer 3R (Table 1).

Oligonucleotide probes complementary to allele-specific *porB* nucleotide sequences were designed from unpublished and published *porB* sequences (EMBL databank accession numbers: m68962, u07193, x65530, x65531, x65534, x67929, x67930, x67931, x67932, x67933, x67934, x67935, x67936, x67937,

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Table 1. *The sequences and locations within the* porB *gene of the synthetic oligonucleotides used for the* porB *semi-nested PCR and* porB *PCR ELISA*

Name	Oligonucleotide base sequence (5′–3′)	Location in *porB* gene*
PCR primers		
23F	AAATCCCTGATTGCCCTGAC	Signal peptide
3R	CAACCAACCGGCAGAAACCA	Conserved region 8
2F	GCCCTGACACTGGCTGCCC	Signal peptide
Oligonucleotide probes		
Class 2	ACTGGGTACTATCGGTCGA	Loop IV
Class 3	GAACCCAAATCAACGATGCC	Conserved region 2
Type 1	ATCGTTGTTGTAGTTTGTAGC	Loop VII
Type 2a	GTTGGTTCTACCAAGGGTAA	Loop VI
Type 2b	TAACACTGGTACTGTCGGCA	Loop VI
Type 4	TGGAGGTCAGGTGGT	Loop I
Type 4/21	GATGCAAAACGCGACAATAC	Loop VII
Type 15	CTGACTGATGCTTCCA	Loop V
Type 22	ATTGGCAGTACCATC	Loop VI

* The positions of the oligonucleotides, relative to the conserved and loop-encoding regions of the *porB* gene are indicated [18, 21].

x67938, x67939). The panel of oligonucleotide probes included: class 2 and class 3 specific probes that were *porB* amplification positive controls and provided limited epidemiological information about non-typable (by PCR ELISA) isolates; seven probes specific for *porB* alleles from serotypes 1, 2a, 2b, 4, 4/21, 15 and 22. Probe types 2a, 2b, 4 and 15 represented the most frequently identified serotypes in England and Wales (22) (Table 1). The PCR primers and 5′ biotinylated oligonucleotide probes were synthesized by Oswell DNA Services, Southampton.

DNA amplification

Reaction components for first round amplification were: 5 μl sample; PCR reaction buffer (Gibco–BRL); 50 μM each of dATP, dCTP, dGTP, dTTP; 3 mM $MgCl_2$; 5 nM of PCR primers 23F and 3R; 1 unit of *Taq* DNA polymerase (Gibco–BRL) in a total volume of 50 μl. The reactions were incubated at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min for 30 cycles followed by a final incubation of 72 °C for 1 min.

The second round reaction components were: 5 μl of the products of first round amplification; PCR reaction buffer; PCR DIG labelling mix (Boehringer–Mannheim); 1·5 mM $MgCl_2$; 1 μM of PCR primers 2F and 3R; 1·25 units of *Taq* DNA polymerase in 50 μl reaction. The reactions were incubated for 30 cycles at 94 °C for 2 min, 60 °C for 2 min and 72 °C for 3 min. At the end of the 30 cycles, the reactions were incubated at 72 °C for a further 3 min. The amplified products (900–1000 bp) [21] were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Solution hybridization and detection by PCR ELISA

The digoxigenin-labelled *porB* genes obtained from the second round amplification were hybridized separately to each of the panel of allele-specific biotin-labelled oligonucleotide probes and detected by colorimetric reaction with the Boehringer–Mannheim PCR ELISA kit, used in accordance with the manufacturer's protocol. A meningococcal *porB* PCR positive control, a negative PCR control, and a negative detection control (sterile deionized water) were included in all assays. Positive reactions were defined as those with A_{405} values more than twice that obtained with the negative detection control. Positive *porB* detection was scored if the amplification product reacted with either the class 2 or the class 3 specific probe and DNA-type was determined by positive reaction with the appropriate probe.

RESULTS

PCR amplification of meningococcal DNA and *porB* typing by PCR ELISA

Depending on the source isolate, amplification of meningococcal DNA with the nested PCR protocol

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Table 2. *The identification of meningococcal DNA in clinical samples using the* porB *PCR ELISA*

Sample*	Meningococcal disease proven by†			Clinically suspected meningococcal disease†	Meningitis caused by other bacteria
	Culture	Latex test or microscopy	Total		
CSF	17/19	14/19	31/38 (0·81)	25/47 (0·53)	0/9 (0·0)
Serum	11/18	2/5	13/23 (0·57)	11/30 (0·37)	—
Whole blood	11/17	2/4	13/21 (0·62)	21/37 (0·57)	—

* Samples were: cerebrospinal fluid (CSF); acute serum taken on admission to hospital (serum) and extracted whole blood DNA (whole blood).

† The number of positive samples out of the total number of each type of sample tested is given, the number in brackets represents the proportion of positives.

gave a single amplicon equivalent in size to either the class 2 or the class 3 OMP gene [21]. Serial dilution of meningococcal suspensions established a detection limit of 1–2 colony forming units for the PCR ELISA using the class 2 and class 3 oligonucleotide probes. Hybridization with the type-specific oligonucleotide probes was compatible with serotype for each of the isolates of known serotype examined by the PCR ELISA.

Specificity of the meningococcal *porB* PCR and PCR ELISA assay

When applied to cell suspensions of other *Neisseria* and *Moraxella* species, the nested PCR protocol amplified products detectable by agaorse gel electrophoresis and ethidium bromide staining from only three species: *N. flavescens* (2/2); *N. gonorrhoeae* (3/3); and *N. lactamica* (5/5). Although these products were of a similar size to those obtained from meningococcal cell suspensions, they did not hybridize with either the class 2 or class 3 specific oligonucleotide probes in the PCR ELISA. None of the specimens tested had positive hybridization reactions with any of the *porB* allele-specific probes.

The *porB* PCR ELISA for the detection of meningococcal DNA in clinical samples

The PCR ELISA results obtained from 82 clinical specimens from cases of proven meningococcal infection and 114 clinical specimens from cases of suspected meningococcal infection are shown in Table 2. In five cases of proven meningococcal infection and five cases of suspected meningococcal infection, at least two types of clinical sample from each patient were tested in the PCR ELISA. A positive PCR ELISA result was obtained from at least one specimen in four of the five proven cases; one negative result was obtained from a proven case for which no CSF specimen was available for testing. Positive PCR ELISA results were obtained from both CSF and whole blood DNA samples taken from two of five suspected cases of meningococcal infection. The assay was also applied to nine clinical specimens from cases of meningitis of other aetiologies: none of these were positive by EtBr staining of agarose gels, on which the reaction products had been separated, or by PCR ELISA.

Type-specific oligonucleotide probe hybridization for *porB* characterization

Serotyping data was available from an appropriate meningococcal isolate for 41 of the clinical specimens from cases of proven meningococcal infection which tested positive by the PCR ELISA. These data were compared with the DNA-type obtained by the PCR ELISA. Six isolates were not serotypable but specimens could be DNA-typed by the *porB* PCR ELISA, five being type 4 and one type 4/21. There was agreement between serotype and *porB* DNA-type data for 32 isolates: 15 type 2a; four type 2b; 11 type 4; and two type 15. One serotype 15 and two serotype 2a isolates hybridized correctly with the class 3 specific probe and the class 2 specific probes, but were not recognized by any of the type specific probes in the PCR ELISA.

The *porB* DNA types of the 57 specimens taken from cases of suspected meningococcal disease that were PCR ELISA positive were as follows: two type 1; 14 type 2a; 10 type 2b; five type 4; three type 4/21; three type 15; and four type 22. Six samples were positive with the class 2 specific probe only; 10

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samples were positive with the class 3 specific probe only. None of the specimens tested that were negative with the class 2 and class 3 probes had positive hybridization reactions with any of the *porB* allele-specific probes.

Application of the *porB* PCR ELISA in an outbreak investigation

The PCR ELISA was used to assist investigation of an outbreak of meningococcal disease in an English secondary school comprising three suspected cases. No isolate was obtained from the first two suspected cases of infection, both of which were fatal, and blood samples from the second and third cases of disease were sent to the MRU for testing in the *porB* PCR ELISA and the *siaD* PCR ELISA [9]. In both cases the invasive organisms were identified as C:2a strains. An isolate was obtained 24 h later from a throat swab taken from the third case. Serological testing confirmed the isolate as C:2a:P1.5. These antigenic characteristics were consistent with strains belonging to the ET-37 complex that are known to cause localized outbreaks of meningococcal disease [23]. As the cases were potentially related, serogroup C vaccination was implemented in the school following this investigation.

DISCUSSION

With the decline in the number of culture-confirmed cases of meningococcal disease, PCR-based methods for the detection of meningococcal DNA in clinical specimens have been developed to improve case ascertainment. A number of different loci have been targeted including the dihydropteroate synthase gene (*dhps*) [5], an insertion sequence IS*1106* [6] and the 16S rRNA gene [7]. Although these tests can aid diagnosis of meningococcal infection, they provide no further epidemiological information.

Antigenic characteristics used as epidemiological markers for meningococcal disease include the serogroup (capsule), serotype (PorB protein) and serosubtype (PorA protein). A *siaD* PCR assay has been developed which identifies the presence of serogroup B or C in meningococci in clinical specimens [9]. Saunders and colleagues amplified and determined the nucleotide sequences of meningococcal *porA* genes from CSF samples [11]. This and subsequent *porA* diagnostic PCRs [10] have been used to determine the subtype of cases of meningococcal infection during clinical trials of vaccine efficacy. In this study we evaluated a semi-nested PCR ELISA for the detection and typing of meningococcal *porB* genes from clinical specimens taken from patients with suspected meningococcal disease. The *porB* gene was chosen for characterization as it encodes the serotype antigen and therefore complements the *siaD* and *porA* methods.

The *porB* PCR ELISA was both sufficiently sensitive and specific to provide reliable *porB* DNA typing information pertinent to epidemiological investigations. Fifty-seven of 114 (50%) clinical samples taken from patients with suspected but unconfirmed meningococcal disease tested positive in the *porB* PCR ELISA. In addition to providing evidence for meningococcal infection in patients these data provided the *porB* DNA type of the putative infecting strains.

The sensitivity of the *porB* PCR ELISA may not be as great as microbiological culture, for several reasons. Firstly, the PCR may be compromised by the different volumes examined in each test, which range from 10–20 ml for blood culture to 50–200 μl for PCR. Further, in this study specimens for PCR testing were often collected some time after the initial specimen that was taken for culture, and specimens taken from a patient several days after the administration of antibiotics may contain less meningococcal DNA [2]. Thirdly, specimens used in this study had been archived frozen and undergone repeated freeze–thaw cycles, possibly leading to the loss of template. This assay has most value in cases where microbiological culture is not possible and is optimally used on fresh clinical samples.

The failure of three of the serotyped organisms to be characterized by PCR ELISA other than as class 2 and class 3 was probably a result of genetic variation in the regions targeted by the probes that did not change the reaction of the PorB proteins with the serotyping mAbs. The insensitivity of some serotyping mAbs has been reported [24, 25] and has been demonstrated to be due in part to genetic variation amongst isolates [20, 21]. Identification of *porB* at a genetic level circumvents the reagent or assay bias of serological methods and in this study *porB* was characterized for six isolates that were non-serotypable with mAbs. This PCR-based approach would permit the characterization of novel *porB* genes, unidentifiable with the current panel of oligonucleotide probes, by application of direct nucleotide sequence determination. The oligonucleotide probe

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panel could be expanded readily once a novel allele sequence was identified.

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REFERENCES

1. Kaczmarski EB. Meningococcal infections in England and Wales. CDR Rev 1997; **7**: R55–9.
2. Cartwright KAV, Reilly S, White DA, Stuart JM. Early treatment with parenteral penicillin in meningococcal disease. BMJ 1992; **305**: 143–7.
3. Wylie PAL, Stevens D, Drake W, Stuart J, Cartwright KAV. Epidemiology and clinical management of meningococcal disease in west Gloucestershire: retrospective, population based study. BMJ 1997; **315**: 774–9.
4. Hart CA, Rogers TRF. Meningococcal disease. J Med Microbiol 1993; **39**: 3–25.
5. Kristiansen B-E, Ask E, Jenkins A, Fermér C, Rådstrøm P, Sköld O. Rapid diagnosis of meningococcal meningitis by polymerase chain reaction. Lancet 1991; **337**: 1568–9.
6. Ni H, Knight AI, Cartwright KAV, Palmer WH, McFadden J. Polymerase chain reaction for diagnosis of meningococcal infection. Lancet 1992; **340**: 1432–4.
7. Rådstrøm P, Backman A, Qian N, Kragsbjerg P, Pahlson C, Olcén P. Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and streptococci using a seminested PCR strategy. J Clin Microbiol 1994; **32**: 2738–44.
8. Davison E, Borrow R, Guiver M, Kaczmarski EB, Fox AJ. The adaptation of the IS*1106* PCR to a PCR ELISA format for the diagnosis of meningococcal infection. Serodiagn Immunother 1996; **8**: 51–6.
9. Borrow R, Claus H, Guiver M, et al. Non-culture diagnosis and serogroup determination of meningococcal B and C infection by a sialyltransferase (*siaD*) PCR ELISA. Epidemiol Infect 1997; **118**: 111–7.
10. Caugant FA, Høiby EA, Frøholm LO, Brandtzaeg P. Polymerase chain reaction for case ascertainment of meningococcal meningitis: application to the cerebrospinal fluids collected in the course of the Norwegian serogroup B protection trial. Scand J Infect Dis 1996; **28**: 149–53.
11. Saunders NB, Zollinger WD, Rao VB. A rapid and sensitive PCR strategy employed for amplification and sequencing of *porA* from a single colony-forming unit of *Neisseria meningitidis*. Gene 1993; **137**: 153–62.
12. Poolman JT, Lind I, Jonsdottir K, Froholm LO, Jones DM, Zanen HC. Meningococcal serotypes and serogroup B disease in north-west Europe. Lancet 1986; **ii**: 555–7.
13. Abdillahi H, Poolman JT. Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. FEMS Microbiol Lett 1987; **48**: 367–71.
14. Guttormsen H-K, Bjerknes R, Halstensen A, Naess A, Høiby EA, Solberg CO. Cross-reacting serum opsonins to meningococci after vaccination. J Infect Dis 1993; **167**: 1314–9.
15. Bjune G, Høiby EA, Gronnesby JK, et al. Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. Lancet 1991; **338**: 1093–6.
16. Sierra GVG, Campa HC, Varcacel NM, et al. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. NIPH Ann 1991; **14**: 195–207.
17. Zollinger WD, Boslego J, Moran E, et al. Meningococcal serogroup B vaccine protection trial and follow-up studies in Chile. The Chilean National Committee for Meningococcal Disease. NIPH Ann 1991; **14**: 211–2.
18. Maiden MCJ, Suker J, McKenna AJ, Bygraves JA, Feavers IM. Comparison of the class 1 outer membrane proteins of eight serological reference strains of *Neisseria meningitidis*. Mol Microbiol 1991; **5**: 727–36.
19. van der Ley P, Heckels JE, Virji M, Hoogerhout P, Poolman JT. Topology of outer membrane proteins in pathogenic *Neisseria* species. Infect Immun 1991; **59**: 2963–71.
20. Bash MC, Lesiak KB, Banks SD, Frasch CE. Analysis of *Neisseria meningitidis* class 3 outer membrane protein gene variable regions and type identification using genetic techniques. Infect Immun 1995; **63**: 1484–90.
21. Feavers IM, Suker J, McKenna AJ, Heath AB, Maiden MCJ. Molecular analysis of the serotyping antigens of *Neisseria meningitidis*. Infect Immun 1992; **60**: 3620–9.
22. Jones DM, Kaczmarski EB. Meningococcal infections in England and Wales. CDR Rev 1995; **2**: R125–30.
23. Wang J-F, Caugant DA, Morelli G, Koumaré B, Achtman M. Antigenic and epidemiological properties of the ET-37 complex of *Neisseria meningitidis*. J Infect Dis 1993; **167**: 1320–9.
24. Poolman JT, Kriz Kuzemenska P, Ashton F, et al. Serotypes and subtypes of *Neisseria meningitidis*: results of an international study comparing sensitivities and specificities of monoclonal antibodies. Clin Diagn Lab Immunol 1995; **2**: 69–72.
25. Wedege E, Caugant DA, Frøholm LO, Zollinger WD. Characterization of serogroup A and B strains of *Neisseria meningitidis* with serotype 4 and 21 monoclonal antibodies and by multilocus enzyme electrophoresis. J Clin Microbiol 1993; **29**: 1486–92.

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