Journal of Molecular Diagnostics, Vol. 10, No. 3, May 2008

DOI: 10.2353/jmoldx.2008.070152

Molecular Characterization of *Neisseria meningitidis* Isolates Using a Resequencing DNA Microarray

Caroline E. Corless, Edward Kaczmarski, Ray Borrow, and Malcolm Guiver

Meningococcal Reference Unit, Health Protection Agency Manchester Laboratory, Manchester Royal Infirmary, Manchester, United Kingdom

Neisseria meningitidis* is a major cause of both meningitis and septicemia. Typically, isolates are characterized by using a combination of immunological phenotyping, using monoclonal and polyclonal antisera, and Sanger nucleotide sequencing of epitope-encoding variable regions, although these methods can be both time-consuming and limited by reagent availability. Herein, we describe and evaluate a novel microarray to define the *porB* and *porA* serotypes of *N. meningitidis* by the resequencing of variable regions in a single hybridization reaction. PCR products for each gene were amplified, pooled in equimolar concentrations, hybridized to the microarray, and analyzed using Affymetrix GeneChip DNA Analysis Software. Resequencing of the microarray data was then validated by comparison with sequencing data. Molecular profiles were generated for 50 isolates that were combinations of phenotypically typeable (ie, PorA and PorB) and non-typeable (PorB only) isolates. Microarray-generated profiles from isolates with a PorB phenotype were concordant with predicted profiles compared with a previously described typing scheme. In addition, 42% (8 of 19) of previously non-typeable samples were assigned a PorB type when tested using the microarray. The remaining isolates were novel types for which no typing antisera are currently available. The *porA* data were 97% concordant with Sanger nucleotide sequencing. These results suggest that that microarray resequencing may be a useful tool for the characterization of meningococci, particularly for those isolates that cannot be phenotyped, offering an alternative to conventional sequencing methods. *(J Mol Diagn 2008, 10: 265–271; DOI:10.2353/jmoldx.2008.070152)

Neisseria meningitidis (meningococcus) is a major cause of meningitis and septicemia causing serious and sometimes fatal endemic and epidemic disease. Strain characterization using genotypic and phenotypic methods is necessary for enhanced surveillance of meningococcal disease before and during the implementation of new meningococcal vaccines to monitor possible antigenic change in the organism and also to ascertain the relatedness of strains in disease clusters.

Historically, cases of meningococcal disease have been characterized by phenotypic methods: reaction of a monoclonal antibody (mAb) or polyclonal antisera with group-, serotype-, and subtype-defining antigens. Serotype falls into two classes, PorB2 or PorB3 outer membrane protein (OMP) as encoded by the *porB* gene; and serosubtype is based on one or two variants of PorA as encoded by the *porA* gene. Sequence analysis of PorA and PorB OMPs has shown three and four surface-exposed loops, respectively, with a high level of amino acid variability.[1,2] Different variable regions (VRs) within each gene are grouped together based on length and sequence similarity, with multiple variants within these groups. PCRs targeting *porA* and *porB* genes have been used in sequence-based typing schemes,[3–5] and sequencing of both genes in combination with sequencing of *fetA* and the seven multi locus sequence typing genes have become the "gold standard" for meningococcal characterization[6]. Sequence-based methods for characterization of strains are increasingly used and are gradually replacing phenotypic methods that have a number of shortcomings, eg, that they are affected by antigenic variability. Also, there is a limited range of available mAbs that can lead to a large proportion of phenotypically non-typeable organisms. Capillary-based sequence typing can overcome this, but *porB* gene sequencing requires multiple sequencing primers for each OMP class.[4,7]

DNA microarrays are devices that permit the rapid generation and analysis of a large amount of DNA sequence data and have recently been reported as having genotyping applications for bacterial and viral pathogens.[8–10] High-throughput, high-density resequencing microarrays such as Affymetrix GeneChip (Affymetrix,

Supported by the Meningitis Research Foundation, United Kingdom (grant MRF 10/00 to C.E.C.).

Accepted for publication January 15, 2008.

Supplemental material for this article can be found on *http://jmd.amjpathol.org*.

The authors declare that that they do not have any affiliations with any organization that may have a direct financial interest in the subject matter or materials disclosed in this article.

Address reprint requests to Caroline E. Corless, Meningococcal Reference Unit, Health Protection Agency Manchester Laboratory, P.O. Box 209, Clinical Sciences Building, Manchester Royal Infirmary, Oxford Road, Manchester, M13 9WL,UK. E-mail: caroline.corless@hpa.org.uk.

Santa Clara, CA) allow the continuous resequencing of up to 300,000 nucleotides (300 kb) of double-stranded DNA sequence (600,000 nucleotides in total) in a single hybridization reaction. However, this would not be a practical approach for meningococcal genetic characterization because the genes that are sequenced are found more than 300 kb apart along the meningococcal genome. Resequencing microarrays can be "customized" according to the requirements of the individual and use DNA of known nucleotide sequence. The technology is based on a 25-nucleotide probe "tiling strategy" that involves examining 25 successive nucleotide sections of DNA. Four probes are designed for each nucleotide position. These are identical at all except the central (13th) position where each of the four possible nucleotides is substituted. When reacted with DNA under test conditions, the variant forming the most stable duplex at the test position will give the best fluorescent signal and identifies the nucleotide at that position. The next nucleotide in sequence is interrogated in the same way with another set of four probes. Both DNA strands are analyzed to maximize base determination confidence. Multiple fragments from different genes can be "tiled" onto a single array by processes of photolithography and combinational chemistry.[11]

This study describes the custom design and evaluation of an Affymetrix meningococcal resequencing microarray (MRM) to determine the *porB* type and *porA* subtype of *N. meningitidis* strains using a single microarray with a single hybridization reaction and to relate these to those data obtained by capillary-based sequencing and phenotyping methods. The microarray also enables identification of new variants in both genes. The MRM was designed to include 74 VR1 and 170 VR2 *porA* variants and 55 Class 2 and 59 Class 3 *porB* variant DNA sequences.

Materials and Methods

Bacterial Strain Selection and Culture Methods

Fifty meningococcal strains comprising those causing invasive disease (isolated from blood culture, $n = 25$; and cerebrospinal fluid, $n = 10$) and carrier isolates ($n = 15$) were selected from among those submitted year to year to the Health Protection Agency Meningococcal Reference Unit (HPA MRU) for phenotypic and genotypic characterization as described.[12] The organisms that were examined covered diverse capsular groups (particularly those associated with invasive disease): B ($n = 32$), C ($n = 13$), 29E ($n = 1$), W135 ($n = 1$), Y ($n = 2$), nongroupable ($n = 1$). For *porB* types, 1 ($n = 2$), 2a ($n = 5$), 2b ($n = 2$), 4 ($n = 13$), 14 ($n = 3$), 15 ($n = 1$), 21 ($n = 2$), and 22 ($n = 3$), along with 19 isolates whose type could not be characterized with the available mAbs; for *porA* subtype VR1, 13 variants belonging to five different families (5, 5-1, 5-2, 5-3, 5-4, 7, 7-1, 7-2, 17-1, 18-1, 19, 19-1, 19-4), and for VR2, 28 variants belonging to 10 different families (2, 2-1, 2-2, 2-7, 2-16, 2-20, 3, 4, 9, 10, 10-1, 10-2, 10-3, 10-4, 10-6, 10-7, 10-8, 10-10, 10-26, 10-27, 13-1, 14, 14-6, 15, 15-11, 16, 23, and 23-2) were examined. The organisms were recovered from Microbank vials (Pro-Lab Diagnostics, Neston, Wirral, UK) that had been stored at −80°C and were cultured overnight on Columbia blood agar (Oxoid, Basingstoke, UK) at 37°C in 5% CO_2.

DNA Extraction, PCR Primer Design, and DNA Amplification

N. meningitidis

Bacterial DNA was extracted as previously described.[13] The *porA* primers were as previously described[14] and amplified VRs 1 and 2 with an amplicon length of 785 bp. The *porB* primers were 23F and 3R as previously described[15] and amplified four VRs (loops I, V, VI, and VII) with amplicon lengths of 1053 bp for *porB*2 and 894 for *porB*3. The PCR master mixture components were standardized in a 50-μl aliquot comprising 5 μl of 10× PCR reaction buffer (Invitrogen Life Technologies, Renfrew, UK), 200 μmol/L each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics, GmbH, Mannheim, Germany), 1.5 mmol/L $MgCl_2$ (Invitrogen Life Technologies), 500 nmol/L each PCR primer, 2.5 units of Platinum TaqDNA Polymerase (Invitrogen Life Technologies) and made up to a volume of 50 μl with sterile injectable water. The thermocycling parameters for *porA* and *porB* PCR assays were 95°C for 5 minutes, then 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. PCR amplicons were visualized by UV irradiation following electrophoresis in a 2% agarose gel with GelStar Nucleic Acid stain (Lonza Biologics, Slough, UK) (1:10,000) at 150 V for 1 hour.

MRM Design

The MRM was designed following the Affymetrix GeneChip system protocols. The number of nucleotides that can be sequenced on an array is calculated by the formula: Number of bases sequenced = Maximum capacity − (number of fragments × 24), with 24 being the first and last 12 nucleotides flanking each discrete fragment tiled on the array. The sequences, inclusive of the 24-nucleotide flanking sequences for each fragment, were converted to a file from which a photolithographic mask was designed for the microarray synthesis process. The MRM design file was the template for data analysis using the designated GeneChip DNA Analysis Software (Affymetrix Inc) and contained a list of all probes tiled on the array and the location of each probe. Also included was a 1000-nucleotide internal control (Tag IQ-EX).

PorB Typing

The *porB* gene sequences to be included on the array were based on those previously identified.[4] To identify additional variant sequences within the four *porB* VRs, 46 class 2 and 79 class 3 gene sequences were kindly provided by Rachel Urwin (Department of Biology, Pennsyl-

Table 1. Alignment of *porA* Variant Sequences for VR2 Family P1.1

VR2 family	DNA sequence
1-0	TGGAAAATGGCG TAGCTAAAAAAGTTG CGGCTGTTGTCG
1-1	TGGAAAATGGCG TAG**T**TAAAAAAGTTG CGGCTGTTGTCG
1-2	TGGAAAATGGCG C**A**ACTAAAAAAGTTG CGGCTGTTGTCG
1-3	TGGAAAATGGCG TAGCTAAAAAAGTT**A** CGGCTGTTGTCG

Nucleotides that do not match the reference sequence (1-0) are marked in bold type and the conserved 12-nucleotide sequences flanking the variable regions are underlined.

vania State University, University Park, PA). Also, a search of *porB* gene sequences that had been submitted to GenBank (National Center for Biotechnology Information, Bethesda, MD) was made. These were aligned in the BioEdit software package using a Clustal W multiple alignment,[16] and a search of previously identified variant sequences[4] was performed. Those not previously identified were designated as new variant sequences and either assigned a three-digit number according to the class and number of the strain (see supplemental Table S1 at *http://jmd.amjpathol.org*) or, if they were identified from the sequences submitted to GenBank as closely homologous with a variant sequence using the nomenclature described,[4] assigned the next letter in the variant scheme. The reference sequences tiled on the array included those described previously[4] plus those newly identified (see Supplemental Table S1 at *http://jmd.amjpathol.org*). These were grouped by OMP class and VR, eg, *porB*2_4VRC, *porB*2_4VRCA, and so forth. In total, there were 114 tiled reference sequences: 24 representing VR1 (9 PorB2 and 15 PorB3), 31 representing VR2 (15 PorB2 and 16 PorB3), 29 representing VR3 (19 PorB2 and 10 PorB3), and 30 representing VR4 (12 PorB2 and 18 PorB3), with a total of 6180 nucleotides (including flanking sequences totalling 2736 nucleotides). A Basic Local Alignment Search Tool Nucleotide (BLASTN, National Center for Biotechnology Information) search of the selected reference sequences was conducted to ensure that they were 100% homologous for the designated gene or variant sequence and to limit the potential for cross-hybridization with similar sequences.

PorA Typing

The *porA* VR sequences (available at *http://neisseria.org/perl/agdbnet/agdbnet.pl?file = poravr.xml*) (also see Supplemental Table S2 at *http://jmd.amjpathol.org*) were aligned in the BioEdit sequence alignment package. Conserved sequence was identified and designated as the flanking sequences 5′ and 3′ of the sequence of the variant to be tiled onto the array. An example of a VR sequence alignment for *porA* VR2 family P1.1 is given in Table 1. In this study there were 74 tiled fragments representing 10 VR1 families and 170 fragments representing 17 VR2 families, with a total of 14,490 nucleotides (including flanking sequences totalling 5856 nucleotides). The reference sequences to be tiled on the array were designated by their variant identifier, for example, *porA*_1-0, *porA*_1-1, and so forth.

Preparation of DNA, Hybridization, and Data Analysis

The amplified PCR products were quantitated, pooled, fragmented and labeled according to the recommended Affymetrix protocol. Microarray hybridization and processing were as per the manufacturer's protocol (Affymetrix Inc.). MRM data files were analyzed using GeneChip DNA Analysis Software version 2.0 following the protocol outlined by Affymetrix Inc with the maximum number of base calls being allowed. The sequence calls were made by comparing the respective hybridization intensities of each of the four probes at a particular position. Multiple alignments for a chosen tiled fragment were performed and homology of the test sample with the reference sequence was clearly indicated, as were multiple and single nucleotide polymorphisms.

Typically the workflow was as follows: day 1, DNA extraction followed by PCR, quantitation, and pooling in equimolar concentrations; day 2, fragmentation of DNA, labeling with biotin, and hybridization to the microarray overnight; day 3, data analysis.

Validation of MRM Data

Capillary-Based DNA Sequencing of porB *and* porA *Isolates*

Forty-seven isolates that had been characterized for *porA* VR1 and VR2 and 10 isolates that were characterized as *porB*3 were selected for capillary-based sequencing. These 10 isolates included three type 4 (VR type profile 4, D, 7, 14a), one type 1 (VR type profile 19, Ac, 7a, 1), one type 14 (VR type profile 19, Db, 7c, 14), one type 4,21 (VR type profile 4, D, 7b, 21), and four that could not be typed using available mAbs. The *porB* and *porA* PCRs were performed as described previously and amplicons visualized by agarose gel electrophoresis. Amplicons were purified of excess oligonucleotides and deoxynucleoside-5′-triphosphates using the Millipore Montage PCR_{96} Plates (Millipore Ltd, Watford, UK) according to the manufacturer's instructions, and the products were resuspended in 60 μl of sterile injectable water (Phoenix Pharmaceuticals, Gloucester, UK). Purified DNA was quantified by agarose gel electrophoresis using a Low DNA Mass Ladder (Invitrogen). The *porB* and *porA* sequencing primers were as previously described.[4,17] The sequencing methodology was as previously described.[18] Raw sequence data were generated using the CEQ 8000 Software (Beckman Coulter, High Wycombe, UK) and analyzed in the Sequencher DNA analysis software version 4.0.5 (Gene Codes Corporation, Ann Arbor, MI). The *porB* sequence data were compared with those generated using the MRM.

Table 2. MRM *porB* VR Genotype Profiles of *N. meningitidis* Serotyped Strains

Serotype	PorB class	No. tested	*porB* VR1	VR2	VR3	VR4
1	3	1	19	Ac	7a	1
1	**3**	**1**	**19**	**Ac**	**341**	**1**
4	3	8	4	D	7	14a
4	3	1	4	B	7	14a
4	3	1	[4]	B	7	14a
4	3	1	[4]	D	7	14a
4	3	1	4	Db	7	14a
4	3	1	4	B	7 days	14a
14	3	3	19	B	7c	14
2a	2	4	C	Eb	2a	C
2a	2	1	Cb	Eb	2a	C
2b	2	1	C	Ea	2b	C
2b	**2**	**1**	**C**	**Near Ea***	**2b**	**C**
15	3	1	A	A	A	Ba
21	3	1	4	D	7b	21
21	**3**	**1**	**329**	**313**	**7b**	**364**
22	2	2	C	Ed	5a	Db
22	**2**	**1**	**C**	**Undefined†**	**Near 5a‡**	**Near Ca§**

Four samples that gave atypical variant profiles for the serotype are in bold type.
*Single-nucleotide polymorphism at position 35 (C to T) amino acid change alanine to valine.
†DNA sequence AACAATGATGCAAAACGTGTTGCAGTAGGTACTGACAATCCTGTT amino acid sequence NNDAKRVAVGTDNPV.
‡polymorphisms at positions 9 to 11 (GAT to ACC) with an amino acid change from aspartic acid to threonine.
§single-nucleotide polymorphism at Ca position 2 (T to A) with an amino acid change from valine to glutamic acid.

Results

PorB Typing

Based on the resequencing of the four *porB* gene VRs and comparison with known *porB2* and *porB*3 variant sequences, the MRM provided genotype profiles for the 50 isolates tested. Of the 31 samples that had been previously PorB-typed by dot blot enzyme-linked immunosorbent assay using a mAb panel, as described previously,[12] 87.1% (27 of 31) of the MRM VR sequence profiles were the same as those previously published for the corresponding type (Table 2).[4,19] Four samples produced MRM VR type profiles, where there was variation from the expected sequence in at least one VR and resulted in an amino acid change. These had been previously serotyped as 1, 2b, 21, and 22 (Table 2). MRM data that were generated from samples with previously characterized PorB types 1, 4, 14, and 4,21 were 100% concordant with that obtained by capillary-based sequencing of representative samples. The variant sequences for these serotypes are published.[4] It was possible to predict serotype in 42% (8 of 19) of samples that could not be PorB-typed at HPA MRU with the available mAb panel. These were PorB2 types 2a ($n = 1$) and PorB3 types 1 ($n = 1$), 4 ($n = 1$), 8/18 ($n = 1$), 14 ($n = 1$), and 17 ($n = 3$) (Table 3). The remaining 11 samples had distinct MRM *por*B profiles for which there are currently no known available typing sera. Eight of these were divided into three distinct groups (Table 3) with the remainder being found to have diverse combinations of VR sequences. This shows that the MRM is useful in identifying unique sequences and emphasizes the diversity of *por*B VR profiles in disease-causing organisms.

PorA Typing

Of the 50 samples resequenced using MRM, 47 had been PorA sequence-typed by capillary-based sequenc-

Table 3. MRM *porB* VR Characterization and Predicted Serotype of Previously Non-Typeable Strains

PorB class*	No.	Serogroup	*porB* VR by MRM 1	2	3	4	Predicted serotype
3	1	B	19	AC	7A	1	1
3	1	B	19	D†	7B	14	14
3	3	B	B	C	7	14B	17
3	1	B	19	AA	10	14A	8/18
2	1	C	C	EB	2A	C	2a
3	1	B	B	DB	7	14A	4
2	4	C	D	EB	2AA/2A	208	None
2	2	NG,W135	D	ED	210	DB	None
3	2	B	Undefined‡	AB	7	4AA	None

*As determined from *porB* PCR amplicon size on gel.
†90.4% homology with VR2-D MRM sequence was CAGGATGTGGGTAACGTGAAG, amino acid translation QDVGNVK.
‡Unable to sequence.

Table 4. Discrepancies between *porA* Results: Capillary-Based Sequencing and MRM Resequencing

MRM ID	Method	*porA* variant	DNA sequence
70	Cap*	4–0	CATGTTGTTGTGAATAAC AAGGTTGCTACTCACGTTCCGGCT
70	MRM†	4–2‡§	CATGTTGTTGTGAATAAC**AAGGTTGCTACTCACGTTCCGGCT**AAGGTTGCTACTCACGTTCCGGCT
59	Cap	10–4	CATTTTGTTCAGAATAAGCAAAATAAGCAAAAT CAGCCGCCTACTCTCGTTCCG
59	MRM	10–10	CATTTTGTTCAGAATAAGCAAAATAAGCAAAAT**AAGCAAAAT**CAGCCGCCTACTCTCGTTCCG
12	Cap	23–2	CATTGGAATACTGTGTATAACACTAACGGTACTACTACTACTACT TTCGTTCCGGCTGTT
12	MRM	23–3	CATTGGAATACTGTGTATAACACTAACGGTACTACTACTACTACT**ACT**TTCGTTCCGGCTGTT

*Where Cap indicates capillary-based sequencing.
†Where MRM indicates MRM microarray resequencing.
‡Bold type indicates sequence differences between *porA* variants.
§Repeated sequences are underlined.

ing of the *porA* gene variable regions (VR1 and VR2). Numbers were aggregated for VR1 and VR2 when calculating concordance between the two methodologies of 96.9% (94 of 97). The three discrepancies were due to limitations of resequencing microarray technology, namely, that accurate interpretation of data obtained from samples where sequences are repeated within a tiled fragment is hindered because of inefficient hybridization (Table 4).

Discussion

The objectives of this study were to design and evaluate a customized resequencing DNA microarray based on Affymetrix GeneChip, that we termed MRM, to accurately determine the *porB* type and *porA* subtype of *N. meningitidis* disease-causing strains in a single hybridization reaction and to relate these to phenotype. By doing this, it is possible to provide a degree of continuity for those phenotypic markers that have historically been used for epidemiological purposes. A further objective was to use the MRM to identify new sequence variants in both genes.

In 2003 to 2004, 49% of isolates submitted to HPA MRU for phenotypic classification could not be PorB-typed.[12] This may be because there is a limited number of mAbs in the routinely used panel or that mAbs that could react with one or more of the PorB epitopes have not been developed. Furthermore, changes in the DNA sequence that encodes an epitope previously recognized by a mAb may lead to it not now being recognized. As such, the existing mAb panel will become less useful over time. As new variants of antigens emerge, there is a need to constantly generate new mAbs. Furthermore, the European consensus is to move toward antigen sequence typing that will result in a decline in the practical demands for new mAb generation. In addition, 45.1% of meningococcal disease cases are diagnosed by the detection of DNA alone and therefore cannot be phenotyped.[12] Taking these data together, no type (phenotype or genotype) is available for 72% of disease-causing meningococci in England and Wales.[12] As such, there is a clear requirement for improved PorB type characterization. Abad et al[5] sequenced strains that could not be PorB-typed. The authors emphasized that genotyping rather than phenotypic PorB typing should be the method of choice for strain characterization. The MRM in this study was designed to include *porB* variant sequences that had been described previously[4] and also to identify those that had not been defined. It provided DNA sequence data for all four *porB* VRs with 100% accuracy when compared with capillary-based sequence data and also samples from known serotypes and profiles previously reported.[4,19]

By comparing MRM profiles from samples that could not be PorB-typed previously, it was possible to predict a type in 42% (8 of 19) of samples (Table 3). Serotyping mAbs are available for 50% (4 of 8) of these samples, which indicates that there may be issues of mAb sensitivity, possibly as a result of "within-type" variation resulting from epitope-encoding gene sequence changes that would reduce the ability of the mAb to bind with the type-specific antigen. The remaining 50% of samples were predicted to belong to types for which mAbs are either not available or are not in routine use at HPA MRU. Furthermore, the data indicate that PorB types 17 (one blood culture and two cerebrospinal fluid isolates) and 8/18 (one blood culture isolate) are causing invasive disease in England and Wales that was previously unrecognized. Also, based on the sequence of the four *porB* VRs within the non-typeable isolates, there are distinct groups for which no typing sera are available (Table 3). These data show the usefulness of the MRM in characterization of isolates that could not be typed using phenotypic methods and demonstrates that there is sequence variation within *porB* VRs for known PorB types. This is not surprising when it has been demonstrated that some epitopes in the surface loops of PorB are under very strong positive selection pressure when exposed to host immune responses and that there is a high level of diversity among *porB*2 and *porB*3 genes.[20] Therefore, as organisms are increasingly exposed to host immune responses, this may result in an increase in non-typeable isolates as amino acid residues in type-defining epitopes change. This diversity of the *porB* gene among meningococcal isolates also highlights the need for strain characterization for epidemiological purposes. Currently, there are a limited number of mAbs available for PorB typing, and nucleotide sequencing has identified a number of uncharacterized types. Without the addition of definitive mAbs for these types, nucleotide sequencing remains the only way to identify and genotype these

strains. The MRM could provide an alternative approach to sequencing gene fragments. Finally, the complexity of the design process of the *porB* portion of the MRM, which used two different molecular nomenclature schemes, one combining letters and numbers and one using numbers only, highlights the necessity for one combined and, therefore, simplified nomenclature.

The MRM data were compared with capillary-based sequencing, which is the current gold standard for *porA* subtyping of meningococci. There was a 97% concordance between the two methodologies. There were three discrepant results that were due to repetitive sequence motifs within the tiled array sequence fragments (Table 4). The identification of variable numbers of repeated sequences is difficult to resolve using probe-based hybridization technology. Any repeated sequence in a test sample will bind to any repeated sequence tiled onto the array; therefore, the accurate determination of repeated sequences is not possible using resequencing microarray technology. This is reflected in the Affymetrix GeneChip CustomSeq Resequencing array Design Guide stating that repetitive elements are removed at the time of array design. Repeated sequences such as those identified in *porA* variants in this study (Table 4) could be identified by running the sequences through a software program such as Repeatmasker (available at *http://www.repeatmasker.org*) and removed from the final microarray design. However, because a "proof of principle" approach was adopted in this study, *porA* variant sequences that were known to include repetitive elements were included in the MRM.

There are limitations to resequencing technology, namely, that due to the nature of the production of microarrays, it is currently expensive to perform, as once was Sanger nucleotide sequencing. However, advances in production technologies, such as increasing the capacity of resequencing microarrays by 10-fold at no additional cost, have increased the number of potential gene targets that could be included. For example, the *fetA* gene would be included in future MRM designs to reflect the recent addition of this gene in meningococcal characterization.[6] A further limitation in the technology is caused by repeated sequences within a tiled fragment that can hinder the accurate interpretation of data. This would limit the future development of a fully comprehensive resequencing microarray *porA* typing scheme, since these types are known to contain repeated fragments. It is therefore critical to remove any repetitive elements during the microarray design process.

In conclusion, the MRM described here could be a useful tool for the determination of the *porB* type and *porA* subtype of meningococcal isolates and also those identified by PCR alone. Technological advances have increased the potential number of gene targets that could be used for genetic characterization. Resequencing microarrays make possible the sequencing of multiple gene targets in a single hybridization reaction with the whole process from DNA extraction to sequence determination taking less than three working days. Providing that the limitations of resequencing technology are taken into consideration, it is useful as a tool for molecular characterization of strains with uncomplicated data analysis. As such, it could offer an alternative to conventional sequencing.

Acknowledgments

Many thanks go to Bernadette McConnell for assistance in manuscript preparation and to Affymetrix UK for assistance with the MRM design and technical and software support.

References

1. 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, 63:1484–1490
2. van der Ley P, Heckels JE, Virji M, Hoogerhout P, Poolman JT: Topology of outer membrane porins in pathogenic *Neisseria* spp. Infect Immun 1991, 59:2963–2971
3. Arhin FF, Moreau F, Coulton JW, Mills EL: Sequencing of *porA* from clinical isolates of *Neisseria meningitidis* defines a subtyping scheme and its genetic regulation. Can J Microbiol 1998, 44:56–63
4. Sacchi CT, Lemos APS, Whitney AM, Solari CA, Brandt ME, Melles CEA, Frasch CE, Mayer LW: Correlation between serological and sequencing analyses of the PorB outer membrane protein in the *Neisseria meningitidis* serotyping system. Clin Diag Lab Immunol 1998, 5:348–354
5. Abad R, Alcala B, Salcedo C, Enriquez R, Uria MJ, Diez P, Vazquez JA: Sequencing of the porB gene: a step toward a true characterization of Neisseria meningitidis. Clin Vaccine Immunol 2006, 13:1087–1091
6. Jolley KA, Brehony C, Maiden MC: Molecular typing of meningococci: recommended for target choice and nomenclature. FEMS Microbiol Rev 2007, 31:89–96
7. Urwin R: Methods in Molecular Medicine, vol 67: Meningococcal Disease. Edited by Pollard AJ, Maiden MCJ. Humana Press Inc, Totowa NJ, 2001, pp 157–172
8. Davignon L, Walter EA, Mueller KM, Barrozo CP, Stenger DA, Lin B, on behalf of the Epidemic Outbreak Surveillance Consortium: Use of resequencing oligonucleotide microarrays for identification of *Streptococcus pyogenes* and associated antibiotic resistance determinants. J Clin Microbiol 2005, 43:5690–5695
9. Lin B, Wang Z, Vora GJ, Thornton JA, Schnur JM, Thach DC, Blaney KM, Ligler AG, Malanoski AP, Santiago J, Walter EA, Agan BK, Metzgar D, Seto D, Daum LT, Kruzelock R, Rowley RK, Hanson EK, Tibbetts C, Stenger DA: Broad-spectrum respiratory tract pathogen identification using resequencing DNA microarrays. Genome Res 2006, 16:527–535
10. Wang Z, Daum LT, Vora GJ, Metzgar D, Walter EA, Canas LC, Malanoski AP, Lin B, Stenger DA: Identifying influenza viruses with resequencing microarrays. Emerg Infect Dis 2006, 12:638–646
11. Barone AD, Beecher JE, Bury PA, Chen C, Doede T, Fidanza JA, McGall GH: Photolithographic synthesis of high-density oligonucleotide probe arrays. Nucleosides Nucleotides Nucleic Acids 2001, 20:525–531
12. Gray SJ, Trotter CL, Ramsay ME, Guiver M, Fox AJ, Borrow R, Mallard RH, Kaczmarski EB: Epidemiology of meningococcal disease in England and Wales 1993/94 to 2003/04: contribution and experiences of the Meningococcal Reference Unit. J Med Microbiol 2006, 55:887–896
13. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB: The simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae and Streptococcus pneumoniae* in suspected cases of meningitis and septicaemia using 'real-time' PCR. J Clin Microbiol 2001, 39:1553–1558
14. Molling P, Jacobsson S, Backman A, Olcen P: Direct and rapid identification and genogrouping of meningococci and porA amplification by LightCycler PCR. J Clin Microbiol 2002, 40:4531–4535

15. Urwin R, Kaczmarski EB, Guiver M, Fox AJ, Maiden MC: Amplification of the meningococcal *porB* gene for non-culture serotype characterization. Epidemiol Infect 1998, 120:257–262
16. Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994, 22:4673–4680
17. Feavers IM, Maiden MCJ: A gonococcal *porA* pseudogene: implications for understanding the evolution and pathogenicity of *Neisseria gonorrhoeae*. Mol Microbiol 1998, 30:647–656
18. Azadan RJ, Fogleman JC, Danielson PB: Capillary electrophoresis sequencing: maximum read length at minimal cost. Biotechniques 2002, 32:24–26, 28
19. Dyet KH, Simmonds RS, Martin DR: Evaluation of *porB* PCR-amplicon restriction endonuclease analysis as a method to determine *porB* variable-region sequences in nonserotypeable meningococci. J Clin Microbiol 2004, 42:1731–1733
20. Urwin R, Holmes EC, Fox AJ, Derrick JP, Maiden MC: Phylogenetic evidence for frequent positive selection and recombination in the meningococcal surface antigen PorB. Mol Biol Evol 2002, 19:1686–1694