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THE USE OF OLIGONUCLEOTIDE PROBES FOR MENINGOCOCCAL SEROTYPE CHARACTERIZATION

Claudio Tavares SACCHI (1), Ana Paula Silva de LEMOS (1), Anne M. WHITNEY (2), Carmo Elias A. MELLES (1), Claude André SOLARI (3), Carl E. FRASCH (4) & Leonard W. MAYER (2)

SUMMARY

In the present study we examine the potential use of oligonucleotide probes to characterize *Neisseria meningitidis* serotypes without the use of monoclonal antibodies (MAbs). Antigenic diversity on PorB protein forms the bases of serotyping method. However, the current panel of MAbs underestimated, by at least 50% the PorB variability, presumably because reagents for several PorB variable regions (VRs) are lacking, or because a number of VR variants are not recognized by serotype-defining MAbs[12]. We analyzed the use of oligonucleotide probes to characterize serotype 10 and serotype 19 of *N. meningitidis*. The *porB* gene sequence for the prototype strain of serotype 10 was determined, aligned with 7 other *porB* sequences from different serotypes, and analysis of individual VRs were performed. The results of DNA probes 21U (VR1-A) and 615U (VR3-B) used against 72 *N. meningitidis* strains confirm that VR1 type A and VR3 type B encode epitopes for serotype-defined MAbs 19 and 10, respectively. The use of probes for characterizing serotypes possible can type 100% of the PorB VR diversity. It is a simple and rapid method specially useful for analysis of large number of samples.

KEYWORDS: *Neisseria meningitidis;* Serotyping, Class 3 protein; *porB* gene.

INTRODUCTION

Most epidemiological investigations of meningococcal disease utilize classification schemes based on differences among meningococcal cell envelope molecules. Those classifications has increased our understanding on the dynamic of *Neisseria meningitidis* infections[4,11,13,14]. All meningococci express either a class 2 or 3 protein, and most strains also express a class 1 outer membrane protein (OMP)[7,16]. Those predominant proteins in the outer membrane function as porins, and antibodies against them are bactericidal[3,7]. Antigenic diversity among these proteins, forms the basis of serotyping and serosubtyping classification[7,10]. There are approximately 20 different serotypes within serogroups B and C and epitopes for 11 of these serotypes are expressed on class 3 protein (PorB).

Sequence analysis of *porB* genes (PorB typing) has shown four regions of variability, designated variable region (VR) 1 through 4. These are located in surface-exposed loops I, V, VI, and VII, respectively[1,3,6,18]. Comparison of PorB amino acid sequences and serological results permitted a rational re-assignment of serotype designation as well as a determination of probable epitope location for serotype-defining MAbs[12]. Serotype characterization of an unknown meningococcal strain include determination of epitope reactivity at regions VR1, VR2, VR3, and VR4 when MAbs are available for those epitopes[12]. Since serotyping becomes a sommation of up to 4 results, the definition of serotype has changed to include the immunological characterization of all 4 VRs. Therefore, the epitope location of serotype-defining MAbs are located on specific VRs[12].

The goal of this study was to examine the potential use of oligonucleotide probes to characterize *N. meningitidis* serotypes without to use MAbs. These probes represent *porB* VR sequences that encode for the surface-exposed serotype-defining epitopes on PorB protein. We examined the use of

(1) Bacteriology Division, Adolfo Lutz Institute, São Paulo, Brazil.
(2) Division of Bacterial and Mycotic Diseases, National Centers for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA.
(3) Bacteriology Department, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil.
(4) Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA.
Correspondence to: Claudio T. Sacchi. Bacteriology Division, Adolfo Lutz Institute, Av. Dr. Arnaldo 351, 01246-902 São Paulo, SP, Brazil. Phone 3061-0111 Extention 2055. e-mail:sacchi@usp.br

probes for serotypes 10 and 19 on a collection of meningococcal strains isolated in Brazil during 1994.

MATERIAL AND METHODS

Meningococcal strains. We selected 72 *N. meningitidis* strains according to their serotypes for analysis (Table 1). These strains were recovered from blood or cerebrospinal fluid samples from patients with systemic disease in Brazil. All selected *N. meningitidis* strains were serogrouped as described previously[13], serotyped and serosubtyped by dot-blotting of whole-cell suspensions as described by WEDEGE et al., 1990. MAbs for serotype 15, 16, 19, and serosubtypes P1.2, P1.3, P1.16 were provided by W.D. Zollinger. MAbs for serosubtype P1.9 was provided by J.T. Poolman, Bilthoven, Netherlands, and mAbs for serotypes 2a, 2b, 4, 10, 17 and serosubtypes P1.4, P1.7, P1.14, P1.15 were produced in Adolfo Lutz Institute.

TABLE 1
Characteristics of the 72 *Neisseria meningitidis* strains used in this study

Strain number	Serogroup: serotype	Serosubtype	Probe VR1-A 21U	Probe VR3-B 615U
N. 243/94	B:4	P1.15	-	-
N.678/94	B:4	P1.15	-	-
N.630/94	B:4	P1.15	-	-
N.722/94	B:4	P1.15	-	-
N.921/94	B:4	P1.7	-	-
N.230/94	B:4	P1.7	-	-
N.334/94	B:4	P1.15	-	-
N.365/94	B:4	P1.15	-	-
N.770/94	B:4	P1.15	-	-
N.145/94	B:4	P1.15	-	-
N.51/94	B:4	P1.7	-	-
N.404/94	B:4	P1.15	-	-
N.950/93	B:4	P1.9	-	-
N.454/94	B:4	P1.7	-	-
N.200/94	B:4	P1.15	-	-
N.555/94	B:4	P1.15	-	-
N.413/94	B:4	P1.15	-	-
N.513/94	B:4,10	P1.9	-	+
N.288/94	B:4,10	P1.9	-	+
N.665/94	B:4,10	P1.9	-	+
N.922/94	B:4,10	nt[a]	-	+
N.25/94	B:4,10	nt	-	+
N.539/94	B:4,10	P1.9	-	+
N.480/94	B:4,10	P1.9	-	+
N.363/94	B:4,10	P1.15	-	+
N.388/94	B:4,10	nt	-	+
N.1002/94	B:4,10	P1.2	-	+
N.734/94	B:4,10	P1.9	-	+
N.771/94	B:4,10	P1.9	-	+
N.420/94	B:4,10	P1.9	-	+
N.676/94	B:4,10	P1.9	-	+
N.527/94	B:4,10	nt	-	+
N.716/94	B:4,10	P1.9	-	+
N.862/94	B:4,10	P1.9	-	+
N.348/94	B:17	P1.14	-	-
N.671/94	B:17	P1.16	-	-
N.925/94	B:17	nt	-	-
N.1053/94	B:17	P1.16	-	-
N.240/94	B:17,10	P1.9	-	+
N.742/94	B:17,10	P1.16	-	+
N.627/94	B:19	nt	+	-
N.29/94	B:19	P1.15	+	-
N.1007/94	B:19	P1.15	+	-
N.434/94	B:19	P1.15	+	-
N.901/93	B:19	P1.9	+	-
N.154/94	B:19	P1.14	+	-
N.40/94	B:19	P1.14	+	-
N.405/94	B:19	nt	+	-
N.438/94	B:19	nt	+	-
N.453/94	B:19	nt	+	-
N.621/94	B:19	P1.15	+	-
N.622/94	B:19	P1.14	+	-
N.651/94	B:19	nt	+	-
N.666/94	B:19	nt	+	-
N.729/94	B:19	P1.7	+	-
N.780/94	B:19	nt	+	-
N.947/94	B:19	P1.14	+	-
N.34/94	B:19,10	P1.4	+	+
N.525/94	B:19,10	P1.16	+	+
N.113/94	B:19,10	P1.16	+	+
N.91/94	B:19,10	P1.9	+	+
N.149/94	B:19,10	P1.16	+	+
N.415/94	B:19,10	P1.15	+	+
N.347/94	B:19,10	P1.16	+	+
N.1119/94	B:19,10	P1.15	+	+
N.1124/94	B:19,10	P1.14	+	+
N.1092/94	B:19,10	nt	+	+
N.528/94	B:19,10	P1.9	+	+
N.163/94	B:19,10	nt	+	+
N.497/94	B:19,10	P1.15	+	+
N.292/94	B:19,10	P1.15	+	+
N.544/94	B:19,10	P1.15	+	+

[a] nt, non-serosubtypeable.

***porB* gene analysis.** To identify PorB amino acid sequences associated with serotype-defining MAbs, from 10 and 19 we selected 7 *porB* genes sequences obtained from GenBank for serotype reference strains that reacted with those MAbs, and sequenced the *porB* gene of the serotype 10 prototype strain (N.34/94), (Table 2). Primers NMP27 (5'-ttgtacggtacaattaaagcaggcgt) and NMP28 (5'-ttagaatttgtgacgcagaccaac) were used to amplify the *porB* gene of N.34/94 [5]. Purification of the PCR product was performed with QIAquick-spin PCR Purification Kit (QIAGEN). Eight oligonucleotide primers [NMP27, F651 (5'-ggcggtgcctataaaagacat), F731 (5'-cgacaatgatgccctgtac) and the reverse strands NMP28, R202 (5'-gttaccgaggtcttcttggcc), R439 (5'-gtagcgtacggaaatgaggcg), R714 (5'-ggtgaatctggtatttctcaat) and, C3RVR1 (5'-cggtttgagagttgtgcg) [19]] were used to sequence the *porB* gene using the Taq Dye-deoxy terminator cycle sequencing kit of Applied Biosystems. The primers were designed to be complement to any to the conserved regions of *porB* gene. The sequencing reactions were purified by using Centri-Sep spin columns (Princeton Separations) and resolved on a 5% acrylamide/8 M urea gel using an ABI model 373A automated DNA sequencing system. DNA sequences obtained from these reactions were aligned, edited and the consensus sequence determined with the University of Winsconsin Genetics Computer Group (GCG) package. The *porB* gene of N.34/94 strain amplification and sequencing were repeated twice, and no variability was found.

Oligonucleotide probes. To verify the correlation between VRs and serotype epitopes 10 and 19 we designed two different VR oligonucleotide probes. Probe 21U represents the VR1-A (5'-cgtagctcacaatggagctcaggcggct) and probe 615U the VR3-B (5'-cgaaattggctttgccaaacgaca). The probes were synthesized on an Applied Biosystems 380B DNA synthesizer and 5'end digoxigenin-labeled according to a standard protocol for digoxigenin-labeling.

Dot blots and hybridization assays. DNA extraction and purification have been previously described [1]. DNA from 21 prototype serotype strains and 72 Brazilian *N. meningitidis* strains was applied to positively charged nylon membranes (Boehringer Mannheim Biochemicals) in 1 µg dots and fixed by UV cross-linking. Filters were stored in plastic bags at -20 °C or used immediately. After prehybridization for 1 h, the probes were added, and hybridization for 16 h was performed at temperatures of 50 °C and 60 °C for the 21U and 615U probes, respectively. The prehybridizations were done at the same temperatures. The Genius kit was used for detection as described above.

RESULTS AND DISCUSSION

The predicted PorB protein sequence of prototype strains N34/94 (for serotype 10), and 6940 (for serotype 19) were aligned with 6 other predicted PorB proteins from prototype reference strains for serotyping (Table 2). Different VR sequences for each VR were identifyed by letters as previously described [12], therefore 1, 4, 3, and 5 different amino acid VR sequences (or types) were defined for VRs 1, 2, 3, and 4 respectively (Table 2).

The predicted PorB protein sequences for N.34/94 strain was characterized as VR1 type A (VR1-A), also present in the other 7 strains and VR2 type D (VR2-D). The VR3 sequence was identical to VR3 type B (VR3-B), also found in strains 126E, M978, 190I, and 6940. In VR4, one unique sequence was found and called as VR type E (VR4-E). For

TABLE 2
Variable region characteristics of the *N. meningitidis* serotype reference strains and their reactivity with oligonucleotide probes.

			VRs[c]				Probes	
							VR1-A	VR3-B
Strain[a]	ACC[b]	Serotype	1	2	3	4	21U	615U
M1080	X65530	19,1	A	A	A	C	+	-
126E	U07191	19,10	A	A	B	A	+	+
M978	U07189	19,10	A	B	B	A	+	+
S3032	X65534	19,7	A	C	A	D	+	-
S3446	U07188	19,14	A	D	C	B	+	-
190I	U07192	19,10	A	B	B	A	+	+
6940	U11030	19,10	A	A	B	A	+	+
N.34/94	U34194	19,10	A	D	B	E	+	+

[a] Meningococcal serotype reference strains.
[b] [ACC] GenBank accession number of *porB* genes.
c Variable regions of *porB* gene.

serotype 19 strain (6940) the VR1 was also type A (VR1-A), the VR2 was type A (VR2-A) as the strains M 1080 and 126E. The VR3 was type B (VR3-B) as the strains 126E, M978, 190I and N.34/94. The VR4 was type A (VR4-A) as the strains M126E, M978, and 190I. All these VR sequences are presented in Table 2 and 3. The VR sequences of PorB protein are located in areas corresponding to predicted outer exposed loops when the protein is folded by using the model for neisserial porin proteins described by VAN DER LEY et al., 1991. The protein sequence of each VR is represented in Table 3.

The probes 615U (VR3-B), and 21U (VR1-A) were hybridized with dot blots containing 1μg of genomic DNA from each of the 72 strains described in Table 1 and the 8 strains on Table 2. The probes hybridized with every serotype 10 and/or 19, confirming that epitope 10 is related with VR3-B while epitope 19 is related with VR1-A. These VRs were refered as VR1-19 and VR3-10 by SACCHI et al. 1998[12].

Serotype is the most practical method to screening large numbers of samples during epidemic, or in field situations, however, the current panel of mAbs underestimate by at least 50% the PorB variability because reagents for several VRs are lacking, or because a number of VR variants are not recognized by serotype-defining MAbs. The sequencing of *porB* gene (VR typing), is important to characterize the precise structure of VR epitopes on PorB molecules and can type 100% of the PorB VR diversity, however, VR typing is not practical for analysis of large numbers of samples. Our hybridization results were concordant with PorB VR analysis and MAb reactivity previously described [12]. Using this method it is possible to characterize 100% of VR amino acid sequences (serotype epitopes), even when MAbs are not available for those epitopes.

RESUMO

O uso de sondas de oligonucleotídeos para caracterização de sorotipos de meningococo

No presente trabalho nós examinamos o uso potencial de sondas de oligonucleotídeos para caracterizar sorotipos de *Neisseria meningitidis* sem o uso de anticorpos monoclonais (MAbs). A diversidade antigênica da proteína PorB forma a base do método de sorotipagem, todavia, o atual painel de MAbs utilizados, sub-estima em no mínimo 50% a diversidade desta proteína devido a falta de reagentes para as várias regiões variáveis (VRs) da proteína PorB ou porque várias variantes das VRs não são reagentes com os MAbs

TABLE 3

Nucleic acid and amino acid sequences for each *porB* gene and PorB protein variable region (VR) type.

VRs	VR Type	DNA sequence	Amino acid sequence	Accession number
		121 156		
VR1	A	GTAGCTCACAATGGAGCTCAGGCGGCTAGCGTTGAA	VAHNGAQAASVE	X65534
		610 633		
VR2	A	CATCAAGTGCAAGAGAACGTGAAT	HQVQENVN	X65530
	B	CATCAAGTACAAGAGGACTTGAAT	HQVQEDLN	U07189
	C	CATCGAGTGCAAGAGGACATAAAT	HRVQEDIN	X65534
	D	CAGAATGTG --- GATAACGTGAAG	QNV.DNVK	U34194
		724 741		
VR3	A	TTGGTT --- GAAGAAAATTAT	LV.EENY	X65534
	B	TTGGCTTTGCCAAACCGACAAT	LALPNDN	U07189
	C	CTGGTT ------- AAAGACAAT	LV..KDN	U07188
		829 864		
VR4	A	AAAGGCTCGTTTGATGATGCAGACTTAAGCAACGAT	KGSFDDADLSND	U07189
	B	AAAGGCTCGTTTGATGATGCAGACTACACCAACGAT	KGSFDDADYTND	U07188
	C	AAAGGCTCGTTTGATGCTACAAACTACAACAACGAT	KGSFDATNYNND	X65530
	D	AAAGGCTCAGTTGATGATGCAAAACGCGACAATACT	KGSVDDAKRDNT	X67934
	E	AAAGGTTTGGTTGATAGTGCAGACTTAAGCAACGAT	KGLVDSADLSND	U34194

We analyzed VR sequences from 7 *porB* genes obtained from GenBank and the new N.34/94 *porB* sequence. The numbers above the sequences refer to the nucleotid position equivalent to the *porB* sequence of S3032 strain (GenBank X65534). Different sequences at the same VR were designated by letters on alphabetic order (VR types). The sequence aligment in this table does not correspond exactly to the VR sequences previously described (BASH et al., 1995). Symbols: -, nucleotide deletion; ., amino acid deletion.

disponíveis. Nós analisamos o uso de sondas de oligonucleotídeos para caracterizar os sorotipos 10 e 19 de *N. meningitidis*. O gene *porB* da cepa protótipo do sorotipo 10 foi sequenciado e alinhado com outras 7 sequências de diferentes sorotipos, e as individuais VRs foram então analisadas. Os resultados com as sondas 21U (VR1-A) e 615U (VR3-B) contra 72 cepas de *N. meningitidis* confirmaram que VR1-A e VR3-B codificam epítopos para os MAbs 19 e 10 respectivamente. É possível o uso de sondas para a caracterização dos sorotipos e podemos tipar 100% da diversidade da VR do gene *porB*. Trata-se de um método simples, rápido, e especialmente útil para a análise de um grande número de amostras.

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