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Evaluation of *porB* PCR-Amplicon Restriction Endonuclease Analysis as a Method To Determine *porB* Variable-Region Sequences in Nonserotypeable Meningococci

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***porB* PCR-amplicon restriction endonuclease analysis is a rapid, simple method developed to assess *porB* variation in nonserotypeable meningococci isolated during New Zealand's epidemic of meningococcal disease. Most nonserotypeable meningococci isolated between 1990 and 1999 inclusively either were type 4 (40.5%) or contained the *porB* variable region 1 (VR1)-19, VR2-D, VR3-7, and VR4-14a sequences (45.1%).**

New Zealand has experienced increased levels of meningococcal disease since mid-1991 (3). Most case isolates were phenotype B:4:P1.4 (serogroup B, serotype 4, serosubtype P1.4), although 13.1% (155 of 1,183) of the B:P1.4 meningococci isolated from 1990 through 1999 were not serotypeable by the use of serotype 1, 2a, 2b, 4, 14, and 15 antibodies (2, 3). It was important to determine why these meningococci were nonserotypeable and if the B:nonserotypeable (nt):P1.4 meningococci were nonserotypeable variants of the B:4:P1.4 meningococci or if they encoded different *porB* sequences.

Restriction fragment length polymorphism (RFLP) analysis of the *porA* and *porB* PCR products is a simple, rapid method that enables all meningococci to be typed (1, 5, 8, 9). Most RFLP-based typing methods are an alternative to serotyping and serosubtyping, although *porA* PCR-amplicon restriction endonuclease analysis (*porA* PCR-AREA) generated subtype-specific restriction profiles (D. R. Martin and S. J. Walker, Proc., Eleventh Int. Pathogenic Neisseria Conf., p. 234, 1998). This report describes the development and application of *porB* PCR-AREA to investigate the variability in *porB* in nonserotypeable meningococci isolated during New Zealand's epidemic.

porB PCR products from 30 New Zealand case isolates with diverse serotypes were sequenced to determine the *porB* variation (Table 1). Primers PorB F (5′-ATCCGCCCTTCAAAA TACACATC-3′) and PorB R (5′-TGGCGCAGACCGACAC C-3′) were used for amplification and sequencing. The amplification reaction mixtures were incubated at 94°C for 2 min, followed by 30 cycles of 94°C for 40 s, 55°C for 40 s, and 70°C for 80 s. Extension was completed at 72°C for 3 min.

All serotype 4 isolates with the P1.4 PorA serosubtype (determined by serosubtyping or DNA-DNA hybridization) had identical *porB* sequences (Table 1). Three type 4 meningococci expressing a PorA serosubtype other than P1.4 had distinct *porB* sequences, suggesting that they are unrelated to the epidemic strain. All B:P1.4 isolates with the same serotype had identical *porB* variable-region (VR) sequences, whereas nonserotypeable isolates had a variety of *porB* sequences (Table 1).

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The *porB* characterization achieved by sequencing cannot be achieved by other methodologies, although time and cost prohibit the use of sequencing for large-scale investigations. In this study the availability of *porB* sequence data enabled the common *porB* sequences to be determined and the restriction enzymes that differentiate between these sequences to be selected. As certain combinations of sequences in VR1 and VR2 were associated with particular serotypes, the *porB* type could be predicted.

Restriction analysis of each of the 30 *porB* PCR products sequenced (Table 1) showed that PCR-AREA could be used as an alternative to sequencing. Restriction of the *porB* PCR product (4.0 μl) was performed in 8-μl reaction mixtures containing 4 U of enzyme. The reaction mixtures were incubated in a 37°C water bath for 4 h. The digestion products were electrophoresed on a 2% agarose gel with 0.5× Tris-borate–EDTA buffer at 5 V/cm for 90 min, stained with 1 μg of ethidium bromide per ml, and visualized under UV light.

AluI and SspI were used in a double digest to differentiate between sequences in *porB* VR1 and VR2 (Fig. 1). AflII was subsequently used to restrict *porB* PCR products containing *porB* VR1-19 and VR2-D to discriminate between *porB* VR4-14 and VR4-14a. Two distinct profiles were found in meningococci with the VR1-19 and VR2-A profile (Fig. 1). The different profiles were due to a silent point mutation at the beginning of *porB* and not to different VR sequences.

When *porB* PCR-AREA was applied to 76 meningococci isolated in 1999, the serotype obtained by *porB* PCR-AREA concurred with the previously established serotype. The restriction profiles for all nonserotypeable meningococci matched the profiles obtained for meningococci whose *porB* sequences were known. Sequencing of *porB* from 12 of the 22 meningococci with the B:nt:P1.4 phenotype confirmed the accuracy of *porB* PCR-AREA, although it was determined that more than two enzymes are required to accurately assess sequences in meningococci containing *porB* VR2-A due to the variability in these *porB* sequences.

***porB* typing.** PCR-AREA was used to determine the *porB* types of all meningococci with the B:nt:P1.4 phenotype that were isolated in New Zealand from 1990 through 1999 (Table 2) except two nonviable case isolates recovered in 1991. It was

TABLE 1. Phenotypes and PorB VR sequences of 30 meningococci isolated from patients with clinical cases of meningococcal disease during 1999

Phenotype	No. of isolates	PorB VR amino acid sequence[a]				*porB* VR type[b] (VR1,VR2,VR3,VR4)
		VR1	VR2	VR3	VR4	
B:4:P1.4	8	EHNGGQVVSVE	QDVDDVK	VEDNY	SFDDADLSND	4,D,7,14a
B:4:nst[c]	4	EHNGGQVVSVE	QDVDDVK	VEDNY	SFDDADLSND	4,D,7,14a
B:4:nst[d]	1	EHNGGQVVSVE	QDVDDVK	VEDNY	SFDDADLSND	4,D,7,14a
B:4:P1.7	1	EHNGGQVVSVE	QDVDDVK	VEDN	SVDDAKRDNT	(4)[e],D,7b,21
B:4:P1.14	1	EHNGGQVVSVE	VRVDKNVN	VEDNY	SFDDADLSND	4,undef[f],7,14a
B:4:P1.14	1	EHNGGQVVSVE	VRVDENVN	VEDNY	SFDDADLSND	4,B,7,14a
B:14:P1.4	3	AHNGAQAASVE	QNVDNVK	VKDN	SFDDADYTND	19,Db,7c,14
B:1:P1.4	4	AHNGAQAASVE	HQVQENVN	VEENY	SFDATNYNND	19,Ac,7a,1
B:15:P1.4	1	AHNGAQAASVE	HRVQEDIN	VEDNY	LVDSADLSND	19,Ab,7,A
B:nt:P1.4	1	EHNGGQVVSVE	QDVDDVK	VEDNY	SFDDADLSND	4,D,7,14a
B:nt:P1.4	1	AHNGAQAASVE	QNVDNVK	VKDN	SFDDADLSND	19,Db,7c,14a
B:nt:P1.4	1	AHNGAQAASVE	QDVDDVK	VEDNY	SFDATNYNND	19,D,7,1
B:nt:P1.4	1	AHNGAQAASVE	HQVQEDLN	ALPNDN	SFDDADLSND	19,Aa,10,14a
B:nt:P1.4	1	DYQDGQVYSVE	QDVDNVK	VEDNY	SFDDADLSND	Undef,Da,7,14a
B:nt:P1.4	1	DYQDGQVYSVE	QDVDNVK	VEDN	SFDDADLSND	Undef,undef,7b,14a

[a] VR sequences are represented by single-letter amino acid codes.
[b] PorB VR types as defined by Sacchi et al. (7).
[c] The *porA* P1.7.4 VR sequence was identified by DNA-DNA hybridization. Not serosubtypeable.
[d] The *porA* P1.7 VR sequence was identified by DNA-DNA hybridization. Not serosubtypeable.
[e] The amino acid sequence is identical to that of VR1-4, but a different nucleotide sequence is present.
[f] Undef, the VR sequence was not described previously.

determined that most meningococci contained the *porB* VR1-4 and VR2-D (40.5%) or the VR1-19 and VR2-D (47.7%) sequences. Restriction with AflII determined that 69 (94.5%) of the meningococci determined to contain *porB* VR1-19 and VR2-D contained the 19, D, 7, and 14a (type 19,D,7,14a) sequences (VR1, VR2, VR3, and VR4, respectively).

Together, serotyping and *porB* PCR-AREA showed that type 4, type 19,D,7,14a, and type 14 are the most common *porB* types found among meningococci with the B:P1.4 phenotype (Table 3). A *porB* PCR product was amplified from all nonserotypeable meningococci, which indicated that they encoded the gene required to express PorB. The inability to type these meningococci is probably not due to the lack of PorB expression, as all 22 B:nt:P1.4 meningococci examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis expressed a band that was consistent with the size of PorB (32 to 35 kDa). Immunoblotting with the type 4 antibody (5DC4C8G8; National Institute for Biological Standards and Control, Potters Bar, England) confirmed that the 32- to 35-kDa bands were PorB. It is most likely that the inability to type 62 of 1,037 (6.0%) meningococci with the *porB* VR1-4 sequence was due to the inability of the type 4 antibody to recognize the epitope expressed on the surface of the bacterium. This result may be

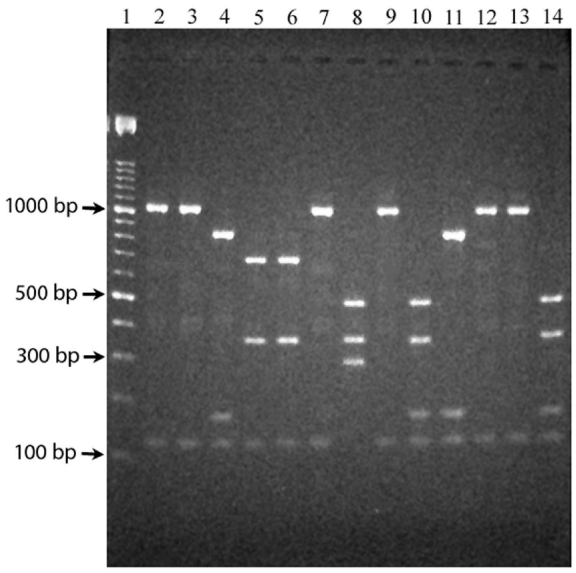

FIG. 1. *porB* PCR-AREA patterns observed following gel electrophoresis of AluI and SspI double digests of the *porB* PCR product. The PCR product was amplified from meningococci with the *porB* VR1-4 and VR2-D sequences (lanes 2, 3, 7, 9, 12, and 13), the *porB* VR1-19 and VR2-D sequences (lanes 4 and 11), the *porB* VR1-19 and VR2-A sequences (lanes 8, 10, and 14), and the *porB* VR1-4 and VR2-B sequences (lanes 5 and 6). Molecular size markers (100 bp; Roche) are shown in lane 1.

TABLE 2. *porB* PCR-AREA VR sequence predictions for isolates of *N. meningitidis* with phenotype B:nt:P1.4 isolated in New Zealand from 1990 through 1999

Yr	No. (%) of type B:nt:P1.4 isolates of the following types[a] predicted by *porB* PCR-AREA:					
	4,B	4,D	19,A	19,D,14a	19,D,14	Total
1990		1 (100)				1
1991		2 (100)				2
1992			1 (100)			1
1993		1 (25)		3 (75)		4
1994		3 (42.9)		4 (57.1)		7
1995		12 (57.1)		9 (42.9)		21
1996	4 (14.8)	5 (18.5)		18 (66.7)		27
1997		13 (32.5)	5 (12.5)	19 (47.5)	3 (7.5)	40
1998	2 (7.4)	13 (48.1)	1 (3.7)	10 (37.0)	1 (3.7)	27
1999	1 (4.3)	12 (52.2)	4 (17.4)	6 (26.1)		23
Total	7 (4.6)	62 (40.5)	11 (7.2)	69 (45.1)	4 (2.6)	153 (100)

[a] VR1,VR2 or VR1,VR2,VR4 types.

TABLE 3. *porB* types of serogroup B meningococci expressing P1.4 PorA isolated in New Zealand from 1990 through 1999 determined by serological typing and *porB* PCR-AREA

Yr	No. (%) of type B:P1.4 types of the following *porB* types:				
	4	14	19,D,14a	Other	Total
1990	3 (60.0)	2 (40.0)	0 (0.0)	0 (0.0)	5
1991	16 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	16
1992	40 (95.2)	1 (2.4)	0 (0.0)	1 (2.4)	42
1993	48 (90.6)	2 (3.8)	3 (5.7)	0 (0.0)	53
1994	87 (91.6)	4 (4.2)	4 (4.2)	0 (0.0)	95
1995	139 (88.5)	9 (5.7)	9 (5.7)	0 (0.0)	157
1996	162 (85.3)	6 (3.2)	18 (9.5)	4 (2.1)	190
1997	201 (85.2)	11 (4.7)	19 (8.1)	5 (2.1)	236
1998	152 (86.9)	7 (4.0)	10 (5.7)	6 (3.4)	175
1999	182 (85.9)	12 (5.7)	6 (2.8)	13 (6.1)	212
Total	1,030 (87.1)	54 (4.6)	69 (5.8)	28 (2.4)	1,181 (100.00)

caused by the masking of the type 4 PorB epitope by meningococcal surface structures or the low sensitivity of the serotype 4 antibody (6). By contrast, 45.1% (69 of 153) of the nonserotypeable meningococci contained the type 19,D,7,14a sequences (Table 2). These meningococci were not serotypeable because the panel of monoclonal antibodies did not include serotype 19 and 7 antibodies.

Our use of three restriction enzymes in *porB* PCR-AREA was justified in the context of New Zealand's epidemic, as it was expected that a limited number of *porB* types would be found. *porB* PCR-AREA differentiated between the type 4,D,7,14a (VR1, VR2, VR3, and VR4, respectively) and the type 4,B,7,14a sequences, although it did not differentiate between the type 4,D,7,14a and type (4),D,7b,21 sequences or the type 4,B,7,14a and type 4,undefined,7,14a sequences. Similarly, the type 19,D,7,14a, type 19,Db,7c,14a, and type 19,D,7,1 sequences were not differentiated. However, *porB* PCR-AREA was able to differentiate between the most common *porB* types identified (Table 1).

Although 73% of the B:nt:P1.4 isolates from England and Wales were categorized as having type 4 *porB*, sequence differences were identified between the isolates (10). By contrast, we found no differences in the sequences of *porB* from serotype 4 and nonserotypeable meningococci with type 4 *porB*. Meningococci causing disease in England and Wales belong to a number of clonal complexes and have a number of different phenotypes (4). In New Zealand the strain causing the majority of cases of disease appears to be highly clonal, which may account for the differences observed when the *porB* sequences from B:nt:P1.4 meningococci in New Zealand and the United Kingdom are compared.

We found that the *porB* PCR-AREA profiles were reproducible and easy to interpret and that they enabled the *porB* types of all meningococci to be determined.

Nucleotide sequence accession numbers. The sequences of type 4,undef,7,14a, type undef,Da,7,14a, and type undef,undef, 7b,14a have been submitted to the GenBank database and have been given accession numbers AY333946, AY333947, and AY333948, respectively.

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