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The genetic structure of *Neisseria meningitidis* populations in Cuba before and after the introduction of a serogroup BC vaccine

Yanet Climent [a,1,*], Rachel Urwin [b,1,2], Daniel Yero [a,1], Isabel Martinez [a], Alejandro Martín [c], Franklin Sotolongo [a], Martin C.J. Maiden [b], Rolando Pajón [c,3]

[a] Department of Molecular Biology, Division of Biotechnology, Finlay Institute, Ave. 27, La Lisa, Havana 11600, Cuba
[b] Department of Zoology, University of Oxford, Oxford, United Kingdom
[c] Center for Genetic Engineering and Biotechnology, Havana, Cuba

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ABSTRACT

We investigated the population genetics in collections of meningococci sampled in Cuba during the period 1983–2005, thereby covering a period before and after the introduction of an antimeningococcal B–C vaccine. A total of 163 case isolates and 210 isolates from healthy carriers were characterized by multilocus sequence typing (MLST) and sequence determination of *porA*, *porB* and *fetA* genes. A total of 56 sequence types (STs) including 28 new STs were identified among these isolates. The analysis of surface antigens revealed variants 3-1 and 3-8 to be prevalent for porB; variant F5-1 was the most common FetA epitope, and variants 19 and 15 corresponded to the prevalent variable regions 1 (VR1) and VR2 PorA epitopes, respectively. The strongest associations between specific surface protein variants and clonal complexes were detected in lineages ST-32 and ST-53. All ST-32 complex isolates possessed porB3 alleles, and the most frequent antigen combination among ST-32 complex isolates was P1.19,15;F5-1. Variants PorB3-64 at PorB and P1.30 at PorA VR2, in combination with the PorA VR1 variants P1.12-1, P1.7 and P1.7-2 as well as the FetA variants F1-2 and F1-7, dominated the ST-53 complex organisms. Furthermore, we observed a statistically significant association between the most frequent *porA*, *porB* and *fetA* alleles and strain invasiveness. Finally, this study showed that the application of VA-MENGOC-BC®, the Cuban antimeningococcal vaccine, reduced the number and frequency of the hypervirulent Clonal Complexes ST-32 and ST-41/44, and also impacted on other lineages. The vaccine also affected the genetic composition of the carrier-associated meningococcal isolates. The number of carrier isolates belonging to hypervirulent lineages decreased significantly after vaccination, and ST-53, a sequence type common in carriers, became the predominant ST.

1. Introduction

A meningococcal disease (MD) epidemic caused mainly by serogroup C meningococci began in Cuba in 1976, increasing the reported incidence for this disease from 0.4 to 5.6 cases per 100,000 inhabitants by 1979. After a nationwide vaccination in 1979 with a capsular polysaccharide A–C vaccine (Merieux Laboratories), however, the incidence of MD continued to climb, with a shift towards serogroup B *Neisseria meningitidis* strains among new cases (Rodríguez et al., 1999). The epidemic peaked in 1983 with a maximum of 14.4 cases per 100,000 inhabitants (Sotolongo et al., 2007). Government efforts to control the disease included funding for the research and development of a serogroup B vaccine, resulting in the product registered as VA-MENGOC-BC® vaccine, with components of outer membrane vesicles (OMV) from serogroup B and purified serogroup C polysaccharide (Sierra et al., 1991). This vaccine is based on outer membrane proteins of the epidemic (homologous) strain (Sierra et al., 1991) that included, among others, PorA, PorB, RmpM, Opa, OpcA and FetA (FrpB) (Uli et al., 2006; Gil et al., 2009). A large, double-blind, randomized, controlled clinical trial using a 2-dose regimen in children 10–14 years old estimated vaccine efficacy in this age group to be 83% [95% confidence interval from 42% to 95%, $P < 0.005$] after 16 months (Sierra et al., 1991).

The vaccination with VA-MENGOC-BC® in Cuba was carried out in 2 stages. The first was a nationwide mass vaccination campaign targeting the high risk population aged 3 months to 24 years,

* Corresponding author. Tel.: +53 7 2716911; fax: +53 7 2047236.
E-mail address: ycliment@finlay.edu.cu (Y. Climent).
[1] These authors equally contributed to this work.
[2] Present address: Department of Biology, The Pennsylvania State University, University Park, United States.
[3] Present address: Center for Immunobiology and Vaccine Development, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609, United States.

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involving over 3 million people and achieving a general coverage of 95% during 1989–1990. The second stage begun in 1991 and continuing to date using a 2-dose schedule: the first dose at 3 month and the second dose at 5 months of age achieving a general coverage over 99% (Sotolongo et al., 2007). In 1991 the vaccine was included in the National Immunization Program (NIP) for all infants and MD incidence decreased to a level of 0.1 cases per 100,000 inhabitants in 2008 (MINSAP, 2008). Meningococcal infection is no longer considered to be a national health problem in Cuba.

Traditionally, isolates of *N. meningitidis* have been identified and characterized phenotypically, based on the recognition of meningococcal surface structures by specific monoclonal or polyclonal antibodies (Abdillahi and Poolman, 1988; Frasch et al., 1985). While serogrouping, serotyping and sero-subtyping, based on the detection of variants of capsular polysaccharide, PorB and PorA respectively, are still used extensively they have often been found to lack resolution, especially when working with endemic strains (Clarke et al., 2001; Vogel et al., 2000). The use of molecular typing methods has revolutionized the epidemiological study of *N. meningitidis*. Approaches such as multilocus enzyme electrophoresis, now replaced by multilocus sequence typing (MLST) (Maiden et al., 1998), have been used to monitor the global distribution and evolution of meningococcal strains (Caugant, 1998; Girard et al., 2006; Caugant, 2008). The discriminatory power of MLST has been enhanced with the inclusion of additional information about the variable regions of PorA, PorB and FetA (Jolley et al., 2007; Urwin et al., 2004), together with pulsed-field gel electrophoresis patterns (Chiou et al., 2006). Currently, a combination of MLST using seven housekeeping genes together with sequence data from PorA, PorB and FetA is considered optimal for dissecting the diversity and dynamics of meningococcal populations (Urwin et al., 2004). For example, such fine-scale typing has been employed to evaluate the impact of mass immunization with a serogroup C vaccine on the risk of emergence of escape variants (Lancellotti et al., 2006), to characterize the risk of fatal MD (Jacobsson et al., 2008), to investigate outbreaks (Feavers et al., 1999) and to establish epidemiological links between cases (Elias et al., 2006).

A sample of *N. meningitidis* isolates was collected in Cuba both before and after the introduction of VA-MENGOC-BC® and comprises isolates from both asymptomatic carriers and patients with meningococcal disease. In the present work, we have used this unique collection to characterize the extent and pattern of genetic diversity of the Cuban meningococcal population using both MLST and sequence data from the major antigenic determinants. In particular, we determined the association of the resulting sequence types (ST) and allelic profiles with carriage status and epidemic potential. The epidemiological impact of the introduction of an OMV vaccine in Cuba, based in these data, is also addressed.

2. Materials and methods

2.1. Meningococcal isolates

A total of 373 meningococcal strains were studied, 163 isolated from MD patients and 210 from healthy carriers (Table 1). The isolates were collected between 1983 and 2005 from different geographic regions of Cuba and preserved at −70 °C. All strains were cultured on Mueller–Hinton agar supplemented with fetal bovine serum and incubated for 18–20 h at 37 °C in a 5% CO_2 atmosphere. The strains were first confirmed as meningococci by Gram staining along with catalase, oxidase and sugar fermentation tests (glucose, lactose, maltose and sucrose). A complete strain list, including their phenotypic characteristics, source and place of isolation is available from the authors on request. The set of strains comprised isolates (113 disease isolates and 85 carrier isolates) from the pre- and peak-vaccination period combined (1983–1991) (Table 1), as well as isolates (50 disease and 125 carrier isolates) from the post-vaccination period (1992–2005).

2.2. Preparation of chromosomal DNA

Meningococcal genomic DNA was prepared from cell suspensions using the Isoquick nucleic acid extraction kit (Orca Research, USA), following the instructions from the manufacturer.

2.3. Multilocus sequence typing

The amplification and sequencing of seven housekeeping genes was performed as previously described (Maiden et al., 1998). The allele numbers for each locus and STs were determined by comparison with the available sequences at the *Neisseria* MLST database (http://neisseria.org/nm/typing/mlst). The STs were assigned to clonal complexes by reference to the *Neisseria* pubMLST isolate database (http://pubmlst.org/neisseria/). Novel ST were submitted to the *Neisseria* PubMLST database for assignment, and the relationships of ST to clonal complexes were established with the eBURST software application (Feil et al., 2004).

Table 1
Genetic diversity of meningococcal isolates collected in Cuba between 1983 and 2005.

Parameter	No. of isolates				
	1983–1991		1992–2005		Total
	Disease	Carrier	Disease	Carrier	
Isolates	113	85	50	125	373
Strain types	39	29	18	47	118
Serogroups[a]	2	2	1	4	5
PorB 2 variants	4	1	3	3	7
PorB 3 variants	20	9	8	10	24
PorA VR1 variants	14	10	7	10	18
PorA VR2 variants	19	14	9	18	24
PorA VR1-VR2 combinations	22	17	10	25	53
FetA VR variants	16	8	10	16	28
Sequence type	27	13	14	16	56
Clonal complexes	7	6	4	10	12
New ST	12	7	4	8	28
Isolates not assigned to a complex	5	1	1	3	10

[a] Including non-groupable strains.

2.4. Genetic characterization of meningococcal strains

PCR amplification and nucleotide sequence determination of the meningococcal *porB* gene was as described previously (Urwin et al., 1998), and *porA* and *fetA* gene sequence determination and identification of the variable regions (VRs) of the PorA and FetA proteins were as reported elsewhere (Thompson et al., 2003; Russell et al., 2004).

2.5. Data storage and statistical analysis

The data were stored on a customized isolate list available at the *N. meningitidis* MLST database. The database automatically generated the strain types according to the recently proposed typing nomenclature (Jolley et al., 2007), in the format serogroup:PorA type:FetA type:sequence type (clonal complex). We used both Pearson χ^2 test and Fisher's exact test as appropriate to assess the relationship between categorical variables, and particularly how carriage/disease rates and period of isolation were related to other variables, such as clonal complex and antigen variants. The level of statistical significance was set at $P < 0.05$ and all tests were two-tailed. All statistical analyses were performed with the statistical software package SPSS, version 11.5.1 (SPSS Inc., Chicago, IL, USA).

To assess differentiation between two different conditions (e.g. populations) using different characters and data partitions (for example "before" and "after" vaccine introduction data subdivisions) a simple metric of differentiation (D) was used as proposed by Jolley et al. (2005).

$$D = 1 - \sum_i \frac{p_{i1}\, p_{i2}}{\bar{p}}$$

where p_{i1} is the frequency of haploptype or polymorphism i in population 1, and $\bar{p}$ is the average frequency of p across the populations. When two populations have identical character frequencies, its value is 0, and 1 when there is no overlap between populations. Whether the statistic was significantly different from 0 was assessed through permutation analysis using 10,000 replicates.

3. Results

3.1. ST assignment

We analyzed 373 Cuban strains; 163 isolated from cases with MD and 210 from carriers (Table 1). From this a total of 56 STs were identified within this sample of Cuban isolates. Twenty eight of the STs identified in this study had not been described previously in the MLST database and were designated ST-6365 to ST-6370, ST-6374 to ST-6377, ST-6379 to ST-6390, ST-6437 to ST-6440, ST-7084 and ST-7086. Forty-seven of the STs were assigned to 12 clonal complexes; nine could not be assigned to any known clonal complex and are denoted here as unassigned STs (UA) (Table 2).

Table 2
Serogroup, strain type, *porB* allele and carrier/disease isolate status, per clonal complex.

Clonal complex	Strain designation (serogroup:PorA:FetA:ST)[a]	*porB* allele	No. of isolates			
			1983–1991		1992–2005	
			Disease	Carrier	Disease	Carrier
ST-8	B: P1.5-2,10: F3-9: ST-153	porB2-3	1		2	
	B: P1.5-2,10-27: F3-9: ST-153	porB2-30			2	
ST-11	ND: P1.5,2: F1-1: ST-11	porB2-2	1			
ST-22	NG: P1.18-1,3: F4-1: ST-22	porB2-23				3
	W135: P1.18-1,3: F4-1: ST-22	porB2-23				2
ST-23	B: P1.5-1,2-2: F5-8: ST-23	porB2-55			1	
	NG: P1.5-1,2-2: F3-6: ST-23	porB2-55				1
ST-32	B: P1.19,15: F3-4: ST-33	porB3-1			4	
	B: P1.19,15: F5-1: ST-2858	porB3-1	1		2	
		porB3-128	1			
		porB3-8	1	1	1	
	B: P1.19,15: F5-1: ST-33	porB3-1	30	2	6	1
		porB3-100		1		
		porB3-120	4			
		porB3-126	1		1	
		porB3-128	2			
		porB3-8	29	41	17	35
	B: P1.19,15: F5-1: ST-34	porB3-8		2		
	B: P1.19,15: F5-1: **ST-6368**	porB3-8				2
	B: P1.19,15: F5-1: **ST-6387**	porB3-8				2
	B: P1.19,15: F5-47: ST-33	porB3-1			2	
	B: P1.19-24,15: F5-1: ST-33	porB3-8	2			
	B: P1.7,30-8: F5-1: ST-33	porB3-8				2
	ND: P1.19,15: F5-1: ST-33	porB3-8		4		
	ND: P1.7-1,1: F5-1: ST-33	porB3-8		1		
		porB3-39		1		
	NG: P1.19,15: F5-1: ST-33	porB3-8		4		1
		porB3-39				1
	Others		12	3	7	2
ST-41/44	C: P1.18-1,34: F1-7: ST-883	porB3-125	2			
	ND: P1.18,15: F1-25: ST-303	porB3-81		2		
	ND: P1.18,25-17: F3-1: ST-303	porB3-81	2			
	ND: P1.18-1,3: F3-6: ST-414	porB3-1			1	
		porB3-2			1	
	ND: P1.22,14-6: F1-28: ST-44	porB3-24	2			

Table 2 (*Continued*)

Clonal complex	Strain designation (serogroup:PorA:FetA:ST)[a]	*porB* allele	No. of isolates			
			1983–1991		1992–2005	
			Disease	Carrier	Disease	Carrier
	Others		11	3	2	1
ST-53	ND: P1.12-1,13: F1-2: ST-53	porB3-64		3		
	ND: P1.12-1,13-1: F1-2: ST-53	porB3-64	1			1
	ND: P1.12-1,13-1: F5-24: ST-53	porB3-64				3
	ND: P1.7,30: F1-7: ST-53	porB3-64				
	ND: P1.7-2,30: F1-5: ST-53	porB3-64				3
	ND: P1.7-2,30: F1-7: ST-53	porB3-64				5
	ND: P1.7-2,30-2: F1-2: ST-53	porB3-64				2
	ND: P1.7-2,30-4: F1-5: ST-53	porB3-64		2		
	NG: P1.12-1,13-1: F1-2: ST-53	porB3-64		1		4
	NG: P1.7,10-1: F1-2: ST-53	porB3-122				2
	NG: P1.7,30: F1-7: ST-53	porB3-64				2
	NG: P1.7-2,30: F1-2: ST-53	porB3-64				2
	NG: P1.7-2,30: F1-7: ST-53	porB3-64				14
	NG: P1.7-2,30-2: F1-7: ST-53	porB3-64		1		2
		porB3-8		1		
	NG: P1.7-2,30-3: F1-7: ST-53	porB3-64				2
	Others			8		14
ST-103	B: P1.5-1,2-2: F1-5: ST-5171	porB2-23	1			
		porB2-25	1			
		porB3-128	1			
	ND: P1.5-1,2-2: F1-5: ST-5171	porB3-8	1			
		porB2-23		1		
	ND: P1.5-2,10-50: F4-3: ST-103	porB3-38				2
	NG: P1.18-1,3: F3-9: ST-103	porB2-22				2
	Z: P1.18-1,3: F3-9: ST-103	porB2-22				1
ST-167	ND: P1.19,15: F5-1: **ST-6388**	porB3-8		1		
	NG: P1.5-1,10-4: F3-4: ST-1624	porB2-55				1
ST-198	NG: P1.18,25-15: F5-5: ST-823	porB3-84				5
ST-254	NG: P1.5-1,2-2: F3-6: **ST-6389**	porB2-55				1
ST-269	B: P1.21,2-2: F3-3: ST-352	porB3-124	1			
	ND: P1.7,13-9: F3-3: **ST-6390**	porB3-81				1
	ND: P1.22,14-13: F1-33: **ST-6384**	porB3-124		1		
UA[b]	B: P1.12,16-11: F1-5: ST-897	porB3-10			1	
	ND: P1.18-1,34: F1-7: **ST-6437**	porB3-125	1			
	ND: P1.19,15: F1-7: **ST-6370**	porB3-128	1			
	ND: P1.19,15: F5-1: **ST-6386**	porB3-8		1		
	ND: P1.22-11,14: F1-51: **ST-6440**	porB3-44				1
	ND: P1.22-11,14: F5-34: **ST-6439**	porB3-44				1
	ND: P1.5-4,2: F5-5: **ST-6438**	porB3-89	1			
	ND: P1.7-2,13: F4-1: **ST-6365**	porB3-127	1			
	ND: P1.7-2,13-2: F4-1: **ST-6365**	porB3-127	1			
	NG: P1.19-1,15: F-1-49: **ST-7086**	porB3-16				1
Total			113	85	50	125

Each new sequence type (ST) is shown in bold.
[a] NG, non-groupable; ND, not determined.
[b] UA, unassigned.

The four most frequent clonal complexes were ST-32 with 229 isolates (61.4%); ST-53 (73 isolates, 19.6%); ST-41/44 (27 isolates, 7.2%) and ST-103 with 10 isolates representing 2.7% of the total population. The eight remaining clonal complexes each occurred at a frequency lower than 2.0%.

3.2. Strain types and antigenic variants among Cuban meningococcal isolates

The genetic variation present in the sample of Cuban meningococcal isolates is shown in Table 1. Serogroup information was available for 308 (82.6%) of the strains, and complete molecular characterization using both MLST and *porA*, *porB* and *fetA* sequences was completed for the set of 373 isolates. A total of 118 unique strain types (serogroup:PorA type:FetA type:ST) was found in the collection, with the greatest genetic diversity observed during the period between 1983 and 1991. The complete data set analyzed here is available at the *N. meningitidis* MLST database.

Meningococcal subtypes are traditionally assigned based on the immunochemical reactivity and sequence of VRs 1 and 2 from PorA, located in the surface-exposed loops I and IV of this protein (van der Ley et al., 1991). In the present study, by sequencing fragments of the *porA* gene, a total of 18 VR1 variants and 24 VR2 variants were observed among the PorA sequences from the 373 isolates (Table 1). There were 53 VR1-VR2 combinations, of which P1.19,15 was the most common (218 isolates, 58.4%).

A total of 28 unique FetA VR sequences were found. The predominant FetA variant was F5-1 (216 strains, 57.9%), found among both disease and carrier isolates (109 and 107, respectively), followed by F1-7, F1-2 and F1-5 with 46 (12.3%), 21 (5.6%) and 18 (4.8%) isolates, respectively. Finally, 31 unique PorB amino acidic sequences were obtained. PorB3 variants were predominant among the sample (94.4%, representing 352 strains) while PorB2

variants represented only 5.6% of the total. Most of the unique PorB3 variants were isolated from patients sampled in the period between 1983 and 1991, representing 83.3% of isolates (Table 1).

3.3. Distribution of antigenic variants and ST among clonal complexes

Overall, ST-11, ST-22, ST-23 and ST-53 clonal complexes appeared to be the most homogeneous within the Cuban population, with each containing only one major ST (corresponding, in each case, to the central ST of the clonal complex). By contrast, the remaining complexes contained multiple STs (Table 2). The strongest associations between surface protein variants and clonal complexes were found for the lineages ST-32 and ST-53.

Clonal complex ST-32, the most frequent in Cuba (61.4% of the isolates), was strongly associated with particular combinations of the four antigenic regions. The most frequent combinations were B:P1.19,15:F5-1:ST-33 with PorB3-8 (122 out of 170 ST-32 clonal complex isolates) or PorB3-1 (39 out of 170 isolates). Within this clonal complex these antigen combinations were also associated with other STs: ST-34 (both isolates), ST-2858 (6 out of 7 isolates), ST-6368 (both isolates) and ST-6387 (both isolates), where the latter two represent newly identified STs. In two cases, isolates with unrelated and new ST exhibited identical antigen profiles: one ST-167 complex isolate was identical to one UA isolate, both being P1.19,15: F5-1 with PorB3-8.

The second most common clonal complex observed was ST-53 (19.6%). In contrast to complex ST-32, the antigenic variability of the ST-53 clonal complex strains was greater primarily for PorA VR1 and FetA. Variants PorB3-64 at PorB and P1.30 at PorA VR2 dominated the ST-53 complex organisms. These variants, in combination with the PorA VR1 variants P1.12-1, P1.7 and P1.7-2 as well as the FetA variants F1-2 and F1-7, were the predominant strain types in that complex.

PorB2 variants were predominant in clonal complexes ST-8 (5 isolates), ST-11 (1 isolate), ST-22 (5 isolates), ST-23 (2 isolates) and ST-254 (1 isolate). PorB2 allele was also found in ST-103 (6 out of 10 isolates) and ST-167 (1 out of 2 isolates). In the specific case of complex ST-22, all five isolates in the sample shared the same VR type (porB2-23). Similarly, all ST-198 complex isolates (5 strains) shared a single porB3 variant (porB3-84).

For the FetA antigen variants there were two strong associations with specific clonal complexes. First, all ST-8 complex isolates had FetA variable region type F3-9, which is also found in complex ST-103 strains. Second, almost all of the F5-5 isolates (5 of 6 isolates) were found in the ST-198 complex and the remaining isolate was in an unassigned clonal complex.

3.4. Association of genotype with clinical status

The analysis of our strain collection by MLST confirmed a high prevalence of clonal complexes ST-32 and ST-41/44 (i.e. hyperinvasive lineages) among disease isolates (Table 3). Similarly, an analysis of antigen profiles also showed statistically significant associations between the most common *porA*, *porB* and *fetA* alleles and disease. Strains with genotype porB3-1, genosubtype P1.19,15 and expressing FetA variant F5-1 ($P < 0.01$ for all alleles) had the highest disease potential. Conversely, there were also alleles and lineages with statistically significant associations with the carrier status, such as PorA VR2 30, porB3-64, FetA F1-2 and clonal complex ST-53 ($P \leq 0.0001$ in all cases). Other abundant alleles in the Cuban strain collection for antigens PorA, PorB and FetA were also predominant in strains isolated from asymptomatic carriers (Table 3).

Table 3
Association of antigen profiles and clonal complexes with clinical status.

Variable[a]	No. of isolates		Disease association rate ratio (CI)[b]	*P* value
	Disease ($n = 163$)	Carrier ($n = 210$)		
PorA (VR1)				
19	117	103	2.64 (1.71–4.08)	<0.001
7-2	8	47	0.18 (0.08–0.39)	<0.001
7	2	16	0.15 (0.03–0.66)	0.0058
12-1	2	13	0.19 (0.04–0.85)	0.0163
PorA (VR2)				
15	118	105	2.62 (1.69–4.06)	<0.001
30	0	33	0.02 (0.001–0.27)	<0.001
13-1	1	10	0.12 (0.02–0.97)	0.0268
30-3	0	8	0.07 (0.004–1.27)	0.0107
30-2	0	7	0.08 (0.005–1.46)	0.0200
14-6	5	0	14.6 (0.8–266.3)	0.0154
PorB				
porB3-8	55	102	0.54 (0.35–0.82)	0.0044
porB3-64	1	67	0.01 (0.002–0.10)	<0.001
porB3-1	56	4	26.9 (9.52–76.3)	<0.001
porB3-24	6	0	17.4 (0.97–310.9)	0.0066
porB3-125	5	0	14.6 (0.8–266.3)	0.0154
porB3-128	5	0	14.6 (0.8–266.3)	0.0154
FetA				
F5-1	109	107	1.94 (1.27–2.97)	0.0022
F1-7	6	40	0.16 (0.07–0.39)	<0.001
F1-2	1	20	0.06 (0.008–0.44)	<0.001
F3-4	7	1	9.38 (1.14–77.0)	0.0237
Clonal complex				
ST-32	123	106	3.02 (1.93–4.72)	<0.001
ST-53	1	72	0.01 (0.002–0.09)	<0.001
ST-41/44	21	6	5.03 (1.98–12.8)	<0.001
ST-8	5	0	14.6 (0.80–266.3)	0.0154

[a] Only significant results are shown.
[b] CI: 95% confidence interval.

3.5. Differentiation between disease and carriage populations

The classification index D was calculated for comparisons of the Cuban carriage and disease-isolate collections for STs, clonal complexes, and the variable regions of PorA, PorB and FetA. Strong differentiation was observed at ST ($D = 0.414$, $P < 0.001$), *porA* VR2 ($D = 0.307$, $P < 0.001$) and *porB* ($D = 0.468$, $P < 0.001$) while values with lower but significant differentiation for clonal complexes ($D = 0.272$, $P < 0.001$), *porA* VR1 ($D = 0.158$, $P < 0.001$) and *fetA* VR ($D = 0.204$, $P < 0.001$) levels. These results indicated that disease and isolate collections represented significantly different sets of STs, as previously established by others in non-vaccinated populations (Jolley et al., 2005) and the existence of hyperinvasive lineages. Therefore, disease and carriage populations, at the ST and antigen sequence levels are significantly differentiated to grant a specific view to each dataset. Furthermore, as there was an obvious bias in the sampled population in the post-vaccination period (with case isolates accounting for roughly a third of the carrier strain set) the temporal analysis had to be performed on each population separately (either carriage or case isolates).

3.6. Effect of vaccination on the genetic structure of the meningococcal population

A temporal association of major surface protein variants and clonal complexes was also statistically significant (Table 4), likely reflecting the impact of vaccination. In the post-vaccination period (1992–2005) there was a significant decrease in the number of strains belonging to hypervirulent clonal complexes ST-32 and ST-41/44 compared to the period comprised between 1983 and 1991, again most likely due to the reduction of the number of cases after the introduction of the vaccine. Most interesting was the fact that the number of carrier isolates belonging to hypervirulent lineages also decreased significantly after vaccination (Table 4). As expected, the frequencies for all major protein variants associated with the carriage state increased significantly between 1992 and 2005. However, PorB variant porB3-8, which was associated with carriers ($P < 0.01$) during the first period, showed a significant reduction for the post-vaccination period ($P < 0.05$ for all isolated, $P < 0.001$ for the carrier population) (Table 4).

4. Discussion

The DNA sequences coding for the surface-exposed VR from PorA, PorB and FetA may provide important information about the emergence and evolution of meningococcal clones in the context of population immunity (Urwin et al., 2004). In this context we have evaluated the effect of mass immunization with a serogroup B–C vaccine on the evolution and structure of *N. meningitidis* populations in Cuba. Genotyping and genosubtyping techniques constitute important tools for epidemiologic monitoring in Cuba after more than 20 years of full vaccine coverage (Sotolongo et al., 2007), particularly since PorA, PorB and FetA proteins are among the most abundant protein components of the VA-MENGOC-BC® vaccine (Uli et al., 2006; Gil et al., 2009). This study represents the first analysis of PorA, PorB and FetA sequence variants among pre- and post-vaccine *N. meningitidis* isolates from Cuba.

PorA has been a commonly targeted antigen during the development of vaccines against serogroup B meningococci derived from locally prevalent epidemic strains (Bjune et al., 1991; Boslego et al., 1995; Cartwright et al., 1999; Martin et al., 2000; Oster et al., 2005; Sierra et al., 1991). Both PorA VR1 and VR2 are highly variable; the PorA sequence database (http://neisseria.org/nm/) comprises 11 and 21 sequence families, respectively, totaling a large number of variants (189 for VR1 and 523 for VR2). Our study also reveals some degree of sequence variation for VR1 and VR2 in the PorA proteins expressed among Cuban meningococcal isolates. However, the most common genosubtype in our sample corresponds to variant P1.19,15, identical to the Cuban vaccine strain. Given that PorA is one of the main targets recognized by the bactericidal antibodies of children immunized with VA-MENGOC-BC® (Milagres et al., 1998), these data help to explain why the number of reported cases of meningococcal disease in Cuba has been very low after the introduction of this vaccine in a mass immunization campaign. PorA P1.19,15 is also prevalent in Malta (Pace et al., 2008), several Brazilian states (de

Table 4
Association of antigen profiles and clonal complexes with the period of isolation.

Variable[a]	No. of isolates (carriers)		First period association rate ratio (CI)[b]	
	1983–1991 $n = 198(85)$	1992–2005 $n = 175(125)$	All isolates	Carrier population
PorA (VR1)				
19	138(59)	82(44)	2.61 (1.71–3.99)***	4.17 (2.31–7.53)***
7-2	13(8)	42(39)	0.22 (0.11–0.43)***	0.23 (0.10–0.52)***
7	4(3)	14(13)	0.24 (0.08–0.73)**	NS
PorA (VR2)				
15	141(61)	82(44)	2.81 (1.83–4.30)***	4.68 (2.57–8.51)***
30	3(3)	30(30)	0.07 (0.02–0.25)***	0.12 (0.03–0.39)***
3	2(0)	10(8)	0.17 (0.04–0.78)*	ND
PorB				
porB3-8	93(58)	64(44)	1.54 (1.01–2.32)*	3.95 (2.20–7.11)***
porB3-64	16(15)	52(52)	0.21 (0.11–0.38)***	0.30 (0.15–0.58)***
porB3-1	42(3)	18(1)	2.35 (1.30–4.26)**	NS
FetA				
F5-1	142(61)	74(46)	3.46 (2.25–5.32)***	4.36 (2.40–7.92)***
F1-7	15(9)	31(31)	0.38 (0.20–0.73)**	0.36 (0.16–0.80)*
F1–2	6(5)	15(15)	0.33 (0.13–0.88)*	NS
F3-9	1(0)	7(3)	0.12 (0.01–1.00)*	ND
Clonal complex				
ST-32	143(60)	86(46)	2.69 (1.75–4.13)***	4.12 (2.28–7.45)***
ST-53	17(16)	56(56)	0.20 (0.11–0.36)***	0.29 (0.150.54)***
ST-41/44	22(5)	5(1)	4.25 (1.57–11.5)**	7.75 (0.89–67.6)*

If any of the four values in the contingency table is zero the association rate ratio was not done (ND). CI: 95% confidence interval.

[a] Only significant results are shown.

[b] NS: not significant, *$P<0.05$, **$P<0.01$ and ***$P<0.001$ as determined by χ^2 or Fisher's exact test.

Filippis and Vicente, 2005) and in some regions of Spain (Vicente et al., 2005). The Cuban vaccine VA-MENGOC-BC® could potentially be used to control outbreaks with meningococcal P1.19,15 clones. A multivalent OMV vaccine would however be needed for broader protection against the endemic heterogenous *N. meningitidis* strains in those countries.

The FetA protein is also a predominant component of meningococcal OMV-based vaccine formulations (Wedege et al., 1998; Vipond et al., 2005; Uli et al., 2006) and was recently included in the molecular typing scheme of *N. meningitidis* (Jolley et al., 2007). PorA and FetA are attractive vaccine candidates, since they are known to be protective and to generate bactericidal responses, both in humans and in animal models of infection and protection (Black et al., 1986; Pettersson et al., 1990; Saukkonen et al., 1989). The PorA VRs, especially VR2, and the FetA VR are relatively long surface-exposed peptides (VR2, 8–24 amino acids long; FetA VR, 20–42 amino acids long) that are easily defined. The molecular characterization of these antigens, which are subject to strong immune selection pressure, undoubtedly pushes forward the design and development of new vaccines. Previous work studying the distribution of surface protein variants among hyperinvasive meningococci has shown that it is possible to predict which combinations of PorA VR regions and FetA variants in a vaccine could be particularly effective (Urwin et al., 2004; Russell et al., 2008). Although in our study the combination PorA VR2/FetA of the Cuban vaccine strain (VR2 type 15 and F5-1) was found in 65.6% of the invasive isolates (107 strains), the fact that the introduction of the vaccine in Cuba dramatically reduced the incidence of meningococcal disease (Sotolongo et al., 2007) indicates that other conserved epitopes are present in the formulation. These conserved epitopes could also be involved in the protection of individuals by VA-MENGOC-BC® against serogroup B strains other than the vaccine type-strain (de Moraes et al., 1992; Morley et al., 2001; Milagres et al., 1998; Noronha et al., 1995).

PorB is a transmembrane protein with eight predicted surface-exposed loops (I to VIII) that are variable in terms of their lengths and amino acid sequences (van der Ley et al., 1991). The two classes of PorB proteins are encoded by either one of their respective *porB* genes, which are mutually exclusive, so that a strain will produce either a class 2 or a class 3 PorB membrane protein (Law et al., 2004). A total of 31 unique *porB* nucleotide sequences were obtained in our sample, and some of these variants added diversity to strains with a unique PorA, FetA and ST combination (Table 2). The three most common *porB* sequences were related to specific clonal complexes, with porB3-8 (153 isolates) and porB3-1 (54 isolates) associated to ST-32, and porB3-64 associated only with isolates from ST-53 complex. Similar results were obtained in a study conducted in China where a strong association between PorB variants and clonal complexes was observed (Yang et al., 2009).

Other studies have detected the association of particular antigenic variants with specific clonal complexes (Caugant et al., 1987; Suker et al., 1994; Wang et al., 1993; Russell et al., 2008). The same pattern is observed within our dataset, although a particular antigenic variant was sometimes associated with different clonal complexes. For example, the predominant FetA type of the ST-32 complex (F5-1) was also found in one ST-167 complex isolate and in another, unassigned strain. Furthermore, the most common PorA type among our ST-32 complex isolates (P1.19,15) was also identified in one isolate from the ST-167 complex and two UA isolates, although these isolates did not share identical FetA and PorB types. Importantly, our results also indicate that there are statistically significant markers of disease potential. We also assessed the relationship between carrier and disease-causing clones, determining the relative virulence potential of particular sequence types, strain types and clonal complexes. A detailed molecular characterization of disease isolates of *N. meningitidis*, correlating the data with their pathogenicity (Yazdankhah et al., 2004) or lethality (Jacobsson et al., 2008) has been carried out by others.

Investigations of the carrier state are clearly warranted to support the introduction of mass vaccination, and to measure its impact (Caugant et al., 2007; Caugant, 2008). Here, the antigenic combination of the vaccine strain was also present in almost one half of carrier strains, a fact that may explain the high efficacy of VA-MENGOC-BC® vaccination for preventing further occurrence of invasive disease in Cuba. Reduction of prevalence among carriage isolates was found to result in a similar reduction in the incidence of meningococcal disease (Ramsay et al., 2003). In Brazil, where the ST-32 complex was responsible for almost 80% of the cases during the 1980s, a carriage study of this hypervirulent lineage revealed that it was carried by only 0.7% of the population (Baethgen et al., 2008). In our data, looking at both PorA and FetA combined, the frequencies of PorA VR2 type 15 and FetA F5-1 goes down in the carrier population after vaccination (Table 4). Interestingly we appreciated that porB3-8 predominantly decreases in the carrier stratum post-vaccination (from data on Tables 2 and 4) when compared with the whole panel or the disease population. This revealing fact probably means that selection forces are different against different antigens (or epitopes contained by them) and even in different compartments: the vaccine may have increased the immune pressure at the mucosal level against porB3-8 more than at the systemic level.

Overall, our data are consistent with previous studies which suggest that meningococcal populations exhibit a highly dynamic population structure (Yazdankhah et al., 2004; Harrison et al., 2006). In particular, the antigens associated with specific clonal complexes rise and fall over time, as predicted by models of pathogen populations structuring by immunological selection (Gupta and Maiden, 2001), and in agreement with a recently described model where competition among lineages, expressed as differences in transmissibility in the presence of host immune selection, do account for the persistence of certain STs in meningococcal populations (Buckee et al., 2008). Our data, unlike previous reports describing meningococcal populations sampled from a completely natural environment (i.e. no vaccine intervention), was collected from a vaccinated population and could thus prove to be very valuable when comparing this model to previously considered alternatives (e.g. the microepidemic model) (Fraser et al., 2005). Our results also take us a step closer towards a detailed understanding of the effects of the introduction of an antimeningococcal vaccine on the target meningococcal population.

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