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Non-culture diagnosis and serogroup determination of meningococcal B and C infection by a sialyltransferase (*siaD*) PCR ELISA

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SUMMARY

Rapid, non-culture, serogroup determination of meningococcal infection is important in contact management where vaccination may be possible. The impending availability of polysaccharide–protein conjugate vaccines for serogroup C disease requires maximal case ascertainment, with serogroup determination, at a time when the number of culture confirmed meningococcal infections is decreasing. A polymerase chain reaction assay (PCR), based on a restriction fragment length polymorphism (RFLP) in the meningococcal serogroup B and C sialyltransferase (*siaD*) gene, was developed to combine the non-culture diagnosis of meningococcal infection from CSF, whole blood and serum with serogroup (B and C) identification. The PCR assay was adapted to an ELISA format incorporating hybridization with serogroup-specific B and C oligonucleotide probes. Specificity for CSFs was 100% and sensitivities were respectively 81, 63 and 30% for CSFs, whole blood and sera. The serogroup-specific PCR ELISA is a significant addition to currently available tests for non-culture diagnosis of meningococcal infection and outbreak investigation.

INTRODUCTION

Following the introduction of the *Haemophilus influenzae* type b conjugate vaccine, *Neisseria meningitidis* is now the major cause of bacterial meningitis in the UK and elsewhere [1, 2]. In England, Wales and Scotland each year, increasing proportions of suspected meningococcal infections remain unconfirmed by culture, due in part to the increasing practice of pre-admission administration of parenteral antibiotics and reluctance to perform lumbar punctures [3]. Evidence for this is provided by the growing differential between the number of culture proven case isolates referred to the Public Health Laboratory Service (PHLS) Meningococcal Reference Unit (MRU) and the number of notified cases of meningococcal disease recorded by the Office of Population Censuses and Surveys (OPCS) [2].

The relative under-ascertainment of laboratory confirmed cases can be compensated to some extent by serodiagnosis [2]. Presence of raised antibody levels enables a retrospective diagnosis of meningococcal infection to be made and anti-polysaccharide antibody estimation can yield useful epidemiological data [2]. Combining these assays is labour intensive and the diagnosis remains retrospective. Ideally a non-culture laboratory method should not only establish the diagnosis but also characterize the infecting organism at least as rapidly and as precisely as conventional methods. In this way the data available would be similar to those obtained by isolation of the organism; yielding epidemiologically reliable information and indicating the possibility for deployment of vaccines for control. With the anticipated availability of a variety of novel meningococcal vaccines, complete ascertainment of cases remains important for

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estimates of vaccine efficacy and an appropriate non-culture method would make an important contribution.

The similarity between the biochemical nature of meningococcal polysialic acid B (α2-8 linked sialic acid) and C (α2-9 linked sialic acid) capsules [4] has hampered the identification of target sequences to differentiate meningococci expressing serogroup B and C capsules. Hybridization occurs between serogroup B, C, W135 and Y capsular genes involved in directing sialic acid synthesis, but not the serogroup-specific sialyltransferases involved in the polymerization of the sialic acid to the polysialic acid chain [5]. Nucleotide sequencing of the sialyltransferase (*siaD*) genes of serogroup B and C meningococci [6] has enabled identification of sequence differences allowing the specific identification of serogroup B or C *siaD* genes.

PCR has been extensively employed in the direct detection of microbiological pathogens from clinical samples including the detection of *N. meningitidis* [7–11]. One such PCR assay for the amplification of the novel meningococcal insertion sequence *IS1106* within CSF specimens [7] has recently been adapted to a colorimetric hybridization assay (PCR ELISA) format [12]. None of the PCRs described to date provides epidemiologically useful information about the causative organism.

This report describes the identification of a *siaD* gene RFLP which appears stable among serogroup B and C meningococci and the development of a serogroup B and C specific PCR based assay for the non-culture diagnosis of meningococcal infection in a colorimetric hybridization assay (PCR ELISA) format. The initial findings include the specificity and sensitivity achieved by the *siaD* PCR ELISA for the detection and determination of the serogroup by examination of meningococcal DNA in CSF, whole blood and serum.

METHODS

Bacterial strains and culture methods

Bacterial strains used were isolates of *Neissera meningitidis* referred to the PHLS MRU (Meningococcal Reference Unit, Manchester Public Health Laboratory, Withington Hospital, Manchester M20 2LR, UK), Neisserial species from the National Collection of Type Cultures (NCTC, 61, Colindale Avenue, London NW9 5HT, UK) and isolates of *Escherichia coli* K1 received from PHLS Food and Enteric Reference Divisions (PHLS Central Public Health Laboratory, 61, Colindale Avenue, London NW9 5HT, UK). Organisms were cultured on blood agar overnight at 37 °C (in an atmosphere of 5% CO_2 if *Neisseria* species). *N. meningitidis* positive control strains for serogroup B and C were H44/76 (B15P1.7, 16) and Men-C11 (C16P1.1).

Bacterial suspensions

Freshly cultured organisms were suspended in sterile water and the suspension adjusted to an A_{650} equal to an O.D. of 0·1 and diluted 1/1000 to yield approximately 100 cells/reaction (5 μl). Dilution series were prepared from these bacterial suspensions for the preparation of 'spiked' CSFs from patients with non-infectious central nervous system (CNS) disease.

Serogrouping by co-agglutination

Serogrouping of *N. meningitidis* isolates was performed by co-agglutination using rabbit polyclonal serogroup-specific antisera as previously described [13].

Treatment of CSF and serum samples

CSF samples were collected from patients with culture proven and suspected meningococcal meningitis and stored at −20 °C. Aliquots (50 μl) were boiled for 15 min, chilled and centrifuged for 5 min at 14000 g. An aliquot (5 μl) of treated CSF was then used directly in a 50 μl PCR reaction. The sera examined were from patients with culture proven and suspected meningococcal disease which had been aliquoted and stored at −20 °C. Aliquots (50 μl) were mixed with 50 μl of 1 × PCR buffer (Gibco Life Technologies, Inchinnan Business Park, Paisley PA4 9RF, UK), boiled for 15 min, rapidly chilled and centrifuged for 5 min at 14000 g. An aliquot (5 μl) of treated serum was then used directly in a 50 μl PCR reaction. DNA was extracted from EDTA bloods (2·4 ml) using an Extractagen 8C machine (VH Bio Ltd, Gosforth, Newcastle upon Tyne NE3 4DB, UK). An aliquot (5 μl) was used directly in a 50 μl PCR reaction.

PCR primers and probes

Degenerate primers and probes (synthesized by Oswel DNA Services, Lab 5005, University of Southampton, Southampton SO16 7PX, UK) complementary to the serogroup B and C *siaD* sequences were designed

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(Primerselect package, DNASTAR Inc., Madison, USA) based upon the published serogroup B *siaD* nucleotide sequence (EMBL Accession No. M64289) [5] and the serogroup C *siaD* nucleotide sequence (personal communication, M. Frosch).

The *siaD* forward and reverse primers were as follows:

5′ AYATWTTGCATGTMSCYTTYCCTGA 3′
5′ AGACATTGGGTWGWRGGKGARAGTAA 3′

The internal probes specific for serogroup B and C *siaD* nucleotide sequences were:

Serogroup B probe
5′ AGGCCTGGCCTCTAGTTCTTTAA 3′
Serogroup C probe
5′ TGGACTGACA*TCGA*CTTCTATTG 3′
Taq I
restriction site

DNA amplification

All PCR amplifications were performed in a final volume of 50 μl containing 2 units of *Taq* DNA polymerase (Gibco Life Technologies), 3·5 mM $MgCl_2$, 5 μl of 10 × PCR buffer (Gibco Life Technologies), deoxynucleoside triphosphates (dNTPs) (200 μM each), two primers (0·5 μM each), 0·02% W-1 detergent (Gibco Life Technologies) and 5 μl of specimen. DNA amplifications were performed using the following cycling parameters: denaturation of 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 25 s, annealing at 52 °C for 40 s, and extension at 72 °C for 1 min. A 5 min extension at 72 °C was included at the end of the final cycle.

An aliquot (10 μl) of the amplified product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide (EtBr) staining. Amplified products were sized in comparison with a 100-bp ladder (Pharmacia Biotech, St Albans, Hertfordshire AL1 3AW, UK) and a positive result recorded if a 460-bp product was present. Negative (water) controls were included in every set of PCR reactions.

Identification of an RFLP in the *siaD* gene

To 15 μl of *siaD* PCR product, 1 μl of *Taq* I (Northumbria Biologicals Ltd (NBL), Nelson Industrial Estate, Cramlington, Northumberland NE23 9BL, UK), 3 μl of 10 × restriction digest buffer (NBL) and 11 μl of deionized, double distilled water were added. Mineral oil (50 μl) was overlaid on each reaction. The reaction digest was then placed overnight at 65 °C. The digests were then analysed by agarose gel electrophoresis and EtBr bromide staining using a 100-bp ladder.

Specificities and sensitivities

To determine the specificity, amplification reactions were performed using boiled preparations from other *Neisseria* species (*N. subflava* ($n = 2$), *N. flavescens*, *N. sicca*, *N. ovis*, *N. caviae*, *N. cinerea*, *N. cuniculi*, *N. dinitrificans*, *N. elongata*, *N. elongata ssp. glycoly*, *N. gonorrhoeae*, *N. lactamica* ($n = 3$), *N. mucosa* var. *heidelberg* and *Moraxella catarrhalis*).

Meningococcal isolates of serogroups other than B or C (NG ($n = 6$), X ($n = 2$), W135 ($n = 3$), Z/29E ($n = 3$), Y ($n = 3$), H ($n = 4$) and A ($n = 3$)) were also examined as well as CSFs from patients with non-meningococcal meningitis (*Streptococcus pneumoniae* ($n = 5$), *E. coli* ($n = 2$), group B streptococcus ($n = 1$), and *H. influenzae* ($n = 7$)). Boiled preparations from two *E. coli* K1 isolates were also examined.

The sensitivity of the PCR was determined using CSF, whole blood and sera specimens from patients with proven meningococcal infection. Cases of proven meningococcal infection were defined as meningococci isolated from a sterile site (CSF or blood) or nasopharyngeal swab, in the presence of clinical symptoms of invasive meningococcal disease, and/or clinical meningococcal infection with detection of Gram-negative diplococci by microscopy or positive latex agglutination.

Solution hybridization by PCR ELISA

Digoxigenin labelling

The amplification reaction mixture was prepared as above except that the dNTPs were used at the following concentrations: 160 μM dATP, dGTP and dCTP, 150 μM dTTP and 8 μM digoxigenin-labelled dUTP.

Digoxigenin detection

Digoxigenin-labelled PCR products were detected according to the Boehringer Mannheim PCR ELISA protocol (Boehringer Mannheim, Lewes, East Sussex BN7 1LG, UK). Briefly, an aliquot of PCR product was denatured and hybridized separately with a serogroup B or C biotinylated capture probes (50 ng/ml of each probe) to allow the immobilization of the probe to a streptavidin-coated microtitre plate. The bound hybrid was detected by an anti-digoxigenin peroxidase conjugate followed by the addition of the

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colorimetric substrate ABTS (2,2 azino-di-(3-ethylbenzthiazoline-sulfonate). Qualitative controls were included in all ELISAs, two positive meningococcal PCR (serogroup B and C) controls, a negative PCR control and a negative detection control.

Positive samples were determined by a calculation of cut off values as follows:

Cut off $= 2 \times A_{405}$ of the negative detection control (water).

The detection limit of the PCR ELISA was determined, using serial dilutions of viable cells of serogroup B (H44/76) and C (Men-C11) meningococci.

RESULTS

Specific amplification of *siaD* gene fragments from serogroup B and C meningococci

Amplification of meningococcal DNA using degenerate primers identified from the *siaD* nucleotide sequence data gave rise to products of 460-bp when visualized by EtBr agarose gel electrophoresis for all isolates of serogroup B and C meningococci examined (Fig. 1).

Differentiation between serogroup B and C amplicons using *Taq* I specific RFLPs

Analysis of the restriction map of the *siaD* sequence revealed two *Taq* I restriction sites unique to the serogroup C *siaD* gene enabling distinction between serogroup B and C meningococci. From the *Taq* I digest of serogroup C *siaD* products the expected DNA fragments of 200, 207 and 53-bp were produced whereas the serogroup B *siaD* product remained at 460-bp (Fig. 1) in agreement with expected size fragments from the restriction map of the serogroup C and B sequences. No amplification products were observed for isolates of serogroups other than B or C with the exception of two non-groupable and one serogroup H isolates which were not associated with clinical disease (data not shown). As can be seen from Figure 1, this RFLP appeared stable among 12 serogroup B and C meningococci of diverse phenotypes.

Colorimetric hybridization assay using serogroup B and C specific biotinylated oligonucleotide probes

Amplification reactions were performed using boiled suspensions of *N. meningitidis*. All serogroup B and C

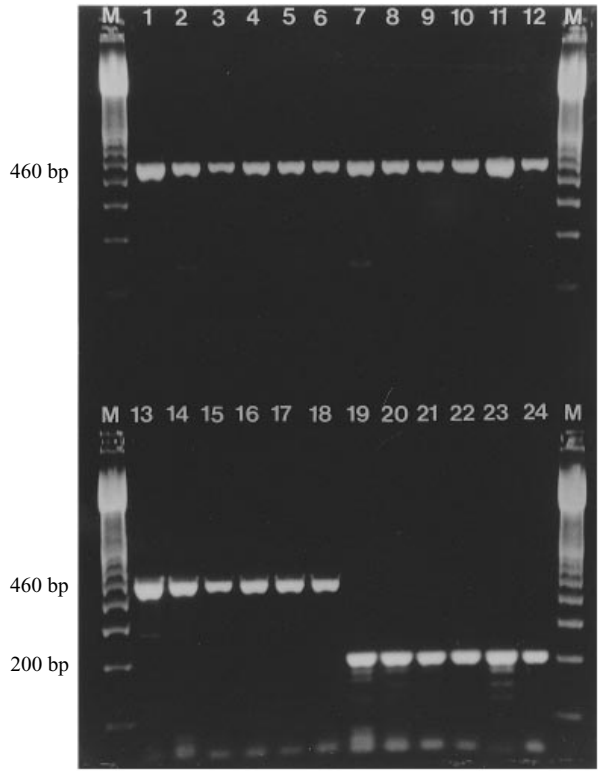

Fig. 1. *Taq* I digests of *siaD* PCR amplicons of serogroup B and C meningococci of diverse phenotypes. Lanes M 100-bp ladder; Lanes 1–12 Uncut *siaD* PCR products; Lanes 13–24 *Taq* I digests; Lanes 1 and 13 B:15:P1.7,16R; Lanes 2 and 14 B:15:P1.15S; Lanes 3 and 15 B:1:P1.5S; Lanes 4 and 16 B:4:P1.10R; Lanes 5 and 17 B:4:P1.4S; Lanes 6 and 18 B:2b:P1.10S; Lanes 7 and 19 C:16:P1.1S; Lanes 8 and 20 C:2b:P1.2,5R; Lanes 9 and 21 C:2a:P1.2,5S; Lanes 10 and 22 C:NT:P1.9S; Lanes 11 and 23 C:2a:NTS; Lanes 12 and 24 C:2a:P1.5S.

meningococci of diverse serotype or serosubtype examined were positive by *siaD* PCR ELISA for their respective serogroup as serologically determined. All meningococci of serogroups other than B or C were negative on PCR ELISA with the exception of a serogroup H isolate. This isolate gave a positive reaction with the serogroup C probe. Two non-groupable (by co-agglutination) meningococci serogrouped by PCR ELISA as serogroup B. All other *Neisseria* species as well as the *E. coli* isolates were negative by *siaD* PCR ELISA.

SiaD PCR ELISA for detection of serogroup B and C meningococci in clinical specimens

Initial estimates show the *siaD* PCR to have a specificity of 100% and a sensitivity of 81% (17/21) for CSFs from proven cases. The sensitivities for

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Table 1. SiaD *analysis of clinical samples from patients with proven meningococcal disease* (*culture-positive, latex positive or Gram-negative diplococci by microscopy*)

Isolate	Latex	GNDC*	Specimen	*SiaD* results
C:NT†:P1.5,2	Negative	No	CSF‡	Negative
C:2a:P1.5			CSF	Negative
B:NT:NT	Negative	Yes	CSF	B
		Yes	CSF	B
	A+C	Yes	CSF	C§
B:14:P1.6			CSF	B
C:2b:P1.5,2	A+C	Yes	CSF	C
B:15:NT	Negative	Yes	CSF	B
		Yes	CSF	B§
B:15:P1.13	Negative	Yes	CSF	B
C:2a:NT	A+C		CSF	C
	B+*E. coli*	Yes	CSF	B§
C:2a:P1.5	A+C	Yes	CSF	C
	A+C	Yes	CSF	C§
B:4:P1.4			CSF	Negative
	A+C	Yes	CSF	C§
		Yes	CSF	B§
		Yes	CSF	B§
B:15:P1.13	Negative	Yes	CSF	B
		Yes	CSF	B§
C:2a:P1.5			CSF	Negative
B:4:P1.15		No	Serum	Negative
C:2a:P1.2			Serum	Negative
C:2b:P1.5,2			Serum	C
C:2a:P1.5			Serum	C
C:2a:P1.5,2			Serum	Negative
C:2a:P1.5,2			Serum	Negative
C:2b:P1.5,2			Serum	Negative
B:NT:P1.15			Serum	Negative
B:4:P1.4			Serum	B
B:NT:P1.15			Serum	Negative
C:2a:P1.5,2			WBDNA‖	C
C:2a:P1.5			WBDNA	C
B:NT:P1.4			WBDNA	Negative
B:4:P1.4			WBDNA	B
C:2a:P1.5,2			WBDNA	Negative
C:2a:P1.2¶			WBDNA	Negative
C:2a:P1.5			WBDNA	C
	A+C		WBDNA	C§

* Gram negative diplococci visualised by microscopy.
† Not serotypable or serosubtypable.
‡ Cerebrospinal fluid.
§ Culture negative, laboratory confirmed cases, serogroup identified by *siaD* PCR ELISA.
‖ Whole blood DNA.
¶ Throat swab (in presence of clinical symptoms of meningococcal disease).

whole blood and sera were 63 (5/8) and 30% (3/10), respectively. None of the CSFs from cases of non-meningococcal disease was found to be positive on the *siaD* PCR ELISA. All *siaD* positive CSFs from proven cases were serogrouped as B or C in complete agreement with the co-agglutination serogroup of the corresponding isolate (Table 1). In 10 cases of laboratory confirmed meningococcal infection, where no culture was obtained (either detection of Gram-negative diplococci by microscopy or positive latex agglutination), the serogroup was identified by *siaD* PCR ELISA (Table 1). Of the specimens from cases of clinically suspected meningococcal infection which were culture and/or latex negative, 43 (6/14), 31 (8/26) and 23% (5/22) were positive from CSFs, whole blood and serum respectively demonstrating the effectiveness of the PCR ELISA. The detection limit of viable cells detectable by the *siaD* PCR ELISA was found to be 1 cell/5 μl, the inoculum for a single PCR reaction.

Application of *siaD* PCR ELISA in outbreak investigations

Both serogroupable and non-groupable isolates were cultured from nasopharyngeal swabs from students investigated following an outbreak of meningococcal disease in a University Hall of Residence caused by a B:15:P1.7, 16 sulphonamide resistant (R) strain. The non-groupable (NG) ($n = 2$) and B ($n = 3$) 15:P1.7, 16R isolates examined here were all identified as serogroup B by *siaD* PCR ELISA. During another outbreak investigation caused by a C:2a:P1.2R strain amongst secondary school children, 3 NG:2a:P1.2R and 3 serogroup C:2a:P1.2R were confirmed as serogroup C.

DISCUSSION

In response to the increasing need for non-culture diagnostic methods for meningococcal infection, several PCR assays have been reported, but so far there are few documented PCRs which provide epidemiological information about the causative organism. Saunders and colleagues [9] described a PCR assay for clinical samples based upon the meningococcal *porA* gene for the confirmation of meningococcal disease with an additional set of primers which distinguishes serosubtype P1.7, 3 meningococci (the prevalent strain of meningococcus in northern Chile in 1993). This PCR assay provided limited epidemio-

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logical information on the serosubtype of this infective organism. Maiden and colleagues [14] have developed a DNA dot-blot for the identification of serosubtype of isolates. This employs a comprehensive panel of probes for each serosubtype and circumvents the drawbacks of serotyping. This technique is now being applied to develop a *porB* serotype-specific ELISA for the non-culture diagnosis of meningococcal infection (personal communication, A. Fox). The variation in the *dhps* gene of *N. meningitidis* has been exploited [15] as a method for combined diagnosis and strain-specific fingerprinting of disease-causing strains in contacts of patients with meningococcal disease. Ni and colleagues [7] developed a PCR for the detection of invasive meningococcal disease based on the *IS1106* insertion sequence which has now been adapted to a colorimetric microtitre hybridization assay to overcome problems with specificity and sensitivity [12].

In this report a colorimetric microtitre hybridization assay (*siaD* PCR ELISA) for the specific identification of serogroup B and C meningococci has been described. This assay is based on a stable RFLP in the *siaD* nucleotide sequence of serogroup B and C meningococci as illustrated from the PCR amplicon restriction endonuclease analysis of serogroup B and C meningococci of diverse phenotypes. The *siaD* PCR ELISA described here is the first PCR assay to provide timely, serogroup-specific information for non-culture proven cases of meningococcal infection which is of public health importance in the management of meningococcal outbreaks.

The *siaD* PCR ELISA is specific for serogroup B and C meningococci; however, a single serogroup H meningococcal isolate, not associated with clinical disease, was amplified and found to react with the serogroup C probe. Meningococci undergo transformation *in vivo*, the mechanism by which meningococci may have originally acquired their capsule (*cps*) gene cluster [6]. Alteration of the meningococcal capsular phenotype has been demonstrated *in vitro* by transformation [16]. Furthermore, it has been established that meningococci may express different capsular types within a single clonal type [17]. It is possible that the serogroup H organism, isolated from the nasopharynx of a healthy individual, has acquired the serogroup C *siaD* gene through transformation with a serogroup C strain simultaneously carried in the nasopharynx. The structure of serogroup H capsular polysaccharide has been determined [18] and shows no antigenic similarities with the serogroup B or C polysaccharides [19].

Meningococci undergo phase variation in capsular expression, particularly following nasopharyngeal colonization and establishment of the carrier state [20]. Down-regulation of capsule expression results in organisms, non-groupable by conventional serological methods, and these are frequently isolated from healthy carriers. The identification of the serogroup status, by this technique, of carried organisms isolated during outbreak investigations will enable better determination of carriage of the outbreak strain, providing improved epidemiological information for management and infection control.

Of the phenotypic markers used for the epidemiological characterization of meningococci, the serogroup is of greatest importance for contact management since serogroup C meningococcal outbreaks can be controlled by vaccination. In the absence of meningococcal cultures, the management of outbreaks of meningococcal disease is hindered by the lack of serogroup information. The instigation of outer membrane protein and polysaccharide–protein conjugate vaccines for serogroup B and C meningococci, respectively, requires maximum case ascertainment and serogroup identification to determine the true incidence of disease and thereby accurately determine efficacy.

In conclusion, this study has demonstrated a highly specific and sensitive test for the detection of serogroup B and C meningococci by the *siaD* PCR ELISA assay for the non-culture diagnosis and confirmation of meningococcal infection. The *siaD* PCR assay additionally provides a timely identification of meningococcal serogroups allowing the effective implementation of appropriate methods for contact management and infection control. The introduction of the serogroup-specific PCR ELISA assay for the non-culture confirmation of meningococcal infection in England, Wales and Scotland will be able to provide a more complete determination of the levels of serogroup B and C infection prior to the introduction of serogroup C conjugate vaccines into the childhood immunization schedule.

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