REPORT

Characterization of N. meningitidis *proteoliposome proteins. Consistency and reproducibility among batches of VA-MENGOC-BC®, assessed by proteomic techniques*

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*I*ntroduction

The cuban vaccine VA-MENGOC-BC® against *Neisseria meningitidis* produced at the Finlay Institute from the B:4:P1.19,15 strain, is composed of proteoliposomes containing outer membrane proteins in which, five main antigens are identified: Por A, Por B, Rmp M, Opa and Opc A. Additionally, the serogroup C polysaccharide is also present in the vaccine [1-3].

Here we describe the analyses of the VA-MENGOC-BC® vaccine proteoliposome by lising proteomic techniques. High reproducibility among manufacturing batches was demonstrated, and minor protein components being also identified.

The main five proteins with intact structure account for 58-65% of the protein content. The are higher than predicted, due to the numerous partial degradation products identified in the samples. Sixty-five species were identified by mass spectrometry (MS), detecting 31 proteins, 26 of which were minor components in the preparation. Two proteins were selected and their respective genes cloned and expressed under the control of the tryptophan promoter. The expressed proteins were evaluated as immunogens in mice, being able to induce a highly functional response.

*M*aterials and methods

Samples under study

The proteoliposome batches evaluated were MPA 018B, C, D and F, previously approved and released by the Quality Control Department at the Finlay Institute. The MPA 018B lot was used to standardize the bidimensional electrophoresis and as a control in all assays.

Sample preparation

A procedure for removing the lipidic components from the sample without affecting its protein content was established. This procedure was applied to all batches under study.

Bidimensional electrophoresis

The study comprised the separation of proteins in the mass range 12-120 kDa, combined with pH ranges 3-10, 4-7 and 6-11. Gels were analyzed with a professional software for bidimensional gels images and with the statistical tools provided by the program.

Protein identification

Proteins were identified through proteolytic digestion with trypsin, followed by mass spectrometry analyses. Several fragmentation experiments were executed (MS/MS) for each protein, providing regions of internal se-quences. This information was employed to identify them by searching in international protein sequence databases.

Cloning, expression and purification in *E.coli* of the genes coding for the *N. meningitidis* proteins NMB0088 and NMB2134

The genes *nmb0088* and *nmb2134*, coding the NMB0088 and NMB2134 proteins, were cloned and expressed the pM-100 vector and oligodeoxynucleotides pairs 7998-7999 and 7742-7743, respectively. Both genes were expressed in the *E.coli* GC 366 strain. Expression experiments were carried out in saline minimal medium M9, supplemented with 1% glycerol, 1% tryptone, 1% casein hydrolysate, 0.1 mM CaCl2, 1 mM MgSO4 and 50 ug/µL ampicillin. Both proteins were obtained in the rupture precipitates, accounting for 50% of the proteins present in those fractions. The NMB2134 and NMB0088 proteins were obtained at 70% and 86% purity, respectively. The immunization experiments were carried out in groups of ten 6 week old Balb/c female mice. All mice received three doses of 20 µg each of the given protein adjuvanted in alum. The quality of the immune response was assessed by ELISA, serum bactericidal activity measurements, and sepsis-induced protection in the infant rat and newborn mice models, respectively.

*R*esults and relevance

The conditions promoting a successful resolution of components of the Cuban vaccine VA-MENGOC-BC® were established by applying bidimensional electrophoresis at three different isoelectric ranges. A reference material and three consecutive production batches were studied. High reproducibility was demonstrated by comparing the maps with the aid of statistical tools. The correlation coefficients between gels ranged from 0.954 to 0.997. From 256 to 297 species were resolved in the range of pI 3-10; 623 to 642 species were detected in the range of pI 4-7 (gels with higher resolution). The five main components constitute 58-65% of the materials resolved by bidimensional elec-

1. Sierra GV, *et al*. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. NIPH Ann Dis (1991); 14(2):195-210.

2. Rodríguez AP, *et al*. The epidemiological impact of antimeningococcal B vaccination in Cuba (1999). Mem Inst Oswaldo Cruz; 94(4):433-40.

3. Campa C, Sierra VG, Gutiérrez MM, Biset G, García LG, Puentes G, *et al*. Method of producing *Neisseria meningitidis* B vaccine, and vaccine produced by method. United States Patent number 5 597 572.

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trophoresis. These components were also detected in numerous resolved species, of lower molecular mass corresponding to degradation products. Seventy-eight species were digested; among them, 15 did not provided useful information, and 63 were identified by mass spectrometry. Up to 29 proteins were identified based on 63 successful determinations (Figure 1 and Table 1). The major components in the preparation were also identified in different spots with molecular weights lower than the theoretical molecular weight predicted for these proteins. For example, the class III outer membrane protein was identified in 13 spots. This phenomenon was also observed for other proteins such as the elongation factor G, identified in four bands, in spite of being a minor component represented by a small dot in the bidimensional electrophoresis gel. Five proteins contained peptides with masses that were 80 units above the expected theoretical values, this suggests the presence of certain modification in the amino acid sequences of these peptides (Table 1). It is relevant to notice that the modified proteins are membrane proteins belonging to the group of the most abundant proteins in the vaccine preparation, also corresponding to the group of *N. meningitidis* proteins widely employed for immunological studies. Two of the proteins identified were considered of potential vaccine interest. Their respective genes were cloned and expressed. Both proteins induced bactericidal titers against the homologous strains and in another three heterologous *N. meningitidis* strain, the animals were protected against two of them. The NMB2134 protein was shown to be highly immunogenic in the newborn mice model, which indicates the induction of an effective immune response during the early developmental stages. The functional response obtained in these immunization experiments is characterized by bactericidal activity and protection in animal models.

Relevance of the study

When conducting this study (from 2001 to 2004), there were no published reports characterizing commercial vaccines by proteomic techniques, particularly by high resolution bidimensional electrophore-

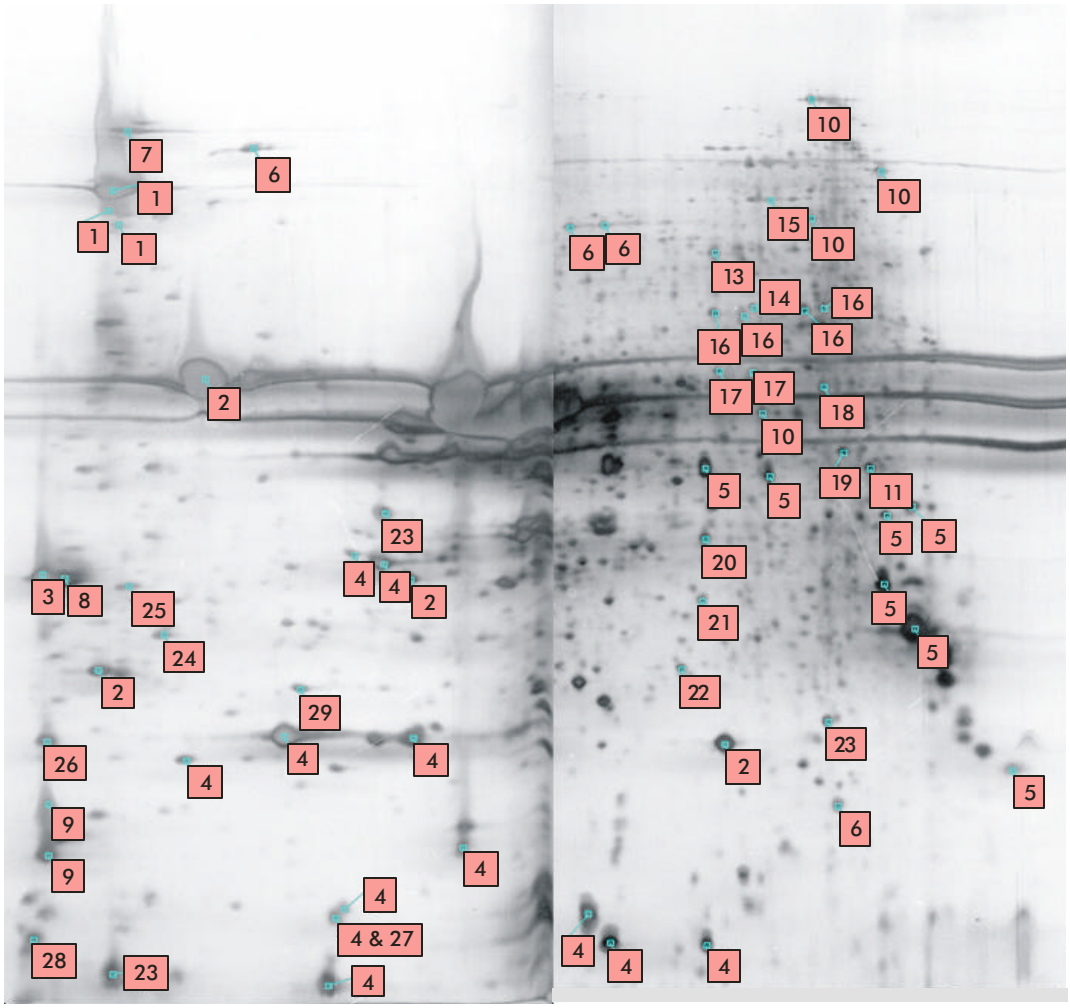

Figure 1. Synthetic image of preparative gels employed for mass spectrometry identification. All the species identified are labeled.

Table 1. Membrane proteins identified in bidimensional gels

No.	Protein/gene	Theoretical molecular weight (kDa)/pI	Gel band Code	Swissprot Accession number	No. peptides found/ No. peptides sequenced	% of coverage
1	Iron-regulated OMP (FrpB)	79/9.45	NMI-38	Q9JXL3	24/4	37
			NMIII-8	Q51132, Q50944, Q51162, Q9JXL3 or Q9JWB8	2/2	3
			NMIII-10	Q51132, Q9JXL3 or Q51133	2/2	5
2	OMP class 1	41/8.73	NMI-115	Q9S3T9	13/4	34
			NMI-270	Q9XBN3	4/2	14
			NMIII-76	Q9R3P0	9/4	29
			NMIII-117	Q9JPJ1	8/3	30
			NMII-769	Q9S3T9	9/3	36
3	OMP class 5c	28/9.68	NMI-233	Q9AE79	10/2	51
			NMIII-72	Q9AE79	12/5	67
4	OMP class 3	34/6.09	NMI-292	P30688	11/4	46
			NMII-847	Q51139	7/2	26
			NMII-863	O68155	6/1	34
			NMII-865	Q9R3T1	7/2	34
			NMIII-135	P30688	9/1	42
			NMIII-149	O53988	2/2	12
			NMIII-163	P30688	6/3	23
			NMIII-171	P30688	3/1	10
			NMIII-173	P30688	4/1	15
			NMIII-182	P30688	6/2	27
			NMIII-61	Q51139	7/3	37
			NMIII-67	P30688	12/4	55
			NMIII-137	P30688	9/1	42
5	OMP class 4 (RMPM or NMB0382)	26/6.00	NMI-414	P38367	9/2	61
			NMII-431	P38367	6/2	28
			NMII-470	P38367	5/1	41
			NMII-483	P38367	4/2	23
			NMII-587	P38367	6/1	39
			NMII-638	P38367	5/3	33
			NMII-779	P38367	4/1	31
6	OMP 85 (OMP85)	88/8.75	NMII-799	O30912 or Q9K1H0	9/2	12
			NMIII-3	O30912 or Q9K1H0	17/2	23
7	Hemoglobin receptor (NMB1668)	89/9.35	NMIII-2	Q9JYA8	31/3	43
8	Opacity protein (OPA)	27/9.45	NMIII-74	O30756	13/2	55
9	Surface protein A NsgA (NSPA or NMB0663)	18/9.64	NMIII-154	Q9RP17	3/3	24
			NMIII-164	Q9RP17	4/4	46
10	Elongation factor G (FUSA or NMB0138)	77/5.08	NMII-14	Q9K1I8	31/2	63
			NMII-56	Q9K1I8	26/2	50
			NMII-95	Q9K1I8	6/2	7
			NMII-340	Q9K1I8	10/2	20
11	Elongation factor TU (TUFB)	43/5.07	NMII-421	Q9K1I7	3/3	11
12	Acetolactate synthase III, large subunit (NMB1577)	63/5.88	NMII-99	Q9JYI0	11/2	25
			NMII-101	Q9JYI0	4/2	8
13	ATP synthase F1 Alpha subunit (NMB1936)	56/5.43	NMII-122	Q9JXQ0	11/2	23
14	Homoserine dehydrogenase (HOM or NMB1228)	47 / 5.31	NMII-188	Q9JR84	3/1	11
15	Putative aminopeptidase (NMB1428)	65/5.31	NMII-86	Q9JYU4	3/3	6
16	3-oxoacyl-(acyl-carrier-protein) synthase II (NMB0219)	43/5.36	NMII-191	Q9K1D8	2/2	5
			NMII-192	Q9K1D8	9/1	45
			NMII-197	Q9K1D8	4/3	16
			NMII-198	Q9K1D8	1/1	5
17	Glyceraldehyde 3- phosphate dehydrogenase (NMB2159)	36/5.40	NMII-277	Q9JX95	2/2	6
			NMII-278	Q9JX95	8/2	32
18	Transaldolase (TAL or NMB0351)	38 / 5.09	NMII-301	Q9K139	12/3	47
19	Electron transfer flavoprotein, alpha subunit (NMB2154)	33/4.99	NMII-401	Q9JXA0	6/1	39
20	Tetrahydropyridine-2-carboxylate N-Succinyltransferase (NMB0335)	30/5.42	NMII-518	Q9K152	8/3	30

Table 1. (Cont.)

No.	Protein/gene	Theoretical molecular weight (kDa)/pI	Gel band Code	Swissprot Accession number	No. peptides found/ No. peptides sequenced	% of coverage
21	DNA-binding response regulator (NMB0595)	25/5.44	NMII-613	Q9JRJ9	4/4	32
22	Putative oxidoreductase (NMB1796)	21/5.73	NMII-705	Q9JY11 or Q9JVV3	3/2	28
23	Putative cysteine synthase (CYSK or NMB0763)	33/6.06	NMII-754	Q9JQL6	7/2	29
			NMIII-48	Q9JQL6	8/2	29
			NMIII-179	Q9JQL6	6/1	30
24	Putative thiol:disulphide interchange protein (NMB0550)	29/8.49	NMIII-104	Q9JR63	8/2	28
25	30S ribosomal protein S2 (RPSB or NMB2101)	27/9.04	NMIII-80	Q9JRG7	9/3	37
26	50S ribosomal L6 (NMB157)	19/9.63	NMIII-139	Q9K1I3	5/1	28
27	50S ribosomal L9 (RPLI or NMB1320)	16/6.61	NMIII-173	Q9JZ31	8/2	59
28	50S ribosomal L11 (NMB0127)	15/9.72	NMIII-175	Q9K1J3	4/1	30
29	50S ribosomal L25 (NMB0876)	21/6.60	NMIII-124	Q9JZW3	11/1	51

sis. There were also no reports on the use of this powerful analytical tool for studying the consistency between production batches.

For the first time the consistency of the production process of the VA-MENGOC-BC® vaccine was evidenced, also demonstrating through high resolution bidimensional electrophoresis its highly complex protein composition. The identification of previously unreported components, like the FrpB (FetA) and NspA proteins, and minor components contributing to the induction of protective immunity, constitutes a significant contribution for developing vaccines based on outer membrane vesicles. This is the first report on the characterization of minor proteins components for such a vaccine. Its relevance is exemplified by the identification of two vaccine candidates against the serogroup B of *N. meningitidis*. Their corresponding genes were cloned and the resulting proteins were purified and used for immunological evaluations in mice; the results further support two patent applications.

This work confers to the Cuban vaccine a high degree of characterization at the molecular level, enabling the identification of the minor components that potentially contribute to the protective effect of the vaccine.