Correlation between Serological and Sequencing Analyses of the PorB Outer Membrane Protein in the *Neisseria meningitidis* Serotyping System

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The current serological typing scheme for *Neisseria meningitidis* is not comprehensive; a proportion of isolates are not serotypeable. DNA sequence analysis and predicted amino acid sequences were used to characterize the structures of variable-region (VR) epitopes on *N. meningitidis* PorB proteins (PorB VR typing). Twenty-six *porB* gene sequences were obtained from GenBank and aligned with 41 new sequences. Primary amino acid structures predicted from those genes were grouped into 30 VR families of related variants that displayed at least 60% similarity. We correlated VR families with monoclonal antibody (MAb) reactivities, establishing a relationship between VR families and epitope locations for 15 serotype-defining MAbs. The current panel of serotype-defining MAbs underestimates by at least 50% the PorB VR variability because reagents for several major VR families are lacking or because a number of VR variants within some families are not recognized by serotype-defining MAbs. These difficulties, also reported for serosubtyping based on the PorA protein, are shown as inconsistent results between serological and sequence analyses, leading to inaccurate strain identification and incomplete epidemiological data. The information from this study enabled the expansion of the panel of MAbs currently available for serotyping, by including MAbs of previously undetermined specificities. Use of the expanded serotype panel enabled us to improve the sensitivity of serotyping by resolving a number of formerly nonserotypeable strains. In most cases, this information can be used to predict the VR family placement of unknown PorB proteins without sequencing the entire *porB* gene. PorB VR typing complements serotyping, and a combination of both techniques may be used for full characterization of meningococcal strains. The present work represents the most complete and integrated data set of PorB VR sequences and MAb reactivities of serogroup B and C meningococci produced to date.

Meningococcal disease has been a significant cause of mortality and morbidity throughout the world (1, 17). Most epidemiological investigations of meningococcal disease utilize classification schemes based on differences among meningococcal cell envelope molecules. All meningococci express PorB, an outer membrane porin protein (OMP); *porB* genes have been assigned to either class 2 or class 3 homology allele groups (11, 21). Most strains also express a class 1 OMP (9, 24), which has been named PorA; its gene is designated *porA*. Variations in PorB and PorA form the basis for meningococcal serotyping and serosubtyping, respectively (9). Antibodies against both proteins are bactericidal, making serotyping results useful not only for epidemiologic surveillance of meningococcal disease, but also for identifying potential vaccine components (4, 8, 19).

Several studies characterizing strains based on serosubtyping and primary structure predicted from *porA* gene sequencing (PorA variable-region [VR] typing), have elucidated the natures, structures, topologies, and reactivities of epitopes in VRs of PorA proteins. A new serosubtyping designation was created on the basis of differences in two VRs located in surface-exposed loops I (VR1) and IV (VR2) of PorA (14, 16, 22, 26). A similar sequence analysis of PorB proteins has shown four regions with a high level of amino acid variability, VR1 through -4, located in loops I, V, VI, and VII, respectively, of the protein (2, 4, 7, 27, 32).

The current panel of 15 serotype-defining monoclonal antibodies (MAbs) reacts with only one PorB VR in any given strain; this finding has historically been interpreted to suggest that a given strain possesses only one immunodominant epitope on the entire PorB protein. Furthermore, 20 to 60% of meningococcal B and C isolates from any given population cannot be serotyped with these reagents, a problem which could distort the serotype prevalence data in defined areas (18, 20, 25). The characterization of VR loops and recent evidence that a serotype-specific sequence can reside in any of four loops suggest that the sensitivity of serotyping could be improved through further antigenic analysis of the PorB protein.

The goal of this study was to expand the number of PorB epitopes available as discriminatory markers on meningococcal strains. This study (i) establishes the nucleotide sequences of *porB* genes in 41 strains, (ii) estimates the degree of variation in each of the four VRs, (iii) establishes a VR type classification scheme for the PorB protein, (iv) identifies the reactivities of all available serotyping MAbs with those strains, and (v) predicts the locations of serotype-defining MAb binding.

MATERIALS AND METHODS

Bacterial strains. *Neisseria meningitidis* strains were obtained from M. Achtman, Max-Planck-Institut fur Molekulare Genetic, Berlin, Germany; O. L.

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Frøholm, Statens Institute for Folkehelse (SIF), Oslo, Norway; P. Kriz, National Institute of Public Health (NIPH), Prague, Czech Republic; and F. E. Ashton, Laboratory Center for Disease Control, Tunney's Pasture, Ottawa, Canada (Table 1). Brazilian *N. meningitidis* strains were recovered from blood or cerebrospinal fluid samples from patients with systemic disease in several states and cities.

Serotyping. All *N. meningitidis* strains were serotyped and serosubtyped by dot blotting of whole-cell suspensions (30) with all MAbs listed below. MAbs for serotypes 2a (F12-7B7/1E10), 2b (F1-9H10/1B3), 4 (F10-2H7/1F7), 7 (F22-8B5/1D10), 17 (F4-3C1/1A6), and 10 (F11-6D12/1C5) and for serosubtypes P1.4 (F11-2A9/1A4), P1.1 (F10-5G6/1B11), P1.23 (F4-1F1/1F3), and P1.15 (F8-8F12/1D6) were produced at the Adolfo Lutz Institute (IAL), São Paulo, Brazil, by the authors. MAbs for serotypes 8 (2725H6) and 15 (1951C8) and for serosubtype P1.2 (1649C7) were provided by C. E. Frasch, Food and Drug Administration (FDA), Bethesda, Md. MAbs for serotypes 2b (2H10-2), 2c (5-1-P2c), 5 (7BG5-H2), 11 (9-1-P11), and 19 (17-1-P19) and for serosubtypes P1.3 (12-1) and P1.16 (3-1-P1.16) were provided by W. D. Zollinger, Walter Reed Army Institute of Research (WRAIR), Washington, D.C. MAbs for serotypes 6 (MN1-B4C), 9 (MN5C10D), and 16 (93E9.1) and serosubtypes P1.6 (MN19D6-10) and P1.9 (MN5A10.7) were provided by J. T. Poolman, University of Amsterdam, Amsterdam, The Netherlands. The MAb for serotype 22 (ATIA5A7/5) was provided by P. Kriz, NIPH, Prague, Czech Republic. We used an additional set of MAbs provided by the National Institute for Biological Standards and Control (NIBSC), Potters Barr, England, for serotypes 1 (MN3C6B-95/680), 2a (5D4-5-95/682), 2b (MN2C3B-95/684), 4 (MN14G21-95/686), 14 (MN5C8C-95/688), 15 (8B5-5-G9-95/690), and 21 (6B11-F2-B5-95/692) and for serosubtypes P1.1 (MN14C2.3-95/694), P1.2 (MN16C10F4-95/696), P1.3 (5G8B2F9-95/698), P1.4 (MN20B9.34-95/700), P1.5 (MN22A9.19-95/702), P1.7 (MN14C11.6-95/706), P1.10 (MN20F4.17-95/710), P1.12 (MN20A7.10-95/712), P1.13 (MN25H10.75-95/714), P1.14 (MN21G3.17-95/716), P1.15 (MN3C5C-95/718), and P1.16 (MN5C11G-95/720).

MAb production and characterization. The methods used for the production of MAbs 7 and 10 were described previously (10). MAbs were prepared against *N. meningitidis* H-276 (870227) and N.34/94 (Table 1). The MAb class and subclass were determined by an enzyme-linked immunosorbent assay using peroxidase-conjugated anti-mouse immunoglobulins as described by the manufacturer (mouse hybridoma subtyping kit; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The cell lines and MAbs for serotypes 7 and 10 are available by request from IAL.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% (wt/vol) polyacrylamide gels as previously described (13). Whole-cell bacterial suspensions were boiled in sample buffer containing 2-mercaptoethanol for 5 min. After electrophoresis, either the gels were stained with Coomassie brilliant blue R-250 or proteins were transferred onto BA85 nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.). Immunoblots and dot blots were probed with appropriate dilutions of ascitic fluid (Table 1). Reactivity was visualized by using horseradish peroxidase-conjugated anti-mouse immunoglobulin G, followed by the substrate 3-amino-9-ethyl-carbazole and hydrogen peroxide (30). The incubation of immunoblots with primary antibodies was performed either without detergent or with 0.25% detergent Empigen BB to restore the antibody-binding capacity of the boiled antigens (15, 29).

DNA extraction and PCR. Cells were harvested from tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 1% (vol/vol) horse serum into 10 ml of 0.1 M NaCl–0.05 M EDTA–0.05 M Tris, pH 8. Cells were lysed with SDS, and high-molecular-weight DNA was extracted as previously described (3). Each DNA sample was examined spectrophotometrically to determine concentration and purity.

Primers P27 and P28 were used to amplify the *porB* gene (7). Each final PCR mixture (50 µl) contained 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim), template DNA at 1 ng μl^{-1}, 10 mM Tris-Cl (pH 8), 50 mM KCl, 1.5 mM MgCl$_2$, 200 µM (each) dATP, dCTP, dGTP, and dTTP, and 0.4 µM concentrations of each primer. Reaction mixtures were first incubated for 5 min at 95°C. Then 5 cycles were performed as follows: 1 min at 94°C, 2 min at the annealing temperature of 60°C, and 20 s at 72°C; this was followed by 25 cycles of 30 s at 90°C, 20 s at 60°C, and 20 s at 72°C. Reaction mixtures were then incubated at 72°C for a further 5 min. PCR products were purified with the High Pure PCR product purification kit (Boehringer Mannheim).

Sequences of *porB* genes. For sequencing the *porB* genes encoding the class 3 protein, 14 primers were used (6 forward and 8 reverse), and for sequencing the *porB* genes encoding the class 2 protein, 11 primers were used (6 forward and 5 reverse). The primers were designed to be complementary to the conserved regions of the *porB* gene (Table 2). Sequencing was performed by using the Taq Dye-deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Sequencing products were purified by using Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.) and resolved on a 5% acrylamide–8 M urea gel with an Applied Biosystems model 373S automated DNA sequencing system. DNA sequences obtained from these reactions were aligned and edited, and consensus sequences were determined with the University of Wisconsin Genetics Computer Group (GCG) package. When a new nucleotide sequence for any VR was found, the *porB* gene amplification and sequencing were repeated once.

Twenty-six *porB* gene nucleotide sequences were obtained from GenBank and

aligned with the 41 new sequences by using GCG sequence analysis software (5). Amino acid alignments were obtained by translating the nucleotide sequences that had been aligned such that codons were in frame. Distance matrices were calculated by using the Distances program of the GCG package (5). An exact amino acid match was scored as 1.5, and any other residue was scored as 0.

Nucleotide sequence accession numbers. The sequences of *porB* genes obtained during the study have been submitted to the GenBank database and have been assigned the accession numbers listed in Table 1.

RESULTS AND DISCUSSION

MAb production and characterization. We produced two new MAbs, F22-8B5/1D10 (IgG2aκ) and F11-6D12/1C5 (IgG2bκ). We characterized these and a third MAb, 17-1-P19 (IgG2bκ), by dot blotting and Western blotting with all strains in Table 1 as antigens. Our results were consistent with those of Tsai et al. (24) in defining the PorB classes for these strains. The dot blot results with these 3 new MAbs in addition to the currently used panel of 15 serotyping MAbs are presented in Table 1 (Proposed serotypes column). The three new MAbs recognized only PorB class 3 proteins and failed to react with any class 2 PorB proteins. Western blotting results with 37 strains known to express class 3 proteins showed that these MAbs bound only in the presence of the detergent Empigen BB (Calbiochem-Novabiochem Corp., La Jolla, Calif.), verifying that the MAb reactivity had been restored. No discrepancies between the two methods were found.

We have renamed MAb F22-8B5/1D10 MAb 7 and have renamed MAb F11-6D12/1C5 MAb 10 to fill the gaps in the serotype scheme designations. MAb 17-1-P19 was renamed MAb 19 since it was developed against the prototype 19 serotype reference strain (32a).

***porB* gene analysis.** To identify PorB amino acid sequences associated with serotype-defining MAb epitopes, we sequenced 41 *porB* genes (Table 1) and obtained 26 additional *porB* sequences from GenBank. The alignment of 67 predicted PorB amino acid sequences identifies the 4 PorB VR sequences on loops I, V, VI, and VII according to the structural model of class 2 and 3 OMPs (26). A distance matrix of amino acid sequences for each of the four VRs identified 60 distinct VR types (11, 16, 17, and 16 different VR types in VR1 to -4, respectively; Table 3).

VR families and VR typing. Type and subtype (identified by letters) were defined as the VR types for PorB and PorA proteins, respectively (23). Serotype and serosubtype were defined solely by MAb reactivity with PorB and PorA proteins, respectively, and were designated by numbers (except for the serotypes 2a, 2b, and 2c). Numbers were reserved for epitopes defined by MAbs, while letters were used for those VR sequences for which no MAbs are currently available.

The 60 VR types were grouped into 30 amino acid sequence families, similar to those described for PorA proteins (22), except that a 60% cutoff was used. Each VR type is a member of a family of related variants that displayed at least 60% amino acid sequence similarity to the prototype. VR families were designated by sequential capital letters for each VR; however, when a particular VR family was established as the epitope for a serotype-defining MAb, the VR family name was the same as that of the MAb recognizing that region (numbers). A prototype for each VR family was defined as the amino acid sequence of the appropriate VR present in the original strain providing the antigen to produce the MAb. For a VR family not recognized by any available MAb, the prototype was defined as the amino acid sequence of the appropriate VR in the strain first analyzed (Table 3). Amino acid sequences within a VR family were distinguished from those of the prototype VR and other variants by lowercase letters given

TABLE 1. Serotype, serosubtype, and PorB VR type characteristics of *N. meningitidis* strains analyzed

Strain	Source[h]	Serogroup	Serotype Original[a]	Serotype Proposed[b]	Serosubtype[f]	Accession no.[c]	PorB protein class[d]	PorB VR type[e] 1	2	3	4
Prototype strains											
M1080	FDA	B	1	19,7,1	P1.7,1	X65530	3	19	Ac	7a	1
B16B6	FDA	B	2a	2a	P1.5,2	X67937	2	C	Eb	2a	C
2996	FDA	B	2b	2b	P1.5,2	X67939	2	C	Ea	2b	C
2396	SIF	B	2c	2c	P1.5,2	**U92911**	2	C	2c	2bb	Db
126E	SIF	C	3	19,10	P1.5,2	U07191	3	19	Ac	10	14a
Z-4008[j]	MPIG	B	4	4,7	P1.4	**U59868**	3	4	D	7	14a
H-276[k]	MPIG	B	4	4,7	P1.10	**U59870**	3	4	D	7	14a
M981	FDA	B	4	4,7	P1−	X65531	3	4	B	7	14a
M992	FDA	B	5	5	P1.7,1	**U92909**	2	Ca	Ec	5	Ca
M990	FDA	B	6	16,6	P1.6	X67936	2	D	16	6	D
M978	FDA	B	8	19,10	P1.7,1	U07189	3	19	Aa	10	14a
M982	FDA	B	9	NT	P1.9	X67938	2	C	E	B	Da
N.34/94	IAL	B	NT[i]	19,10	P1.4	U34194	3	19	Db	10	Aa
M136	SIF	B	11	16,11	P1−	**U95366**	2	D	16	11	D
S3032	SIF	B	12	19,7	P1.12,16	X65534	3	19	Ab	7a	A
S3446	SIF	B	14	19,14	P1.23,14	U07188	3	19	Db	7c	14
H.44/76	SIF	B	15	15	P1.7,16	X83428	3	A	A	A	Ba
BB393	FDA	B	15	15	P1.3	**U62905**	3	A	A	A	Ba
H355	FDA	B	15	15	P1.15	M68962	3	A	A	A	B
60E	FDA	C	16	16,2b[g]	P1.7,1	**U92908**	2	C	16	2ba	Db
6557	FDA	B	17	17,7	P1.23,14	U07190	3	B	C	7	14b
190I	FDA	B	18	19,10	P1.6	U07192	3	19	Aa	10	14a
6940	FDA	B	19	19,10	P1.6	U11030	3	19	Ac	10	14a
35E	SIF	C	20	2c	P1.1	X67940	2	C	2c	2bb	Db
M1027	FDA	A	21	4,21	P1−	**U59869**	3	(4)	D	7b	(21)
503/93	NIPH	B	22	NT	nt	**U92906**	2	C	Ed	5a	Db
Additional strains											
94010	LCDCTP	C	2a	2a	P1.5,2	**U92907**	2	C	Eb	2a	C
M986	FDA	B	2a	2a	P1.5,2	**U92912**	2	C	Eb	2a	C
N.446/95	IAL	C	2b	2b	P1.10	**U92910**	2	C	Ea	2b	C
N.781/95	IAL	C	2b	2b	P1.10	**U92913**	2	C	Ea	2b	C
N.862/95	IAL	C	2b	2b	nt	**U94962**	2	C	Ea	2b	C
N.1302/95	IAL	B	2b	2b	P1.5	**U92905**	2	C	Ea	2b	C
N.459/93	IAL	B	4	4,7	P1.7,1	**U92903**	3	4	Da	7	14a
N.585/94	IAL	B	4	4,7	P1.13	**U92899**	3	4	B	7	14a
N.300/94	IAL	B	4	4,7	P1.9	**U59866**	3	4	B	7	14a
CU385	FDA	B	4	4,7	P1.15	L03786	3	4	B	7	14a
N.1281/95	IAL	B	4	4,7	nt	**AF012017**	3	4	B	7	14a
N.150/88	IAL	B	4	4,7	P1.15	**U59872**	3	4	D	7	14a
N.44/89	IAL	B	4	4,7	P1.15	**U59871**	3	4	D	7	14a
N.155/94	IAL	B	4	4,7	P1.15	**U94960**	3	4	D	7	14a
N.610/93	IAL	B	4	4,7	P1.15	**U59867**	3	4	D	7	14a
N.314/95	IAL	B	4	4,7	P1.15	**AF012015**	3	4	D	7	14a
N.454/95	IAL	B	4	4,7	P1.15	**AF012013**	3	4	D	7	14a
N.45/95	IAL	B	4	4,7	P1.15	**AF012014**	3	4	D	7	14a
501	CDC	B	4,15	4,15	nt	**AF002250**	3	4	A	A	Ba
N.294/97	IAL	B	15	15	P1.16	**AF012018**	3	A	A	A	Ba
N.109/93	IAL	B	4	4,10	P1.9	**U92904**	3	4b	D	10	14a
N.23/97	IAL	B	4	4,10	P1.15	**AF001321**	3	4	D	10	14a
N.520/95	IAL	B	4	4,10	P1.15	**AF012016**	3	4	B	10	14c
N.163/94	IAL	B	8	19,10	P1.6	**U92902**	3	19	Aa	10	14a
N.19/93	IAL	B	7	7	nt	**U92901**	3	A	D	7d	14a
N.105/93	IAL	B	17	17,7	nt	**U92900**	3	B	C	7	14b
N.1342/96	IAL	B	17	17,7	P1.7,16	**AF001322**	3	B	C	7	14b
N.1329/96	IAL	B	17	17,7	P1.7,16	**AF001319**	3	B	C	7	14b
N.405/94	IAL	B	19	19,7,1	nt	**U94961**	3	19	Ac	7a	1
N.1434/96	IAL	B	19	19,14	P1.23,3	**AF001323**	3	19	Db	7c	14
N.1450/96	IAL	B	19	19,14	P1.23,3	**AF001320**	3	19	Db	7c	14
N.1454/96	IAL	B	19	19,14	P1.23,3	**AF001318**	3	19	Db	7c	14
N.257/93	IAL	B	NT	NT	nt	**U92914**	2	Cb	Ed	C	Ca
J129	GB	B	4	(4),(7)[h]	P1.15	X67933	3	4a	D	7	14a
BB1350	GB	B	4	(4),(7)	UK	U07193	3	4	D	7	14a
PM36	GB	B	4	(4),(7)	P1.15	X79464	3	4	B	7	Bb
PM40	GB	B	4	(4),(7)	P1.2	X78579	3	4	B	7	14a
B54	GB	A	4,21	(4),(21)	P1.X,9	X67934	3	4	D	7b	21
B227	GB	A	4,21	(4),(21)	P1.5,9	X67935	3	4	D	7b	21
G1960	GB	B	15	(15)	P1.7,16	X67932	3	Aa	A	A	Ba
MC58	GB	B	15	(15)	UK	X65532	3	A	A	A	Ba

[a] Original serotypes were defined by Frasch et al. (9) by using rabbit immunosera and MAbs.

[b] Proposed serotypes were characterized by dot blotting reactions using serotype-defining MAbs, including the new MAbs 7, 10, and 19 as described in the text. Because we did not have access to eight meningococcal strains from sequences obtained from the GenBank, the serotyping of those strains was not repeated in our laboratory; the serotypes based on predicted reactions with the serotype-defining MAbs are presented in parentheses.

[c] Boldface GenBank accession numbers are those for which the nucleotide sequences were determined in this work.

[d] PorB protein classes were defined by SDS-PAGE (24).

[e] PorB VR types were defined by a comparison of MAb reactivities and amino acid sequence homology as described in the text.

[f] P1−, no expression of PorA class 1 protein by SDS-PAGE; nt, nonserosubtypeable; UK, unknown serosubtype.

[g] VR3-2ba can only be detected by dot blotting using serotype-defining MAb 2b (F1-9H10/1B3) or 1082E7.

[h] MPIG, Max-Planck-Institut Fur Molekulare Genetic; LCDCTP, Laboratory Center for Disease Control, Tunney's Pasture. GB, GenBank.

[i] NT, nonserotypeable.

[j] 882066.

[k] 870227.

TABLE 2. Nucleotide sequences of primers used for *porB* gene
amplification and sequencing

Primer[a]	Nucleotide sequence
porB gene	
(F) P27 ....TTG TAC GGT ACA ATT AAA GCA GGC GT
(R) P28...........................TTA GAA TTT GTG ACG CAG ACC AAC
Class 2 protein-encoding *porB* gene	
(F) 2F327 ....GGG TAC TAT CGG TCG TGT AGA AAG
(F) 2F657 ....GTA CGT TTC TGT TGC CGG TCA G
(F) 2F505 ....GCC GGT TTC TTC GGT CAA TAC GCA G
(F) 2F476 ....CTT ACC ACG CCG GTC TGA AAT ACG
(F) 2F777 ....CGT AAC GCC TCG CGT TTC TTA CGC
(R) 2R327 ....CTT TCT ACA CGA CCG ATA GTA CCC
(R) 2R177 ....GAC TGG CGG TTA CCC CAG CCG CTG
(R) 2R476 ....CGT ATT TCA GAC CGG CGT GGT AAG
(R) 2R777 ....GCG TAA GAA ACG CGA GGC GTT ACG
Class 3 protein-encoding *porB* gene	
(F) 273...........................CGA CAT CAA TCC TTG GGA TAG C
(F) 392...........................GCG TAC AAT ACG CGC TTA ACG A
(F) 685...........................CGC TTC GGC AAC GTA ACG CCC C
(F) 731...........................CGA CAA TGA TGC CCT GTA C
(F) 651...........................GGC GGT GCC TAT AAA AGA CAT
(R) 439...........................GTA GCG TAC GGA AAT GAG GCG
(R) C3RVR1 ....CGG TTT GAG AGT TGT GCG
(R) 714...........................GGT GAA TCT GGT ATT TCT CAA T
(R) 294...........................GCT ATC CCA AGG ATT GAT GTC G
(R) 414...........................GTC GTT AAG CGC GTA TTG TAC G
(R) 700...........................TTA CGT TGC CGA AGC GGT ATG C
(R) 202...........................GTT ACC GAG GTC TTC TTG GCC

[a] Primers P27 and P28 were used to amplify the *porB* gene (7). Primer
C3RVR1 was described previously (32). (F) and (R), forward and reverse ori-
entations of primers, respectively, in relation to the direction of gene transcrip-
tion.

in the order of elucidation (e.g., 7, 7a or A, Aa); however, since
the detection of those variants was not made by immunochem-
ical reactions, we used the additional letters only as part of the
VR typing system and not as part of the serotyping designa-
tion. A VR amino acid sequence variant with less than 60%
sequence relatedness to the prototype of any VR family was
assigned to a new VR family, in order of elucidation. When
nucleotide changes produced no difference in the amino acid
sequence, the same prototype letter or number designation
was placed in brackets, e.g., VR type A, [A]; VR type 4, [4].

Relationships between MAb reactivity and PorB VR family.
The comparison of all available serotype-defining MAb reac-
tivities and amino acid sequences of VR families is presented
in Table 3. All strains of a given serotype possessed amino acid
sequences characteristic of their respective family at the
epitope-defining VR; only strains with amino acid sequences at
least 60% related (same family) in the same VR reacted with
a particular MAb. We then correlated VR families with MAb
reactivities, establishing a relationship between VR families
and epitope locations for all serotype-defining MAbs (Table
3). In most cases, a sequence change in one VR loop occurred
independently of changes in any other loops. Some exceptions
were noted; for example, VR2-B or VR2-D was almost exclu-
sively present in combination with VR1-4.

A comparison of PorB amino acid sequences and serological
results permitted a rational reassignment of serotype designa-
tions as well as a determination of probable epitope locations
for serotype-defining MAbs. The serotype characterization of
an unknown meningococcal strain included a determination of
epitope reactivities at regions VR1, VR2, VR3, and VR4 when
MAbs were available for those epitopes. For example, the

serotype designation 19,7,1 for strain M1080 indicates the
PorB protein with sequences of VR family 19 in VR1 (VR1-
19), VR family 7 in VR3 (VR3-7), and VR family 1 in VR4
(VR4-1). There is no available MAb for VR family A on VR2
(VR2-A) (Tables 1 and 3). Since serotyping becomes a sum-
mation of up to four results, we have expanded the definition
of serotyping to include the immunological characterization of
VR1 to -4 of the meningococcal PorB protein.

The probable epitope location for serotype 15 strains could
most likely have been identified as VR1, VR3, or VR4 by using
nucleotide probes (2). Our *porB* nucleotide sequence and the
immunological results for meningococcal strain 501 showed
that the epitope location for serotype 15 was not VR1. Strain
501 has VR1-4 and reacted by dot blotting and Western blot-
ting with serotype-defining MAbs 4 and 15. For serotype 17,
the probable epitope location could most likely have been VR1
or VR2. Additional studies are necessary to locate the VR
epitope for serotype-defining MAb 15 within either VR2-A,
VR3-A, or VR4-B and to locate that for serotype-defining
MAb 17 within either VR1-B or VR2-C of the PorB class 3
protein (Table 1).

We determined 14 previously unknown epitope locations by
correlating VR types with MAb reactivities: serotype 1 (VR4-
1), serotype 2a (VR3-2a), serotype 2b (VR3-2b), serotype 2c
(VR2-2c), serotype 4 (VR1-4), serotype 5 (VR3-5), serotype 6
(VR3-6), serotype 7 (VR3-7), serotype 10 (VR3-10), serotype
11 (VR3-11), serotype 14 (VR4-14), serotype 16 (VR2-16),
serotype 19 (VR1-19), and serotype 21 (VR4-21) (Table 1).

Epitope mapping studies of PorA proteins have shown that
a single amino acid change may or may not modify recognition
by the relevant serosubtype antibody (16, 22, 23, 28, 31). A
similar effect was observed with PorB proteins and serotype-
defining MAbs. For example, amino acid substitutions in
VR1-4a and VR1-4b were not close to the apex on loop I, and
reactivity with serotype-defining MAb 4 was not affected. On
the other hand, serotype-defining MAb 7 reacts with VR3-7,
VR3-7a, and VR3-7d strains but not with VR3-7b or VR3-7c
isolates, where a tyrosine on the top of loop VI has been
deleted (Table 3).

We also observed differences in specificity between serotype-
defining MAbs produced by different laboratories. The
VR3-2b family possesses three VR types: VR3-2b, VR3-2ba,
and VR3-2bb. The difference between VR3-2b and VR3-2ba is
a lysine-to-glutamic acid substitution on the top of loop VI. For
two serotype 2b MAbs, F1-9H10/1B3 (IAL) and 1082E7
(FDA), the substitution did not abolish antibody binding in dot
blot reactions, while serotype-defining MAbs MN2C3B
(NIBSC) and 2H10-2 (WRAIR) failed to react with VR3-2ba
strains.

Serotypes to be removed. An analysis of results in Table 1
suggested that new MAbs 7, 10, and 19 identify epitopes on
class 3 proteins that are shared among some meningococcal
strains of different serotypes. In the past these MAbs would
have been disqualified because the previous dogma stated that
each serotype-defining MAb should recognize only one sero-
type. For this reason, serotype-defining MAbs 2c and 16 have
not been considered suitable for serotyping. Since we now
appreciate that a given MAb can recognize a VR common to
several serotypes, these MAbs can be incorporated and used to
improve the sensitivity and discriminatory power of serotyping
(Table 1). New serotype-defining MAbs and VR sequences can
be easily identified and incorporated into the system by using
Tables 1 and 3. Epitope-predicted sequence information can
be used as a guide for future serotype-defining MAb produc-
tion.

Some reference strains, previously defined as having differ-

TABLE 3. Characteristics of *porB* gene VR nucleotide sequences, PorB protein VR families, and types of *N. meningitidis*

Loop	PorB VR type[a]	PorB protein class[b]	Reactivity with serotype-defining MAb[c]	Strain	Sequence[d]		Accession no.
					VR nucleotides	VR amino acids	
I	VR1-4	3	+	M981	124 156 GAGCACAATGGAGGTCAGGTGGTTAGCGTTGAA	42 52 EHNGGQVVSVE	X65531
	VR1-[4]	3	+	M1027	..A.............................	....	U59869
	VR1-4a	3	(+)	J129	........G.......................	..K........	X67933
	VR1-4b	3	+	N.109/93	A...............................	K..........	U92904
	VR1-19	3	+	6940	GCTCACAATGGAGCTCAGGCGGCTAGCGTTGAA	AHNGAQAASVE	U11030
	VR1-A	3	NAA	H355	TTTCACCAGAACGGCCAAGTTACTGAAGTTACA	FHQNGQVTEVT	M68962
	VR1-Aa	3	NAA	G1960	...........................G..	..........A	X67932
	VR1-B	3	NAA	6557	TATTACGAAGACGGCAAAGCTGCTGAAGTTACA	YYEDGKAAEVT	U07190
	VR1-C	2	NAA	B16B6	19 54 GATGCTGGTACATATAAAGCTCAAGGCGGAAAATCT	7 18 DAGTYKAQGGKS	X67937
	VR1-Ca	2	NAA	M992	.......C..........A................	..A...D.....	U92909
	VR1-Cb	2	NAA	N.257/93	.......A..........................	..D........	U92914
	VR1-D	2	NAA	M990	AATCAAGGCGTAAAAACC	NQGVKT	X67936
V	VR2-A	3	NAA	H355	610 633 CATCAAGTGCAAGAGGGCTTGAAT	204 211 HQVQEGLN	M68962
	VR2-Aa	3	NAA	M978	........A.......A.......	.....D..	U07189
	VR2-Ab	3	NAA	S3032	....G...........A.A.A...	.R...DI.	X65534
	VR2-Ac	3	NAA	M1080	..............AA.G.....	.....NV.	X65530
	VR2-B	3	NAA	M981	GTGCGGGTGGATGAGAACGTGAAT	VRVDENVN	X65531
	VR2-C	3	NAA	6557	GAGCAGATAGATAACGTGAAG	EQIDNVK	U07190
	VR2-D	3	NAA	J129	CAGGATGTGGATGACGTGAAG	QDVDDVK	X67933
	VR2-Da	3	NAA	N.459/93	........A............	...N...	U92903
	VR2-Db	3	NAA	N.34/94	...A........A........	.N..N..	U34194
	VR2-2c	2	+	2396	556 603 AACACTGATGCAGAACGTGTTGCAGTAGGTGCTACAGATGCCAATCCT	186 201 NTDAERVAVGATDANP	U92911
	VR2-16	2	+	60E	AACAAGGATGCAGAACGTGTTGCAGCAGGTACTTCAGGTGCCTATGCT	NKDAERVAAGTSGAYA	U92908
	VR2-E	2	NAA	M982	AACAATGATGCAGAACGTGTTGCAGTAAATACTCCAAATGCCCATGCT	NNDAERVAVNTPNAHA	X67938
	VR2-Ea	2	NAA	2996	..AG..A.....................G...........C..	KDN........A...P	X67939
	VR2-Eb	2	NAA	B16B6	....C...................G...........C..	.T.........A...P	X67937
	VR2-Ec	2	NAA	M992	...G...............A............	.D........T....	U92909
	VR2-Ed	2	NAA	N.257/93	..AG.TA.......G.......GG....A..GG.......C..	KDN......G.TG..P	U92914
VI	VR3-7	3	+	H-276	727 741 GTTGAAGACAATTAT	243 247 VEDNY	U59870
	VR3-7a	3	+	S3032	........A......	..E..	X65534
	VR3-7b	3	−	B54	...........---	....-	X67934
	VR3-7c	3	−	S3446	...A........---	.K..-	U07188
	VR3-7d	3	+	N.19/93	...A..........	.K...	U92901
	VR3-10	3	+	N.34/94	GCTTTGCCAAACGACAAT	ALPNDN	U34194
	VR3-A	3	NAA	H355	ACTGATGCTTCCAAT	TDASN	M68962
	VR3-2a	2	+	B16B6	694 717 AACAACGAGGTTGGTTCTACCAAG	232 239 NNEVGSTK	X67937
	VR3-2b	2	+	2996	AATAACACTGGTACTGTCGGCAAACAA	NNTGTVGKQ	X67939
	VR3-2ba	2	−/+	60E	....................G.....	.......E.	U92908
	VR3-2bb	2	−	35E	.........T..G.......G.....	...VS..E.	X67940
	VR3-5	2	+	M992	AACAACGACGGTTCTGCCAATAAT	NNDGSANN	U92909
	VR3-5a	2	−	503/93	........T...A........C.A	....T..Q	U92906
	VR3-6	2	+	M990	CAA	Q	X67936
	VR3-11	2	+	M136	AACAACGAAGGTGCTGCTAAACAA	NNEGAAKQ	U95366
	VR3-B	2	NAA	M982	AACAACGAGGTTGGTAATCTCAATATT	NNEVGNLNI	X67938
	VR3-C	2	NAA	N.257/93	AATAACACCGGTGTTGCCGCCGATCAA	NNTGVAADQ	U92914

TABLE 3—*Continued*

Loop	PorB VR type[a]	PorB protein class[b]	Reactivity with serotype-defining MAb[c]	Strain	Sequence[d]		Accession no.
					VR nucleotides	VR amino acids	
VII	VR4-1	3	+	M1080	835 864 TCGTTTGATGCTACAAACTACAACAACGAT	279 288 SFDATNYNND	X65530
	VR4-14	3	+	S3446	TCGTTTGATGATGCAGACTACACCAACGAT	SFDDADYTND	U07188
	VR4-14a	3	−	M978	....................TA.G......	......LS..	U07189
	VR4-14b	3	−	6557	................CG.TA...TAC.	......RY.T	U07190
	VR4-14c	3	−	N.520/95	.........A.........TA.G......	...N..LS..	AF01201
	VR4-21	3	+	M1027	TCGGTTGATGATGCAAAACGCGACAATACT	SVDDAKRDNT	U59869
	VR4-[21]	3	+	B54	..A.........................	....	X67934
	VR4-A[e]	3	NAA	S3032	TTGGTTGATAGTGCAGACTACACCAACGAT	LVDSADYTND	X65534
	VR4-Aa	3	NAA	N.34/94	..................TA.G......	......LS..	U34194
	VR4-B	3	NAA	H355	TTGGTTGATAATGCAGACATAGGCAACGAA	LVDNADIGNE	M68962
	VR4-Ba	3	NAA	MC58	........G..................	...D......	X65532
	VR4-Bb	3	NAA	PM36	.C.T.......................	SF........	X79464
	VR4-C	2	NAA	B16B6	826 840 GTGAAAGACGCAAAT	277 281 VKDAN	X67937
	VR4-Ca	2	NAA	M992	............G.	....S	U92909
	VR4-D	2	NAA	M990	GAGAAAGTAGCACGT	EKVAR	X67936
	VR4-Da	2	NAA	M982	.......C.......	..A..	X67938
	VR4-Db	2	NAA	35E	A......C.......	K.A..	X67940

[a] PorB VR types were defined by comparison of MAb reactivities and amino acid sequence similarity as described in the text. Brackets indicate that nucleotide sequence differences produce no amino acid changes with respect to the prototype.

[b] PorB protein classes were defined by SDS-PAGE (24).

[c] Reactivity with serotype-defining MAbs was evaluated by dot blotting and immunoblotting reactions; the results were 100% concordant. Immunoblot reactions were positive only in the presence of detergent Empigen BB. +, positive reaction; −, negative reaction; −/+, the reaction can be negative or positive depending on the MAb used; see text. Parentheses indicate that the reactivity is not based in experimental evidence but rather is predicted. NAA, no antibody available.

[d] The numbers above the sequences refer to the nucleotide or amino acid VR position, based on the *porB* gene sequence of B16B6 strain for the class 2 protein and S3032 strain for the class 3 protein; conserved regions of the loops were not considered part of the VRs. −, nucleotide or amino acid deletion; ., nucleotide or amino acid identical to that of the prototype.

[e] For serotype 12, the corresponding epitope is most probably VR4-A(2), but since there is no MAb 12 available, we did not consider this designation.

ent serotypes, have identical VR amino acid sequences, or VR types, at all four VRs of their PorB proteins (Table 1). One example is serotypes 2c and 20, and another is serotypes 3, 8, 18, and 19 (2; this study). The VR sequences for serotypes 8 and 18 are identical at all four VRs, as are those for serotypes 3 and 19. Serotypes 3 and 19 are identical to serotypes 8 and 18 at VR1, -3, and -4, but they differ by two amino acids at VR2. It is not clear whether this difference of two amino acids is sufficient to generate an epitope that could discriminate these two serosubtypes (Tables 1 and 3). In addition, serotype-defining MAb 8 failed to recognize any PorB protein epitope by Western blotting and was not further considered. It should be noted that the type 8 MAb reactivity may be to a very conformational epitope that requires more renaturing than usually occurs even with Empigen BB. We suggest keeping serotypes 2c and 19, since specific MAbs for these prototypes are available, and removing from the serotype scheme serotypes 3, 8, 18, and 20.

The MAb defining new serotype 22 (12) recognized the prototype strain by dot blotting and the corresponding PorB class 2 protein by immunoblotting. Because the PorB VR sequences of the prototype strain are VR1-C, VR2-Ed, and VR4-Db, we predict that MAb 22 would react with similar PorB VR sequences such as those present on strains B16B6, N.257/93, and 60E (Table 1). However, MAb 22 failed to react with any of these strains, suggesting that MAb 22 may not be directed against any of the four VRs of PorB. Since we are basing the serotype system on reactivity with PorB VR epitopes, we dropped this serotype from consideration.

Two MAbs failed to recognize strains of reported homologous specificities. MAb MN14G21 (serotype-defining MAb 4)

did not recognize any of the serotype 4 strains listed in Table 3 with the exception of strain M1027; on the other hand, MAb MN5C10D (serotype-defining MAb 9) reacted in dot blots with whole-cell suspensions from all strains listed in Table 3. We did not analyze these MAbs further.

Serotyping versus PorB VR typing. The 30 PorB VR families are observed on both class 2 (14 families) and class 3 (16 families) proteins. The current MAb panel identifies only 50 and 25% of these families, respectively. With the addition of the three new MAbs (MAbs 7, 10, and 19), we were able to identify 44% of the PorB class 3 protein families (Table 3).

To examine the effects of the serotyping panel expansion on serotyping sensitivity, we tested 113 nonserotypeable strains expressing the class 3 PorB, which represented 11% of 1,047 meningococcal strains isolated in Brazil during 1994. The percentage of nonserotypeable strains was reduced to 3% ($n = 31$) when the three new MAbs were added to the 13-member serotyping panel (MAbs 1, 2a, 2b, 2c, 4, 5, 6, 7, 10, 11, 14, 15, 16, 17, 19, and 21). We believe that the preparation and characterization of additional MAbs for serotyping will increase the discriminatory power of this system further.

PorB VR typing is important to characterize the precise structures of VR epitopes on PorB molecules. These data demonstrate that the current panel of MAbs underestimates this variability by at least 50% because reagents for several major VR families are lacking or because a number of VR types are not recognized by serotype-defining MAbs. These difficulties, also reported for serosubtyping (6), are shown as inconsistent results between serological and sequence analyses, leading to inaccurate strain identification and incomplete epidemiological data.

In addition to its importance for epidemiological analysis and classification, serology is the most practical method to screen large numbers of samples during epidemics or to screen samples in field situations where specialized equipment is not accessible. PorB and PorA sequencing (VR typing) can be done only in specialized laboratories, is not currently practical for large numbers of samples, and is laborious and expensive. These concerns warrant improvements in serology systems based upon the production of new MAbs, based in turn on the use of VR sequence data such as that presented here.

In conclusion, we determined that the reactivities of serotype-defining MAbs could be correlated with PorB VR sequences. This information enabled us to expand the panel of MAbs currently available for serotyping by including MAbs of previously undetermined specificities. Use of the expanded serotype panel enabled us to improve the sensitivity of serotyping by resolving a number of formerly nonserotypeable strains. Furthermore, this information can be used to predict the family placement of unknown PorB proteins, without sequencing the entire *porB* gene. PorB VR typing complements serotyping results, and a combination of both techniques may be used to fully characterize meningococcal strains. The present work represents the most complete and integrated set of PorB VR sequences and MAb reactivities of meningococci produced to date.

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