Full title: Production and control of a Brazilian tailor-made meningococcal B vaccine

Short title: Brazilian meningococcal B vaccine production

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Abstract

Meningococcal disease has been a public health problem in Brazil since the serogroups A and C epidemics occurred in the 1970s. The Oswaldo Cruz Foundation in Brazil has been working to develop a serogroup B meningococcal vaccine composed of detergent-treated outer membrane vesicles (OMV) and detoxified endotoxin (dLOS) from *Neisseria meningitidis* serogroup B prevalent strains. Experimental vaccine were produced in a pilot-scale under Good Manufacturing Practice condition (GMP). Physicochemical and biological controls were established based on what has been reported previously for OMV vaccines. The developed vaccine contained the main class 1, 2, 3, and 5 proteins, some minor iron-regulated proteins, and 5–10% residual lipopolysaccharide related to total protein content. The dLOS was added, as a vaccine component, in half of the total protein amount. The pyrogenicity of the final products, based on the residual LOS in OMVs, varied from 1.01 to 2.24 ng LOS/kg rabbit. Three experimental vaccines, were highly immunogenic in mice with better performance using higher antigen concentrations.

Keywords: Meningococcal disease

Outer Membrane Vesicles (OMVs)

LOS (lipooligosaccharide)

dLOS (detoxified lipooligosaccharide)

37 INTRODUCTION

38 Meningococcal disease remains a worldwide health problem and has been a public health
39 problem in Brazil since the groups A and C epidemics of the 1970s. Serogroup B caused 80%
40 of the meningococcal disease cases throughout the 1980s with a significant number of cases in
41 children < 2-years-of-age [1,2].

42 The, B:4,7:P1.19,15, L3,7,8 phenotype was responsible for the 1988 São Paulo epidemic,
43 continued to represent the predominant B phenotype from 2000 to 2004, and seems to be the
44 main strain today [3,4].

45 The other prevailing phenotypes identified were B:4,7:P1.7, L3,7 and B:15:P1.7,16, which have
46 high frequencies in the southern states of Brazil. These three prevalent serosubtypes belong to
47 the same ET-5 electrophoretic type [5].

48 The emergence of W135, which is associated with the ST-11 complex, was reported in São
49 Paulo, Rio de Janeiro, and Rio Grande do Sul. Serogroup C was responsible for 75% of the
50 cases identified in 2010, 17% by serogroup B, 6% by serogroup W135, and 2% for serogroup
51 Y [1].

52 The Brazilian Ministry of Health decided the use a Cuban vaccine (VAMENGOC-BC®) in the
53 southern region of Brazil in the 1990s when they were trying to control the meningococcal B
54 epidemic. Case control studies to estimate vaccine efficacy in Brazil were performed from 1989
55 to 1990. The results indicate that the vaccine generally did not provide protection against
56 heterologous strains and only induced modest protection in young children [6,7,8].

57 Based on the results of the use of the Cuban vaccine in Brazil, three Brazilian research
58 institutions (Bio-Manguinhos/Fiocruz, Institutos Butantan, and Adolfo Lutz) have collaborated
59 to develop a serogroup B meningococcal vaccine composed of detergent-treated OMVs and

detoxified lipooligosaccharide (dLOS) from Brazilian prevalent strains. The Brazilian approach has focused on developing a tailor-made vaccine that have been developed and successfully proven efficacious against serogroup B meningococcal disease in Cuba, Norway, and New Zealand. The inclusion of dLOS as a vaccine component, was based on the fact that other subcapsular antigens, in addition to OMV, may benefit cross-immunity within different serotypes of meningo B strains [9,10].

Preliminary pre-clinical data showed animal protection induction of experimental vaccines from an intraperitoneal challenge model in mice, and protective bactericidal antibody against vaccine strains [11].

The study presented here describes a vaccine standardized production and control under Good Manufacturing Practice (GMP) conditions in a pilot-scale process. The results indicate production and controls compatible with OMV vaccines and potential use of the developed product for clinical studies for safety and immunogenicity assessment [12,13].

MATERIAL AND METHODS

VACCINE PREPARATION

VACCINE STRAINS

The N44/89 (B:4,7:P1.19,15, L3,7,9) and N603/95 (B:4,7:P1.7.1, L3,7) strains were provided by the Adolfo Lutz Institute, São Paulo, Brazil.

BIOMASS HARVEST

The production process included cultivation of the vaccine strains in a 150 L bioreactor (B Braun Model Biostat UD 100) using Catlin MC.6 with 20 μM Fe^{+3} and 42 μM ethylene

diamine-di-(*O*-hydroxy-phenylacetic acid) (EDDHA) to produce the OMVs. Lipooligosaccharide (LOS) was produced in the same way described for OMVs without EDDHA. Bacterial growth was inactivated with heat (56°C/30 min), and the biomass was separated by continuous flow centrifugation (LAPX 4045GP-31G; Alfa Laval) [14].

OMV ANTIGENS

The LOS-depleted OMVs were obtained from the culture supernatant, and concentrated by tangential ultrafiltration in a Centrasette stainless steel cassette holder using a polyethersulfone Centrasette cassette (0.9-m²) and a molecular weight cutoff of 100 kDa (Pall Corp., Port Washington, NY, USA). The concentrate was treated with 2% (w/v) sodium desoxycholate (DOC) and LOS-depleted OMVs were harvested by ultracentrifugation (100,000g for 2 h), in water for injection containing 3% sterile-filtered sucrose using a 0.22-µm Millipak®-200 polyvinylidene fluoride membrane (PVDF; Millipore, Billerica, MA, USA) [11].

DETOXIFIED LOS ANTIGEN

LOS from N44/89 strain, was obtained from the culture biomass after hexadecyl trimethyl ammonium bromide (Cetavlon-Fluka Analytical code 52370) treatment using the hot phenol/water method (Gotschlich,1969; Westphal,1965). The extracted LOS was further purified by gel filtration chromatography using 20 mM Tris-HCl, pH 8.5, containing 0.5% DOC and 5 mM EDTA as the mobile phase on a single Sephacryl HR S-300 column (ID: 5.0 µm L: 100.0 cm). The LOS sample solution applied to the column was limited to 4% of bed volume and a LOS mass not greater than 600 mg [15].The detoxified LOS (dLOS) was obtained by treatment of raw material with 0.20 M NaOH in a water bath at 60°C for 150 min [16,17].

102 VACCINE FORMULATION

103 Vaccine 1: [25 µg OMV protein (12.5 µg from N44/89 + 12.5 µg from N603/95 + 12.5 µg
104 dLOS from N44/89) + Al(OH)₃/2 mg/mL]. Vaccine 2: [50 µg OMV protein (25 µg from N44/89
105 + 25 µg from N603/95 + 25 µg of dLOS from N44/89 + Al(OH)₃/2 mg/mL]. Vaccine 3: [100
106 µg OMV protein (50 µg from N44/89 + 50 µg from N603/95 + 50 µg of dLOS from N44/89 +
107 Al(OH)₃/2 mg/mL]. The final quality control tests were performed in the Quality Control
108 Department of Bio-Manguinhos (Rio de Janeiro, Brazil). Vamengoc BC® and DTP vaccine as
109 the reference in pyrogen test .

110 VACCINE RESPONSE STUDIES

111 STUDY GROUPS

112 Swiss Webster male and female mice (weight, 12–17 g) were obtained from Oswaldo Cruz
113 Foundation breeding unit (Rio de Janeiro, Brazil) and caged with free access to food and water
114 in a room maintained at 22–24°C with a 12 h:12 h light/dark cycle in the Bio-Manguinhos
115 Experimental Animal Facility. The animal procedures were conducted according to the
116 Oswaldo Cruz Foundation Ethical Committee for animal using (CEUA/FIOCRUZ number
117 LW65/14). Groups of 15 mice were immunized intramuscularly with 0.2 mL of the final
118 experimental product (diluted 1:10 in phosphate buffered solution (PBS). A three-dose
119 immunization schedule of 14 days intervals was used. Groups of five mice were bled (retro-
120 orbital via) before each dose and 15 days after the third immunization.

121 OMV-ENZYMELINKED IMMUNOSORBENT ASSAY (ELISA)

122 To detect anti-OMV total IgG, 96-well plates (ref. no. 3590; Corning-Costar, Corning, NY,
123 USA) were coated with 100 µL/well of OMV (4 µg/mL) from N44/89 or N603/95 strains. Sera

samples were diluted in two-fold serial dilutions in TBS with 0.05% Tween 20 (Merck, Schuchardt, Germany) and 5% FCS (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 4°C. The plates were incubated with anti-mouse IgG conjugated with alkaline phosphatase (whole molecule) (Sigma-Aldrich A-3688) diluted 1:2000. All sera were titrated in duplicate, and the titers were determined at an absorbance of 405 nm using a VERSA max tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). As the antibody standard, a positive post vaccination serum was used in all experiments. The observed optical density was transformed to arbitrary units per milliliter by a sigmoidal standard curve (logit-log transformation) calculated from the values (1000 EU/mL) of the reference serum [18].

LOS ELISA ASSAY

Microtiter plates (Imm lux ref 1000; Dynex Technologies, Chantilly, VA, USA) were coated overnight at room temperature with 100 µL/well of 10 µg/mL LOS-DOC micelles dissolved in PBS with 0.1% DOC. TBS with 5% bovine serum albumin (BSA) (Sigma-Aldrich ref A9418) was used as the blocking buffer for 2 h at 37°C. Sera samples were titrated in duplicate in TBS with 5% BSA and incubated for 3 h at 37°C. Conjugated antibodies (Sigma-Aldrich A-3688) were diluted 1:1000 and incubated for 2 h at 37°C. The reaction was developed for 20 min with a phosphatase substrate (Sigma-Aldrich S0942). All sera were titrated in duplicate and titers were determined with absorbance at 405 nm in VERSAmax tunable microplate reader – Molecular Devices. The total IgG antibody responses (EU/mL) against OMVs and LOS, were presented as geometric mean concentrations (GMC) of five serum samples (pool of five mice) by using a 4-parameter logistic curve-fitting analysis with SoftMaxPro software and documentation - Molecular Devices, against the standard curve generated by in-house serum with 1000 EU/mL of arbitrary units. All p-values were calculated with Kruskal Wallis test. Differences were considered as statistically significant at $p < 0.05$ [18,19].

SERUM BACTERICIDAL ASSAY (SBA)

The bacterial strains, was streaked on Columbia blood agar (Merck 1.10455.0500), plated with 5% horse blood (CBA), and incubated overnight at 37°C in 5% CO₂. The next morning, 10–20 colonies, were subculture on another CBA plate and incubated for 4 h at 37°C in 5% CO₂. After 4 h, the bacteria were suspended in a bactericidal buffer (Hanks balanced salt solution) containing 0.5% BSA (Sigma, Poole, UK) and 0.5 U/mL heparin (CP Pharmaceuticals, Wrexham, UK). The bacteria concentration was adjusted to 2×10^5 colony forming units (cfu)/mL. Equal volumes (10 µL) of the bacterial suspension and human complement were added to 20 µL heat-inactivated test serum serially diluted two-fold in bactericidal buffer and added to 96-well U-bottom microtiter plates (Greiner, Frickenhausen, Germany). Human plasma from a suitable donor (Research Ethics Committee, Hemorio Hospital, Rio de Janeiro, Brazil No. 118/07) was used as the complement source. The plasma was used in the assay after a 60 min incubation at 25% of the final concentration. The number of bacteria was determined at time zero, by allowing 10 µL of the reaction mixture to flow 8–10 cm in lanes down a CBA plate (the tilt method). After a 60-min incubation of the reaction mixture at 37°C, 10 µL was removed from each well and plated on CBA. The colonies were counted after a 37°C overnight incubation in 5% CO₂. SBA titers were expressed as the reciprocal of the final serum dilution step that produces a 50% mortality after 60 min compared to the number of bacteria at time zero. Three in-house control sera (low, medium, and high titers) were used as controls for the assays in each target strain. The test was performed using vaccine strains as the target [20,21].

TEST FOR TOXICITY

The experimental vaccines were tested by intraperitoneal injection of one human dose, but not more than 1 mL of experimental vaccine into 5 mice (weight, 17–22 g) diluted in diluent. Two guinea pigs (weight, 250–350 g) were also injected (i.p.) with the equivalent of 10 human doses

in a volume < 5 mL. The vaccines tested were considered innocuous if the animals survived at least 7 days without showing significant toxic signs. Cuban (VAMENGOC-BC®) and DTP (Butantan Institute, São Paulo, Brazil) were used as the reference of licensed vaccines for human use, in Brazil [22].

RABBIT PYROGEN TEST

Briefly, the tested vaccines in various human-dose dilutions were given intravenously to three rabbits (weight, 2.5–3.0 kg) at a rate of 1 mL/kg of rabbit body weight. Cuban (VAMENGOC-BC®) and DTP (Butantan Institute, São Paulo, Brazil) were used as a reference of licensed vaccines for human use, in Brazil. Rectal temperature was measured with an indwelling rectal thermometer and recorded 5 h after injecting the sample. The preparations were considered approved if the summed temperature variations of the three rabbits did not exceed 1.15°C [22].

STERILITY TEST

According to the European Pharmacopoeia, eight vials of each vaccine containing five doses each in Al(OH)₃ vaccine diluent, pooled, and filtered through a 0.22-µm PVDF membrane to fluidize the thioglycolate (TGC) and soy-bean casein media (TSB). The TGC and TSB were incubated at 30–35°C for 24–48 h and 20–25°C for 10–15 days, respectively [23].

PHYSICO-CHEMICAL CONTROLS

PROTEIN CONCENTRATION

Briefly, the OMVs were mixed in the copper reagent (4% CuSO₄·5H₂O) with sodium dodecyl sulfate (SDS) (1% w/v) for 10 min. Sample absorption was measured after adding Folin Ciocalteu's phenol reagent and incubating for 45-min at room temperature. Protein content was

determined in Beckman DU530 spectrophotometer at 660 nm against a BSA standard curve (Pierce, Rockford, IL, USA) [24].

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) PATTERN

The protein profile of the OMV preparations, before vaccine formulation, was analyzed by 12% PAGE in the presence of 2% (w/v) SDS. The proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue, and analyzed by densitometry. The stained class protein bands were determined by scanning the gel, the relative amount of each protein was calculated using Quantity One software (densitometer GS-800; Bio-Rad Laboratories, Hercules, CA, USA), and was presented as a percentage of total protein [25].

LOS AND dLOS ELECTROPHORESIS PATTERN

The LOS samples (LOS and dLOS) were treated for 5 min at 100°C in 0.05 M Tris-HCl buffer (pH 6.8), 2% (w/v) SDS, 10% (w/v) sucrose, and 0.01% bromophenol blue and fractionated on an SDS-polyacrylamide gel (8 × 7.3 cm by 0.75 mm) containing 4% and 16.5% acrylamide in the stacking and separating gels, respectively. The electrophoresis was performed at 15 mA in the stacking gel and 20 mA in the separating gel until the tracking dye had traveled about 10 cm. The SDS-PAGE fractionated LOS preparation was stained using the conventional silver staining method of Tsai and Frasch, 1982. A protein standard (16,949–2,512 kDa) was used to estimate the molecular weights of LOS and dLOS. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue [26]. HP Scanjet G4050 Scanner was used, to scan the gel, with the following parameters: Output Type: Gray Scale; File Type: Unzipped Tif (*.tif) image; Output Resolution: 1200 dpi (dot per inch). The gel image file was analyzed using the Image Master 1D Prime V3.01 software (Pharmacia Biotech, San Francisco, CA, USA)

216 **STRUCTURAL CHARACTERIZATION OF dLOS**

217 Detoxified LOS, which is *O*-deacylated (dLOS), was hydrolyzed in acetate buffer at pH 4.5. It
 218 was purified by gel-filtration chromatography using a Bio-Gel P-4 column. The dLOS
 219 electrospray ionization/mass spectroscopy analysis was performed with a Thermo Finnigan
 220 LCQ Deca XP ion trap mass spectrometer (ThermoScientific, Waltham, MA, USA). Capillary
 221 temperature was 200°C, capillary voltage was 45 KV, and mass range *m/z* was 250–2000 [27].

222 **RESIDUAL LOS IN THE OMV PREPARATIONS**

223 LOS was quantified by determining 2-keto-3-deoxyoctonic acid (KDO) content using a mass
 224 conversion factor value of 20. OMVs from each strain, were diluted in 1 M Tris-HCl, 0.5%
 225 SDS, and 0.05 M CaCl₂ buffer, at pH 7.5 following incubation for 5 min at 100°C. The protein
 226 in this suspension was digested with proteinase K for 4 h at 56°C. KDO content was measured
 227 using the thiobarbituric acid procedure. Briefly, a standard aqueous (0.1 mg/mL) solution of
 228 KDO (Sigma ref K2755) was prepared and stored at –20°C. This standard solution was diluted
 229 to prepare five different concentrations. The digested samples were hydrolyzed in trifluoroacetic
 230 acid and treated in sequence with periodic acid, sodium arsenite, and thiobarbituric acid and
 231 heated in a boiling water bath for 10 min. KDO content was determined by measuring optical
 232 density in a Beckman DU530 spectrophotometer (Beckman, Brea, CA, USA) at 548 nm against
 233 a KDO standard curve [28].

234 **pH**

235 pH was determined in each experimental vaccine in its diluent according to the United States
 236 Pharmacopeia using standard buffer solutions from the Physicochemical Quality Control of
 237 Bio-Manguinhos [23].

238 **Sucrose content**

The quantity of sucrose in the experimental vaccines diluted in water was analyzed by high-performance liquid chromatography (HPLC; Waters, Milford, MA, USA) using a Shodex Sugar SC1011 column (300 × 8 mm) at 75°C and degasified Milli-Q water as the mobile phase at a flow rate of 0.8 mL/min. The sucrose amount was evaluated with a refractive index detector (Waters IR 410 Channel 2) [29].

Aluminum hydroxide adsorption

The experimental vaccines were centrifuged (4,000 rpm/20 min/4°C), and protein content was measured in the supernatant. The test was verified by > 80% adsorption of the total vaccine protein concentration [22].

Electron microscopy

Droplets of LOS-depleted OMVs from the N44/89 strain (1 mg protein/mL) in distilled water were applied to glow-discharged, carbon-filmed grids for 1 min and negatively stained with 0.5% (w/v) phosphotungstic acid, pH 7.0 for 1 min. The preparations were examined under an electron microscope operated at 100 keV (JEM 1010; Jeol, Tokyo, Japan) [30].

Sialic acid content

Residual sialic acid was quantified by HPLC using a pulsed amperometric detector. Before HPLC, the OMV DOC treated, was hydrolyzed with 0.2 M HCl at 100°C for 60 min. HPLC analyses were performed on a Dionex I.C.S.–3000i, with a LC50 gradient pump and an ED50 electrochemical detector with a gold working electrode. A borate trap was used for the mobile phase followed by an amino trap for sample clean up. The analysis was performed using a Carbowac PA10 guard and analytical column. Data were acquired, based on the standard *N*-acetyl neuraminic acid peak at 5.1 min. The mobile phase was 0.1 M NaOH: 0.1 M sodium acetate (45:55) at a flow rate of 0.8 mL/min. Total run time was 30 min [31].

262 **Aluminum content**

263 Aluminum from the experimental vaccines was evaluated accordingly to European
264 Pharmacopoeia [32].

265 **Statistical analysis**

266 The Kruskal–Wallis test was used to evaluate differences between the experimental vaccines 0
267 (before vaccination) and 15 days after the last dose. Differences were considered significant at
268 $p < 0.05$. The ELISA and SBA data of the two strains were compared with the Mann–Whitney
269 test. All data were analyzed using Statgraphics Plus Professional ver. 4.1 software
270 (Statgraphics, Warrenton, VA, USA).

271 **RESULTS**

272 **PHYSICOCHEMICAL CONTROLS**

273 The experimental formulations were sterile, more than 80% of vaccine antigens adsorbed in Al
274 (OH)₃, had appropriate pH, sialic acid, aluminum, and antigen concentrations in accordance
275 with the established quantitative limits. The OMVs in residual LOS varied from 5 to 10% of
276 the OMV protein (Table 1).

277

278 Table 1–Physicochemical and biological controls for the tested vaccines.

Test	Control method	Quantitative limits	Vaccine 1 ^a	Vaccine 2 ^b	Vaccine 3 ^c
Protein (µg/mL)	Modified Folin-Lowry	Protein content (±10%)	23.6	47.4	98.53
dLOS (µg/mL)	Osborn	dLOS content (±10%)	12.5	25	50
Residual LOS (µg/100 µg ptn)	Osborn	5–10	4.3	4.3	4.55
Group B polysaccharide (µg/100 µg ptn)	HPI-PAD	< 0.25	0.01	0.01	0.01
Aluminum (mg/mL)	Atomic adsorption	< 1.25	0.76	0.78	0.85
Identity	Dot-Blot assay	Reactivity with specific monoclonal antibody 60–90 kDa: 13–21% Class 1: 13–17% Class 3: 22–35% Class 4: 6–16% Class 5: 7–14%	Pass	Pass	Pass
Antigen standard	SDS-PAGE		Pass	Pass	Pass
Degree of binding to adjuvant (%)	Modified Folin-Lowry	> 80	94.5	94.8	98.53
Sucrose (mg/mL)	HPLC	26–38	30.52	29.94	29.5
pH	Determination by pH-meter	6.5–7.8	7.1	7.1	7.2
Sterility testing	Membrane-filtration method (Ph. Eur.)	Pass	Pass	Pass	Pass
Abnormal toxicity	Safety test (Ph. Eur.)	Pass	Pass	Pass	Pass
Pyrogenicity	Rabbit method	≤1:4000	1:1000	1:1000	1:2000
Dilution (ng protein/Kg rabbit)		Pass	23.6	47.4	49.3

279 ^aVaccine 1: 25 µg outer membrane vesicles (OMV) protein (N44/89 + N603/95) + 12.5 µg dLOS + 2 mg Al (OH)₃/mL

280 ^bVaccine 2: 50 µg OMV protein (N44/89 + N603/95) + 25 µg dLOS + 2 mg Al (OH)₃/mL

281 ^cVaccine 3: 100 µg OMV protein (N44/89 + N603/95) + 50 µg dLOS + 2 mg Al (OH)₃/mL

The base peak was identified as a multiple-charge ion $[M+2H-H_2O]^{+2}$ at m/z 1387.9, after charge deconvolution, showed molecular weight compatible with a dihexosamine backbone, carrying 1,4' diphosphate groups (PP) and *N,N*-diacylated by 3-hydroxy-tetradecanoyl fatty chains. No variation of mass was observed for OS core moiety, (Figure 1).

Figure 1: Electrospray ionization/mass spectroscopy (ESI/MS) of O-deacylated lipooligosaccharide (LOS) from N44/89 *Neisseria meningitidis* B, strain.

A second minority ion was also analyzed. A sodiated-protonated ion $[M+H+Na]^{+2}$ at m/z 1367.87. It had mass compatible with a diacylated lipid A and OS core described previously (m/z 1387.9), but differs in their phospho groups, showing only a monophosphorylated (P) form. Native LOS was also analyzed showing molecular weight compatible with and hexacylated structure carrying two units of phosphoethanolamine (data not shown).

MORPHOLOGY

The morphology of the final N44/89 preparation obtained by electron microscopy is presented in Figure 2. The OMV range in size and morphology after sterile filtration were around 80–100 nm.

Figure 2: Electron microscopy of outer membrane vesicles (OMV) from the N44/89 strain (1 mg/mL) in distilled water negatively stained with 0.5% (w/v) phosphotungstic acid, pH 7.0 The preparations were examined under an electron microscope (JEM 1010; Jeol, Tokyo, Japan).

300 **BIOLOGICAL CONTROLS**

301 **VACCINES IMMUNE RESPONSE IN MICE**

302 The three experimental vaccines induced significant increases in total IgG ($p < 0.001$) in
 303 response to the OMVs (Table 2) of both Brazilian prevalent strains with no difference between
 304 them. Seroconversion to LOS (300 and 1,000-fold) was detected 15 days after the third dose
 305 for vaccines 2 and 3, respectively.

Table 2: Total IgG geometric mean concentrations (GMCs) against vaccine components from the N44/89 and N603/95 strains before and after immunizing mice with three doses of a vaccine as determined by enzyme-linked immunosorbent assay.

Mice total antibody by ELISA against OMVs from strains N44/89 and N603/95 and LOS from N44/89 strain									
Study time	N44/89 (B:4,7:P1.19,15, L1,3,7,8) ^d			N603/95(B:4:P1,7,1, L,3,7) ^e			LOS (B:4,7:P1.19,15, L1,3,7,8) ^f		
	25 µg /mL ^a	50 µg /mL ^b	100 µg /mL ^c	25 µg /mL ^a	50 µg /mL ^b	100 µg /mL ^c	25 µg /mL ^a	50 µg /mL ^b	100 µg /mL ^c
Day 0	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Before 2 nd dose (Day 15)	263 ± 32	868 ± 40	185 ± 40	113 ± 8.28	446 ± 44	448 ± 12	<DL	<DL	<DL
Before 3 rd dose (Day 30)	3 508 ± 339	7027 ± 182	11 596 ± 649	4128 ± 478	7008 ± 215	11 061 ± 253	<DL	<DL	<DL
After 3 rd dose (Day 45)	14 946 ± 1 138	8415 ± 497	16 262 ± 191	14 368 ± 115	11 781 ± 203	10 279 ± 243	<DL	334.6 ± 16.5	1002 ± 142.3

^aVaccine 1: 25 µg OMV protein (N44/89 + N603/95) + 12.5 µg detoxified endotoxin (dLOS) + 2 mg Al(OH)₃/mL

^bVaccine 2: 50 µg OMV protein (N44/89 + N603/95) + 25 µg dLOS + 2 mg Al (OH)₃/mL

^cVaccine 3: 100 µg OMV protein (N44/89 + N603/95) + 50 µg dLOS + 2 mg Al (OH)₃/mL

^dOMV from N44/89 as coating antigen; ^eOMV from N603/95; ^fLOS as coating antigen.

^gN° of subjects: three pools of five mice each in the indicated group: GMC ± standard error; NE – non-evaluated; <DL below detection limit.

315 Vaccine 1 did not induce a LOS response in mice at the same evaluation time, suggesting that
 316 the immune antigen response was concentration dependent. Considering a fourfold increase in
 317 bactericidal titres, after 3 doses of vaccines as a immunogenicity parameter, the experimental
 318 vaccines were very immunogenic in mice. The response for N44/89 strain was stronger than for
 319 N603/95 even in the vaccine with the lowest concentration of vaccine components. Based on
 320 the bactericidal activity of pre immune sera, the response to N44/89 was 4 and 8 times higher
 321 than N603/95 in the intermediate and maximum antigen vaccines concentration, respectively.
 322 In addition, the bactericidal titers were significantly higher for N44/89 than what was observed
 323 to N603/95 after the third dose (Table 3).

324

Table 3: Serum bactericidal assay (SBA) antibody titers (reciprocal serum dilution $\geq 50\%$ killing) against group B vaccine strains before and after immunizing mice with a three-dose vaccination schedule using phase II trial meningococcal experimental vaccines.

Bactericidal titers indicated as reciprocal sera dilution $\geq 50\%$ killing ^a						
Study time point	N44/89 (B:4,7:P1.19,15:P5.5,7: L1,3,7,8) ^e			N603/95(B:4:P1,7,1: P5.5,7: L,3,7) ^f		
Experimental Vaccines	25 μg /mL ^b	50 μg /mL ^c	100 μg /mL ^d	25 μg /mL ^b	50 μg /mL ^c	100 μg /mL ^d
Pre-immune sera (Day 0)	2	2	2	2	2	2
After 3 rd dose (Day 45)	256 (128–256)	256 (128–256)	512 (256–512)	2 (2–2)	64 (32–64)	64 (32–64)

^aSera from three-five pools of five mouse (GMT)

^bVaccine 1: 25 μg OMV protein (N44/89 + N603/95) + 12.5 μg detoxified endotoxin (dLOS) + 2 mg Al (OH)₃/mL

^cVaccine 2: 50 μg OMV protein (N44/89 + N603/95) + 25 μg dLOS + 2 mg Al (OH)₃/mL

^dVaccine 3: 100 μg OMV protein (N44/89 + N603/95) + 50 μg dLOS + 2 mg Al (OH)₃/mL

^eN44/89 as SBA target strain.

^fN603/95 as SBA target strain

335 TOXICITY AND PYROGENICITY

336 Both mice and guinea pigs survived to 7 days of time observation without any sign of toxicity,
 337 such weight loss, due to experimental preparations and reference vaccines administration.
 338 According to the WHO requirements for polysaccharide vaccines against *N. meningitidis*, the
 339 final product should not induce fever in rabbits at a 1:4,000 dilution of the human dose.
 340 Vaccines with 25 and 50 µg protein/mL were approved in rabbits when the human dose was
 341 diluted to 1:1,000. A vaccine with 100 µg protein/mL was approved in rabbits diluted 1:2,000
 342 of the human dose. Cuban and DTP vaccines were approved in 1:4000 of human dose. In
 343 terms of minimum pyrogenic dose based on protein amount, the experimental preparations 1,
 344 2 and 3, were respectively 23,6, 47,4 and 49,3 ng of protein per kg of rabbit. The reference
 345 vaccines were 12,5 ng of protein per kg of rabbit for VAMENGOC-BC® and the DTP
 346 vaccines. The experimental vaccines were less pyrogenic than the references. Raw and dLOS,
 347 were tested in the rabbit pyrogen test at the highest quantity of the third (50 µg/mL)
 348 experimental vaccine. After a 30 min NaOH treatment, a sharp fall in pyrogenicity of the raw
 349 LOS was detected. The dLOS samples from the 120 and 150 min treatments were approved
 350 by the rabbit pyrogen test at dilutions of 1:200 and 1:100, respectively (Table 4).

351

Table 4: Vaccines toxicity and “in vivo” pyrogen test.

Tested formulation	Abnormal Toxicity ^a	“In vivo” pyrogen test ^b .
Vaccine 01 ^c		Pass
Dilution	Pass	1:1000
ng OMV protein/ Kg rabbit		23,6
Vaccine 02 ^d		Pass
Dilution	Pass	1:1000
ng OMV protein/ Kg rabbit		47,4
Vaccine 03 ^e		Pass
Dilution	Pass	1:2000
ng OMV protein/ Kg rabbit		49,3
VAMENGOC-BC [®]		Pass
Dilution	Pass	1:4000
ng OMV protein/ Kg rabbit		12,5
DTP vaccine		Pass
Dilution	Pass	1:4000
raw LOS		Not
50 000ugLOS/ Kg rabbit	Not applied	Tested
Detoxified LOS (dLOS)		Pass
Dilution after 120 min of NaOH treatment	Not applied	1:200
Detoxified LOS (dLOS)		500 ng/ Kg rabbit
Dilution after 150 min of NaOH treatment	Not applied	Pass
		1:100
		500 ng/ Kg rabbit
		Pass

PROTEIN AND LOS ELETROPHORETIC PATTERNS

The iron starvation strategy for vaccine strain growth induced IRP expression in OMV outer membrane from both vaccine strains in the molecular weight range between 60 and 90kDa. Por A and other class proteins were well expressed in OMV from N44/89 and N603/95 (Figure 3).

Figure 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 µg/outer membrane vesicle [OMV] protein) protein profile of vaccine strains OMV (A) N603/95 and (B) N44/89. Molecular weight (MW); (NP) OMV before sodium deoxy cholate (DOC) treatment; (P) OMV after DOC treatment; (BF) vaccine bulk before sterile filtration; (AF) vaccine bulk after sterile filtration.

Table 5 shows the percentages of expression of the main OMV proteins of the vaccine strains used as reproducibility parameter of the production process of this vaccine component.

Table 5: Class protein proportion (%) of vaccine bulk after sterile filtration by densitometry

Protein (%)	N44/89	N603/95
Por A	23.54	8.10
Por B	28.84	15.26
Class 4	7.63	1.94
Class 5	15.95	9.67

The structural changes in Lipid A from raw LOS after the NaOH treatment are shown in the SDS profiles. Both molecules had two bands but the molecular weights of dLOS were lower than those shown in LOS profile before alkali detoxification (Figure 4).

Figure 4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis lipooligosaccharide (LOS) profile from N44/89 biomass. MW, protein molecular weight standards of 16.949–2.512 kDa; LOS, native endotoxin; dLOS, detoxified endotoxin.

DISCUSSION

The poor immunogenicity of serogroup B polysaccharide has focused on the study of subcapsular antigens as an alternative approach to have an effective vaccine against meningococcal disease caused by this serogroup [10,11, 33].

In countries in which meningococcal disease is caused by a specific strain of meningococcus B, OMV vaccines have been shown to be effective. Their serotype specificity restricts the induction of cross-immunity among the wide variety of pathogenic strains of this serogroup indicating the preferred use of these products for outbreaks control [34]. The OMV vaccines

induce protective antibodies mainly against Por A, considered their major immunodominant antigen, which is the main target for SBA activity. The vaccine high efficacy is dependent of the similarity between Por A from vaccine and circulating strains. However, minor antigens as other outer membrane proteins and LOS, acting synergistically with Por A can contribute with vaccine efficacy [35,36]. The use of native OMV conformation as vaccine component, requires a detergent treatment to decrease LOS concentration aiming its decrease toxicity. LOS is an additional subcapsular antigen in OMV and has been studied to broaden coverage and cross-reactivity of serogroup B meningococcal vaccines (zolliner). Despite successfully decreasing toxicity, the outer membrane components that contribute to induce the immune response can also be affected. It was very important the establishment of all physico-chemical controls of vaccine components according to the literature, that will presented to Brazilian regulatory agency to go forwards in clinical trials (Table 1),.

The Brazilian vaccine against *N.meningitidis* serogroup B has in its composition, outer membrane vesicles (OMV) from Brazilian prevalent strains. They were obtained from bacteria growth with free iron starvation using ethylene diamine-di-(O-hydroxy-phenylacetic acid) (EDDHA) as iron chelator. This approach aimed to simulate the condition of availability of free iron in the blood of human host. The EDDHA chelating and release iron constants are similar to transferrin constants, which is the protein carrier of this element, in the blood. The presence of iron attached to protein carriers or EDDHA in bioreactor, induces the expression of iron regulated proteins (IRPs) on *N.meningitidis* OMV membrane that may be important in vaccine-induced immune response and increase of bacteria pathogenicity. Outer membrane from *B.pertussis* obtained from growth under iron starvation conditions, showed higher expression of proteins regulated by this ion as well as decrease in LPS phosphorylation degree compared to standard molecule [37, 38, 39,40].

The mass spectrometry data showed a severe reduction of mass comparing native and detoxified LOS. The observed mass variation was attributed to the hydrolysis of fatty acyl esters and partial hydrolysis of phospho groups, which generated a less toxic molecule (Figure 1).

Detergent-treated OMVs mainly induce immune response to parental strains homologous to Por A, which provides limited cross-protection of these vaccines to different serosubtypes and serogroups. The expectation of an increase in vaccine cross-reactivity using LOS as additional component was based on previous studies. The L3, 7, and 8 Brazilian prevalent strain immunotypes are expressed on a high percentage of serogroup B strains associated with epidemics [41]. The expectation of an increase in vaccine cross-reactivity using LOS as additional component must be evaluated in human clinical trials of the developed vaccine [42]. A significant increase in total IgG to OMV was observed, in mice, for both vaccine strains after immunization with the three experimental preparations, compared to pre immune values. It can be used as a primary vaccine potency control parameter. There was no difference between three vaccine concentrations. Total IgG produced against LOS increased 15 days after the third dose only for the most concentrated vaccines. Moreover, the increase was concentration-dependent, as the highest LOS concentration induced the highest total IgG concentration. Total IgG produced against LOS did not indicate a different role for residual LOS on OMVs and added dLOS in the vaccine immune response (Table 2). The role of each of these molecules in inducing a protective response against meningococcus should be evaluated in human clinical studies.

However the use of LOS as vaccine component, demands decrease of its toxicity not to increase OMV intrinsically pyrogenicity. A NaOH treatment is required to remove the ester-linked fatty acid tails from lipid A, which are responsible for the toxicity. dLOS as a potential target of serogroup B meningococcal vaccines is based on its contribution in bactericidal and opsonic activities of induced antibodies in meningococcal disease and after immunization with

OMV vaccines [36, 37,38]. The samples of LOS and dLOS were analyzed by SDS-PAGE, presenting difference in electrophoretic profile. The difference observed was attributed to Lipid A reduction degree of acylation, after NaOH treatment, which was confirmed by ESI-MS analysis (Figure 2).

The SBA titers to N44/89 induced in mice were protective after the first dose and remained protective for 15 days after the third dose of all preparations with no increase after the third dose. On the other hand, the SBA titers to the N603/95 strain were protective only after the second dose, suggesting that the third dose was important. In addition, the N603/95 titers were significantly lower than for N44/89 using all vaccines. The values remained at lower levels for 15 days after the third dose (Table 3).

Human and murine-specific protecting antibodies are mainly directed against two long, hypervariable, extracellular regions in Por A loops 1 and 4. However, other minor proteins and LOS are antigens in the total immune response induced by OMV vaccines. Although Por A is the dominant antigen, other outer membrane components may act synergistically during the induced immune response against meningococcal B. Antibody affinity maturation was induced in response to the dominant antigen but was seriously delayed in response to a subdominant antigen, resulting in less functional serum IgG. The expression of minor proteins and their localization in the outer membrane are mandatory to activate the complement cascade via the classical pathway, which is the first step to eliminate the bacteria [35]. The Por A from N603/95 (P1.7.1) may have been a subdominant antigen compared to that of P1.19.15 from N44/89 during the immune response to the experimental vaccines, which may contributed to SBA lower titres to N603/95 strain. The weaker immune response observed to the N603/95 strain may have been caused by weaker or less accessibility of B cell epitopes due to physical and chemical properties, such as hydrophobicity, length, or the combination of OMVs from the vaccine strains [21].

The Brazilian vaccine development approach, uses multiple antigens from B prevalent strains with the aim of broadening vaccine coverage (Figure 3 and 4). The combination of prevalent vaccine strains with different PorA (P1.19.15 and P1.7.1 from N44/89 and N603/95 respectively), and LOS immunotypes 3, 7, and 8, shared among different B strain serosubtypes followed the tailor-made vaccine concept based on Brazilian meningococcal disease epidemiology [41].

The rabbit pyrogen test has been used historically to measure pyrogenicity potential of human and animal vaccines. However, whole cell and OMV based vaccines, which intrinsically contain relatively high concentration of pyrogenic material, has been a source of great concern. Several studies have been reported to define the best biological tests that can be used as safety parameters for clinical trial [43].

The pyrogenicity evaluation of OMV based vaccines has been shown that the way of production of these structures as well as the characteristics of the rabbit's strains can affect the test response what has hampered the establishment of the acceptable safety range for new vaccines, in the pyrogen test "in vivo". Although the results showed here are based on protein concentration, the minimum pyrogenic dose established in pyrogenio test "in vivo" considers all pyrogens. In this way, to experimental vaccines are being considered all vaccine components contributing to the "in vivo" pyrogen test results [9,10].

Experimental vaccines showed minimum pyrogenic dose between 23 and 50 ng of protein per rabbit body weight. The differences observed for the vaccine with 25ug and 50ug of OMV protein /mL can be attributed to inherent "in vivo" pyrogenic test variation. Although the vaccine with the highest concentration (100ug/mL protein), also presented 50ng ng of protein per rabbit body weight as minimum pyrogenic dose, it was achieved with double dilution compared to vaccines with lower concentration of OMV protein. The results suggested that

OMV concentrations higher than 50ng of protein per kg of rabbit body weight, the pyrogenicity activity seems to concentration dependent (Table 4).

Most of OMV based vaccines clinical studies, used 50 or 100 µg of OMV protein/mL but none of them, combining dLOS and OMVs from two strains, as the Brazilian B vaccine [33,34]. In the Brazilian developed vaccines, residual LOS from OMVs, plus dLOS from the N44/89 strain composed the total amount of LOS. To assess the contribution of dLOS in experimental preparations, it was also evaluated in the pyrogenic test "in vivo". The test performed with raw and detoxified LOS showed a decrease of 1×10^6 in LOS pyrogenicity activity which may attributed to de Lipid A desacetillation [9,10].

In the development of the Brazilian vaccine against meningococcus B, as for other producers, we used as a reference the WHO requeriments for meningococcal polysaccharide vaccines. The vaccine should be approved in rabbit pirogen test, in the dilution of 1:4000 of human-dose or 25 ng polysaccharide/rabbit kg body weight as the minimum pyrogenic dose of *N.meningitidis* polysaccharide. According to what was established by the WHO, all tested vaccines would be safe for clinical trials in humans as they have passed the pyrogenicity test in rabbits at dilutions less than 1: 4000 of the probable human dose. The OMV-based vaccine, VAMENGOC-BC®, and the DTP vaccine officially included in Brazilian Immunization Program calendar were used as references in the "in vivo" pyrogenic test to compare the experimental vaccine pyrogenic potential. Both vaccines, would be approved by WHO for clinical trials. However, their potential pyrogenicity were higher than what was observed for the three experimental vaccines in rabbits [44].

The production approach applied to obtain OMVs from Brazilian prevalent strains was not much different from that described for OMV vaccines. The experimental preparations were approved by rabbit pyrogen test results of $< 20,000$ EU/mL vaccine. Residual LOS toxicity in

505 the OMVs was 5–10 µg/100 µg total protein, which is in accordance with previous studies
506 [45,46, 47].

507 These study results indicate that the use of the Brazilian meningococcal B tested vaccines are
508 in accordance with the literature for similar products and clinical trials studies can be
509 considered.

510 **CONFLIT OF INTEREST**

511 All authors have no conflict of interest to declare.

512

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516 **AUTHOR CONTRIBUTIONS**

517 All authors contributed equally in the elaboration of this article.

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Supporting information

S1 - Figure 1: Electrospray ionization/mass spectroscopy (ESI/MS) of O-deacylated lipooligosaccharide (LOS) from N44/89 *Neisseria meningitidis* B, strain.

(File: Figure 1 PLoS One Ellen Jessouroun.tif)

S2 - Figure 2: Electron microscopy of outer membrane vesicles (OMV) from the N44/89 strain (1 mg/mL) in distilled water negatively stained with 0.5% (w/v) phosphotungstic acid, pH 7.0. The preparations were examined under an electron microscope (JEM 1010; Jeol, Tokyo, Japan).

(File: Figure 2 PLoS One Ellen Jessouroun_300dpi.tif)

S3 - Figure 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 µg/outer membrane vesicle [OMV] protein) protein profile of vaccine strains OMV (A) N603/95 and (B) N44/89. Molecular weight (MW); (NP) OMV before sodium deoxy cholate (DOC) treatment; (P) OMV after DOC treatment; (BF) vaccine bulk before sterile filtration; (AF) vaccine bulk after sterile filtration.

(File: Figure 3 PLoS One Ellen Jessouroun .tiff)

673 Figure 4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis lipooligosaccharide
 674 (LOS) profile from N44/89 biomass. MW, protein molecular weight standards of 16.949–2.512
 675 kDa; LOS, native endotoxin; dLOS, detoxified endotoxin.
 676 **(File: Figure 4 PLOs One Ellen Jessouroun.tiff)**

Figure

Figure

Figure

LOS dLOS

This figure shows a Western blot with two lanes labeled 'LOS' and 'dLOS' at the bottom. The 'LOS' lane contains two prominent dark brown bands at the bottom, corresponding to the 8159kDa and 6214kDa markers. The 'dLOS' lane shows a very faint band at the 8159kDa position and no visible band at the 6214kDa position.

Figure