VA-MENGOC-BC Vaccination Induces Serum and Mucosal Anti Neisseria gonorrhoeae Immune Responses and Reduces the Incidence of Gonorrhea

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VA-MENGOC-BC™ vaccination induces serum and mucosal anti *Neisseria gonorrhoeae* immune responses and reduces the incidence of Gonorrhea

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Abstract

Background: Overall there are over 30 different sexually transmitted infections (STI) with *Neisseria gonorrhoeae* being the third most frequent with a reported 78 million cases per year. Gonococcal infection causes genital inflammation which can be a risk factor for others STI, particularly human immunodeficiency virus. Gonorrhea is a treatable disease, but recently an increase in antibiotic resistance has been of concern. There are currently no vaccines available. However, parenteral vaccination with anti *N. meningitidis* serogroup B vaccine has been reported to decrease the incidence of gonococcal burden in New Zealand

and in Cuba despite the fact that parenteral vaccination is not deemed to induce mucosal IgA. Here we explore possible mechanisms of protection against gonococcal infection through parenteral meningococcal B vaccination.

Methods: Ninety-two serum, saliva and oropharyngeal swabs samples of young adults (healthy and Neisseria carriers) of the internal higher school were obtained. They have been vaccinated with VA-MENGOC-BC™ (MBV) during their infancy and boosted with a third dose during this study. Serum and saliva samples were analyzed by ELISA and Western Blot to measured IgG and IgA antibodies against *N. meningitidis* and *N. gonorrhoeae* antigens. *N. meningitidis* carriers were determined by standard microbiological test. In addition we reviewed epidemiologic data for *N. meningitidis* and *N. gonorrhoeae* infections in Cuba.

Results: Epidemiological data show the influence of MBV over gonorrhea incidence suggesting to be dependent of sexual arrival age of vaccinees; but not over syphilis. Laboratorial data permit the detection of 70 and 22 non-carriers and carriers of *N. meningitidis*, respectively. Serum anti MBV antigens (PL) responses were boosted by a third dose and were independent of carriage stages; but saliva anti PL IgA responses were only present and were significant induced in carriers subjects. Carriers boosted with a third dose of MBV induced similar anti-gonococcal and -PL saliva IgA and serum IgG responses; meanwhile, serum anti-gonococcal IgG was significant lower. In saliva, at least two gonococcal antigens, was identified by western blot. Lastly, gonococcal specific mucosal IgA antibody responses, in addition to the serum IgG antibodies, might contributed to the reduction of the incidence of *N. gonorrhoeae*. We hypothesize that this might have contributed to the observed reductions of the incidence of *N. gonorrhoeae*.

Conclusion: These results suggest a mechanism for the influence of a Proteoliposome-based meningococcal BC vaccine on gonococcal incidence.

Introduction

There are over 30 different sexual transmitted infections (STI). The World Health Organization estimates one million infections occur every day worldwide [1]. Out of 357 million STI contracted annually, *Neisseria gonorrhoeae* accounts for 78 million cases annually among people aged 15-49 years[2].

Neisseria is a large genus of bacteria that colonize the human mucosal surfaces. Only two of 11 human species are pathogens: *N. meningitidis* and *N. gonorrhoeae* which causes bacteraemia, septicaemia and meningoencephalitis, and gonorrhea, respectively.

Gonorrhea may be asymptomatic, a localized infection or a disease associated with multiple complications. Women often have asymptomatic infections but symptomatic infections can result in purulent vaginal discharge, dysuria, intermenstrual bleeding, and menorrhagia. Complications in woman are common and include pelvic inflammatory disease with subsequent increased risk of ectopic pregnancy, low fertility or sterility. In men, the hallmark symptoms include urethral mucopurulent discharge and dysuria. Infection can predispose to HIV infection[3,4].

Gonorrhea has been treated successfully by use of antimicrobials for the past 70 to 80 years. However, internationally, there is now a high prevalence of *N. gonorrhoeae* strains with resistance to most antimicrobials previously and currently widely available for treatment (e.g., sulphonamides, penicillins, earlier cephalosporins, tetracyclines, macrolides, and fluoroquinolones). The recent occurrence of treatment failures with extended-spectrum cephalosporins (ESCs) cefixime, and ceftriaxone and the emergence of gonococcal strains exhibiting high-level clinical resistance to all ESCs, combined with resistance to nearly all

other available therapeutic antimicrobial, have caused great concern[5]. No vaccine is currently licensed to prevent gonorrhea. However, there are recently reports of some vaccines being active against *N. gonorrhoeae*[6,7]. However, there is epidemiological evidence that vaccinations against the related organism *N. meningitidis* may provide some protection against gonorrhoea. In addition, some results in mice are supportive of efficacy[8].

There are several vaccines against *N. meningitidis*. These include the older plain polysaccharide vaccines, conjugate polysaccharide vaccines and most recently protein based vaccines against serogroup B meningococcus. The latter include the outer membrane proteins vaccine absorbed onto aluminium hydroxide against serogroup B meningococcus. One vaccine, that was developed in Cuba (VA-MENGOC-BC™) which also contains C polysaccharide from *N. meningitidis* serogroup C, was licenced in 1989 after the successful efficacy trial and included in National Immunization Program schedule in Cuba in 1991. There are also two other licensed meningococcal B protein based vaccines (Trumenba and Bexsero, the latter also including an outer membrane vesicle component (OMV))[9].

With regard to the Cuban vaccine, it is known that that VA-MENGOC-BC™ immunization by intramuscular route (i.m) induces a preferential Th (helper) 1 pattern of immune response characterized by induction of IgG and IgG1 with opsonophagocytic and some bactericidal activity but not a mucosal specific IgA response[10]. The Th2 pattern normally induced by $Al(OH)_3$ containing vaccines is changed to a preferential Th1 immune response when given with OMV. Cross-priming and cytotoxic T lymphocyte (CTL) responses are induced in mice[11,12].

Of note, the existence of cross-reactive proteins between *N. meningitidis* and *N. gonorrhoeae* has been reported[13,14]. Herein, we investigated the potential for cross-protection against gonorrhoea by VA-MENGOC-BC™.

VA-MENGOC-BC™ is a complex proteoliposome (PL, OMV) based vaccine which has the potential to induce a cross-reactive response against *N. gonorrhoeae*. This was initially postulated by our group in humans[15].

N. gonorrhoeae is an obligate human pathogen which has hampered vaccine development. Nevertheless, a chimpanzee model has been used[11,16,17,18] and mice can be made partially susceptible to infection by 17-β-estradiol treatment[19].

One of the shortcomings of a parenteral immunization approach for gonorrhoea prevention is its relatively poor ability to induce genital-tract-specific IgA[20,21]. IgA is considered the antibody most important in protecting the genital tract from infection, as its presence is correlated with a protective role against *Chlamydia* and HIV[22,23], although IgG also has some role in protecting the mucosa. Of note is that intranasal immunization has been promising in terms of eliciting genital-tract antigen-specific IgA and IgG in mice[24,25], primates[26], and humans[27,28]. Given the increasing burden of gonococcal infections and the increasing proportion of these infections with resistant organisms, the possibility of gonococcal protection by meningococcal vaccination needs to be more explored.

Materials and Method

The present study has two components: an ecological study and laboratory-based one.

Epidemiological data. *N. meningitidis* and *N. gonorrhoeae* incidence data from the statistic report of the Public Health Ministry[29] of Cuba were reviewed from all vaccinated population

against *N. meningitidis* between 1970 and 2018, as well as data from the Finlay Institute related to the vaccination program for VA-MENGOC-BC™ in Cuba[30].

LABORATORY-BASED STUDY

Preparation of membrane extract of *N. gonorrhoeae*. The *N. gonorrhoea* ATCC 19424 strain was culture in chocolate agar plates at 3-10% CO_2, and 35-36°C with high humidity. They were then transferred to 3 mL liquid media for 1 h and afterward, transferred to 500 mL Erlenmeyer flask which was placed in a shaker agitator overnight at 35-36°C. They were then centrifuged at 3000 rpm and the pellet extracted in 5 mL of lithium chloride buffer (200 mM LiCL, 100 mM $C_2H_3O_2Li$, pH 6.0) over glass spheres for 2 h at 50ºC under shaker. Specimens were then centrifuged at 13000 rpm for 20 min and then the supernatant centrifuged at 40000 rpm for another 2 h. The pellet containing the outer membrane proteins was then resuspended in distilled water with 0.02% sodium azide and maintained at 4ºC until used.

Preparation of Proteoliposome (PL). PLs from outer membrane vesicles detergent-extracted from *N. meningitidis* serogroup B were prepared and supplied as ethanol precipitate by the vaccine production unit of Finlay Institute, Havana, Cuba, as for *N. meningitidis* serogroup B vaccine as referred by J del Campo *et al*[31].

Samples. Ninety-two (45 male and 47 female) young adults of 18 ± 2 years old students of an high school that have been previously vaccinated with VA-MENGOC-BC™ when they were 3 and 6 months of age by intramuscular route 6-weeks apart as established in the National Immunization Program at that time participated in this study.

Immunization. A third (booster) dose of VA-MENGOC-BC™ was administered by intramuscular route at the beginning of this study. Parotid saliva and sera were obtained at 9 and 21 days after the booster dose, respectively. The protocols were approved by Finlay ethical committee and each volunteers received information and signed the informed document. *N. meningitidis* carrier stages were determined at the beginning of the study by collecting pharyngeal samples as described by Sotolongo[32]. Briefly, swabs from the posterior oropharyngeal area with sterile applicators and immediately applied to Mueller Hinton (Merck) plates, supplemented with 5% of foetal bovine serum (Hyclone) and vancomycin, nystatin y colistine. The plates were incubated at 37ºC for 24-48 hours at 5% of CO_2. *Neisseria* identification was performed as referred by Sotolongo and API NH (bioMérieux) and API NH (bioMérieux)[33.]

Analysis of antibody responses by ELISA. Maxisorp 96-well plates (Nunc) were coated with 100 µL of PL form *N. meningitidis* (20 µg/mL) or membrane extracted proteins from *N. gonorrhoeae* (5 µg/mL) in 0.05 M carbonate buffer, pH 9.6 for 4 h at room temperature, followed by overnight incubation at 4ºC. The plates were blocked with 2% BSA in PBS for 30 min at 37ºC. Sera and saliva were diluted 1:100 and 1:2, respectively and incubated for 1 h at 37ºC. After washing with 0.05% Tween-20, plates were incubated 1 h at 37ºC with goat anti-human immunoglobulin (Ig) G, or -human IgA (Southern Biotechnology Associates, Inc., Birmingham, AL) coupled to horseradish peroxidase in 1% BSA-PBS buffer. The plates were then washed with 0.05% Tween-20 and developed using 100 µL of 1 mg/mL *o*-phenylenediamine dihydrochloride (Sigma) in 0.1 M citrate buffer (pH 4.5) in the presence of 0.04% H_2O_2. The reaction was stopped with 0.1 M H_2SO_4. Negative and positive controls for serum and saliva were used in each plaque. Each plate was accepted when: the average of the

serum positive control replicates ranged from 0.4-1.0 and negative control ranged from 0.01 to 0.1 and in saliva the positive control ranged between 0.2-0.6 and the negative between 0.05 and 0.1. If the results of the controls on each plate were not among those values the plate would be rejected and the samples would be repeated. The absorbance was read at 492 nm and expressed as optical density (OD).

Analysis of human antibody response by Western blot. Bacterium extracts from *N. meningitidis* and *N. gonorrhoeae* were separated in polyacrylamide gel and then transferred to nitrocellulose. This was run at 180 V for 1 h. Nitrocellulose was blocked overnight at 4ºC in with PBS and BSA 1%. Saliva and sera samples were diluted as for ELISA in PBS 0.1% Tween 20 and 1% BSA were incubated for 1 h at 37ºC. After washing peroxidase conjugated anti human IgA (SIGMA) or IgG (SIGMA) was added and incubated 1 h at 37ºC. After washing the blot was developed with dimethylformamide and the reaction was stopped with water.

Statistical analysis. Graphics processing and analysis of results was performed by GraphPad Prism version 5.00 (GraphPad Software, USA). Results were analyzed by Two way ANOVA, followed by Bonferroni post-test. Statistical significance was considered when $p<0.001$.

Results

EPIDEMIOLOGIC DATA

Neisseria gonorrhoeae incidence decreased following meningococcal BC vaccine interventions performed in Cuba

The incidence of *N. gonorrhoeae* between 1970 to 2018 increased at the same time as that for *N. meningitidis* in the 80's with two peaks in 1989 with 40,129 cases and in 1995 with 45,000

cases. Between 1982 to 1999 the incidence was over 20,000 cases *per* year (Fig. 1A). After this period, vaccination was begun with VA-MENGOC-BC™ to protect against B and C serogroups. As gonorrhea is a sexual transmitted disease we were focus in the sexual aged (≥12 years olds). Then, an immediate (vaccination of sexual age subjects), more or less immediate (vaccination of younger than 12 that arrive kinetically to 12 years old) and mediate (vaccination in < 6 years old) influence of OMV. Of note, there was a parallel immediate reduction of 50% of the cases (~20,000 patients) of gonorrhea after the efficacy trial followed of the campaign of 1988-90 where 803,402 (15-19 years old, but the other where more than 12 years old) in the of sexual active population was vaccinated (Fig. 1A). From 1993-1995 the gonorrhea incidence increased again. This likely reflects the aging of the unvaccinated young children into the sexually active population. Recall that we would not expect an influence of meningococcal B vaccination in the population less than 14 years of age (1,050,483) and the population >19 years old which was not vaccinated. The long term (mediate) influence are beginning to reflect cases vaccinated with ages less than 6 years olds during the campaign of 1988-1990, which at 1996 and afterward begin a sexually active life. The same mediate influence occurred for those vaccinated into the routine National Immunization Program with real total vaccine coverage that could influence at least 12 years later (Fig. 1A). A decline in both gonorrhoea and syphilis incidences was observed between 2000 to 2010, but after that date, the rate of syphilis continued to increase but gonorrhea decreased (Fig. 1B). This is compatible with a possible impact of against gonorrhea of the infant VA-MENGOC-BC™ program as these children then became sexually active. Overall, these results support our previous reports of the influence of a Proteoliposome-based meningococcal BC vaccine on gonococcal disease risk[12] .

LABORATORY-STUDY RESULTS

Neisseria meningitidis carrier stages influence the systemic and mucosal cross-recognition between *N. meningitidis* and *N. gonorrhoeae* antigens

VA-MENGOC-BC™ vaccinated subjects induce serum anti *N. meningitidis* PL IgG but minimal specific IgA. All the 22 subjects that have been carriers of *N. meningitidis* induce significant higher levels of mucosal specific IgA after a third boost with VA-MENGOC-BC™ vaccination; but the serum specific IgG response of 70 non-carriers were similar than carriers samples (Fig. 2A). All 22 carriers samples recognize *N. meningitidis* and *N. gonorrhoeae* antigens at serum IgG and saliva IgA levels. Note that while the specific IgA response were lower than serum IgG response they were positive at the same level against PL and gonococcal antigens. As expected the serum IgG crosreactive response was higher against *N. meningitidis* than *N. gonorrhoeae* antigens (Fig. 2B). To identified the possible protein target by specific saliva IgA, a western blot was performed. Saliva IgA recognized several antigens as IgG did in particular the Class 4 (RmpM) and p70 proteins as a sample representation (Fig. 2C). Overall, *N. meningitidis* serogroup carrier stages influence the quality of the immune response induce by parenteral VA-MENGOC-BC™ vaccine adding to anti PL and anti *N. gonorrhoeae* IgG the specific anti IgA mucosal responses.

Discussion

The development of anti-meningococcal OMV vaccine allowed control of meningococcal epidemic in Cuba. Besides having a 95% reduction of meningococcal morbidity and mortality, there was also an apparent impact on the incidence of the related species *N. gonorrhoeae.*

One interesting question is why there was an epidemic of both meningococcal and gonococcal diseases at the same time? A simplistic answer is that both are diseases caused by human restricted bacteria of the same genus and probably the immune response surveillance that control both diseases was affected at the same time. If one believes this, then it is easy to answer a second question - why did both decline with only one vaccine? The decline of gonorrhea cases in the entire country (gonorrhea is an obligatory declaration disease in Cuba) might be an indirect proof of cross-reactive immune response[34]. Our use of an A plus C plain polysaccharide vaccine eliminated the circulation of the C serogroup and probably caused expansion of the B serogroup which then predominated during the rest of the Cuban outbreak. The complex composition of the PL[35,36,37] as protective antigenic core of VA-MENGOC-BC™ and its adjuvant properties[10] are likely involved in the induction of cross-protection as these proteins have a structural homology with *N. gonorrhoeae* and have been described as capable of producing a cross-reactive response[38]. Another possibility is based on the structural homology that exists between commensal species of the same genus such as *N. lactamica* since it is closely related to both *N. meningitidis* and *N. gonorrhoeae* and it frequently colonizes the upper respiratory tract[39]. In addition, genetic analyses of both pathogenic and non-pathogenic *Neisseria* show they are closely related, with evolutionary studies suggesting frequent exchange of genetic material including virulence genes[40]. Support for this cross-reactivity hypothesis is also provided by data from New Zealand where a decline of gonococcal incidence was also observed after use of a related PL (OMV) vaccine MeNZB™[41].

The third question is why we observed two peaks in gonorrhea-incidence behavior in Cuba? The possibility of an immediate and parallel effect could be consequence of the vaccination

of 65% (15 to 19 years old) plus 28% (≥14 years old). This decrease could be potentiating by the later less immediate impact of the vaccination of 75% (6 to 14 years old). On the other hand, the 90's correspond to the 'special period' in Cuba with serious limitations in several areas including the health system and an increased in the gonorrhea incidence was observed which was similar to syphilis incidence, another sexual transmitted disease. One additional possible explanation is the increased attention and registration of infection transmitted disease due to the interest of our ministry of health to pay more attention to Human Immunodeficiency Virus (HIV) since 1993. The total accessibility of condom was since 2003 as one effective measure to block the HIV transmission. The second decrease could be due to the influence of the arrival of the cohort of meningococcal vaccinated 3 months to 6 years olds to a sexually active age. A continuous decline in gonorrhea incidence was observed since 2000; but after that 2010 syphilis continues to increase but not gonorrhea. This is compatible with a possible impact of against gonorrhea of the infant VA-MENGOC-BC™ program as these children became sexually active. Overall, these results support our previous reports[14] of the influence of a PL-based meningococcal BC vaccine over gonococcal incidence.

The second decrease could be due to the influence of the arrival of the cohort of 75.3% of meningococcal vaccinated 3 months to 6 years olds to sexually active age. This would imply that long-lasting vaccine induced at cellular levels in babies, children, and pre-teenagers[42,43] and the new mechanisms (a Th1 response with opsonophagocytic antibodies, in addition to the bactericidal dogma) of protection postulated[8] means that this vaccine, until now, has not required a third dose to effectively control meningococcal disease in Cuban population.

Changes in serotypes and subtypes in post-vaccination patients and carriers can be interpreted as an expression of broad-spectrum vaccine-induced immune response, as epidemic serotypes

and subtypes were eliminated or decreased and non-typable and non-sub-typable serotypes and subtypes increased in patients and carriers. Serotypes and subtypes different from the vaccine strain did neither increase in patients nor carriers[44,45].

Carriage could be presented in human as acute, chronic or occurring in cycles normally in more than 10% of the population increasing during outbreak. We postulate that natural carriage in totally immunized population could induce mucosal immune response influencing gonorrhea incidence at sexual initiation. Our demonstration that *Neisseria* carriage induces significantly more mucosal anti PL IgA and both IgG and IgA that are cross-reactive with several gonococcal proteins supports this hypothesis of mucosal cross-protection. In addition, we noted that the anti-meningococcal salivary IgA responses increase with age and/or meningococcal carriages[46].

Changes in serotypes and subtypes in post-vaccination patients and carriers can be interpreted as an expression of broad-spectrum vaccine-induced immune response, as epidemic serotypes and subtypes were eliminated or decreased and non-typable and non-sub-typable serotypes and subtypes increased in patients and carriers. Serotypes and subtypes different from the vaccine strain did neither increase in patients nor carriers. The vaccine was effective against homologous strains and also against heterologous strains. Frequency and diversity of hypervirulent clonal complexes (ST-32 and ST-41/44) in patients and carriers decreased after vaccination and were replaced by the ST-53 complex, which represents a positive change[46,47,48].

We already demonstrated that neither VA-MENGOC-BC™ nor AFPL1 or AFCo1 by i.m route induce mucosal specific IgA in mice[8]. Similar results were demonstrated using the Norwegian MenBVac or MeNZB where no change or very modest increases in salivary

antibody responses shortly after vaccination[49,50]. There are some studies underway evaluating the use lipo-oligosaccharides to protect against gonorrhea infection[51,52] recognizing that polysaccharides might induce cross-reactive IgA[53,54]. The development of single time vaccinations strategy that combines parenteral and mucosal routes has also been proposed[55,56]. This might provide the opportunity to increase vaccine coverage and mucosal immune response which might result in increased mucosal immune response against gonorrhea infection.

Lastly, while the mechanism of protection against gonococcal infection is not fully understood, the induction of functional antibody responses such as complement mediated bacterial killing, inhibition of binding to reproductive tract epithelial cells and stimulation of opsonophagocytosis are generally considered to be likely contributors[43].

In summary, IgA mucosal response in addition to the Th1 driving IgG response against gonococcal infection is important and carriers state influences the mucosal the mucosal response induce by VA-MENGOC-BC™. Further research is necessary to define the optimal vaccination strategy against *Neisseria gonorrhoeae.*

Conflicts of Interest

The authors have no conflicting interests.

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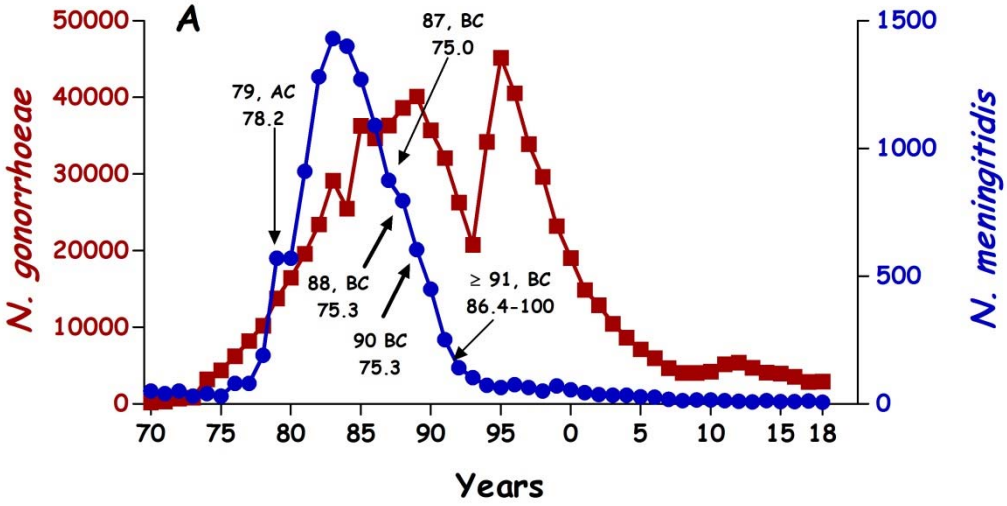
Fig. 1. Incidence of pathogenic *Neisseria* species and vaccination impact in Cuba from 1970 to 2018. A, Epidemic outbreak in Cuba in the 80's with the main years of specific actions and the *N. gonorrhoeae* incidence. In 1979 the plain AC polysaccharides Sanofi vaccine with coverage of 78.2% was applied. In 1987 the anti-meningococcal BC vaccine efficacy trial followed by vaccination campaigns at 1988 and 1990 with coverages of 75.0 and 75.3%, respectively was applied. The vaccine was introduced in the national vaccination program since 1991 with converges of 86.4% in 1991 to 100% in most of the following years. Decline of *N. gonorrhoeae* incidence by the effect meningococcal vaccine over gonorrhea might be: immediate (parallel decline immunization of high % of sexual active population using BC vaccine) of the efficacy and campaign (65.5 plus 28% of vaccinees) trials; the more or less immediate (action over 39% of campaign vaccination groups with age between 6 to 14 years old below or in sexual active population) and the mediate influence (action over 33% of less than 6 years old or those vaccinates during infancies since 1991) represented in the below table. Of note that AC vaccination did not influence at any time over gonorrhoea incidence. B, Comparison of Gonorrhea and Syphilis incidences since 1987 to 2019. y, year; m, month; AC, vaccine against plain *N. meningitidis* serogroup A and C; BC, anti-meningococcal BC vaccine (VA-MENGOC-BC™); NIP, national immunization program of Cuba.



Year		79	87	88-90	Since 91
Action		AC	BC	BC campaign	NIP
Coverage %		78.2	75.0	75.3	84-100
Vaccinate		3 245 046	754 014	2 692 446	All new born
Age		< 20 y	< 19 y	3 m-19 y	3-5.5 m
Possible effect over Gonorrhea	Immediate	No	49 387.92 (65.5%) 15-19 y	754 015 (28%) > 13 y	No
	± Immediate		No	1 050 483, 39%, 6-14 y	No
	Mediate		No	887 948, 33%, < 6 y	All

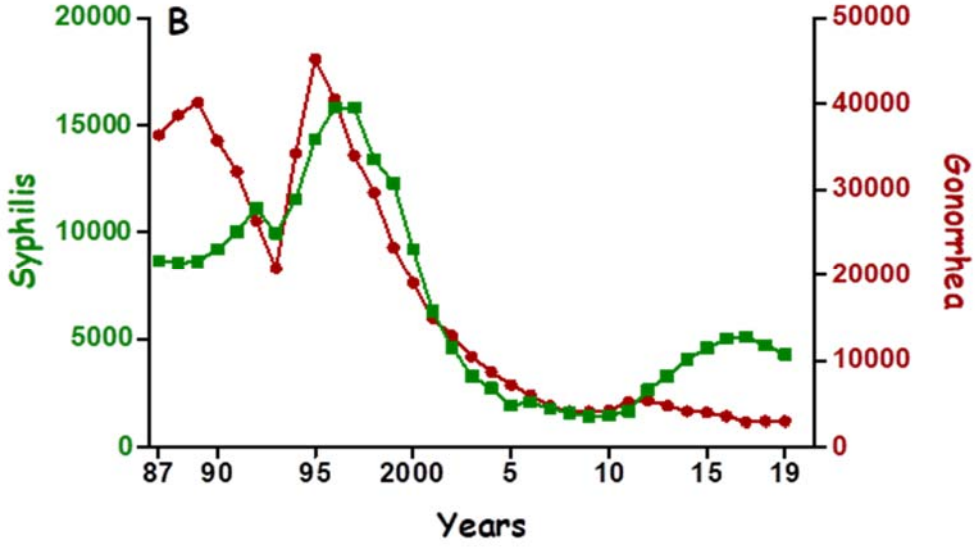
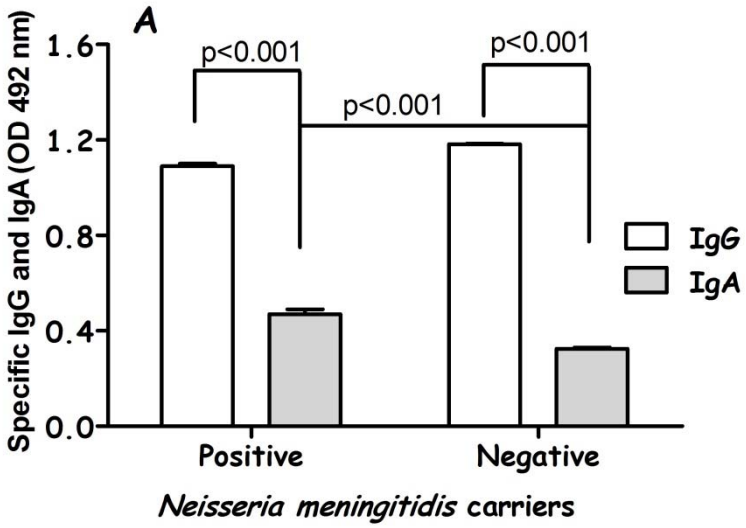
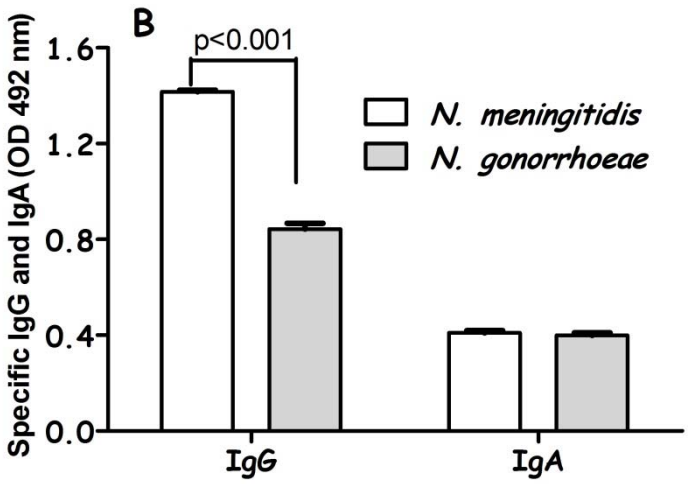

B
20000
15000
10000
5000
0
Syphilis
50000
40000
30000
20000
10000
0
Gonorrhea
87
90
95
2000
5
10
15
19
Years

Fig. 2. Significant increase of specific IgA in human *Neisseria meningitidis* carriers after an additional dose of VA-MENGOC-BC™. A, anti Proteoliposome (PL, vaccine antigens) serum IgG and saliva IgA in human positive (n = 22) or negative (n = 70) ***Neisseria*** carriers samples by ELISA. B, serum and saliva samples from carriers recognize both, *N. meningitidis* serogroup B and *N. gonorrhoeae* antigens by ELISA. C, representative samples of serum and saliva from carriers recognize both, *N. meningitidis* serogroup B and *N. gonorrhoeae* antigens by Western Blot. NmB, *N. meningitidis* B; Ng, *N. gonorrhoeae*. Statistical analyses were done by 2 way ANOVA followed by Bonferroni post-test using Graph Pad Prism 5 software.





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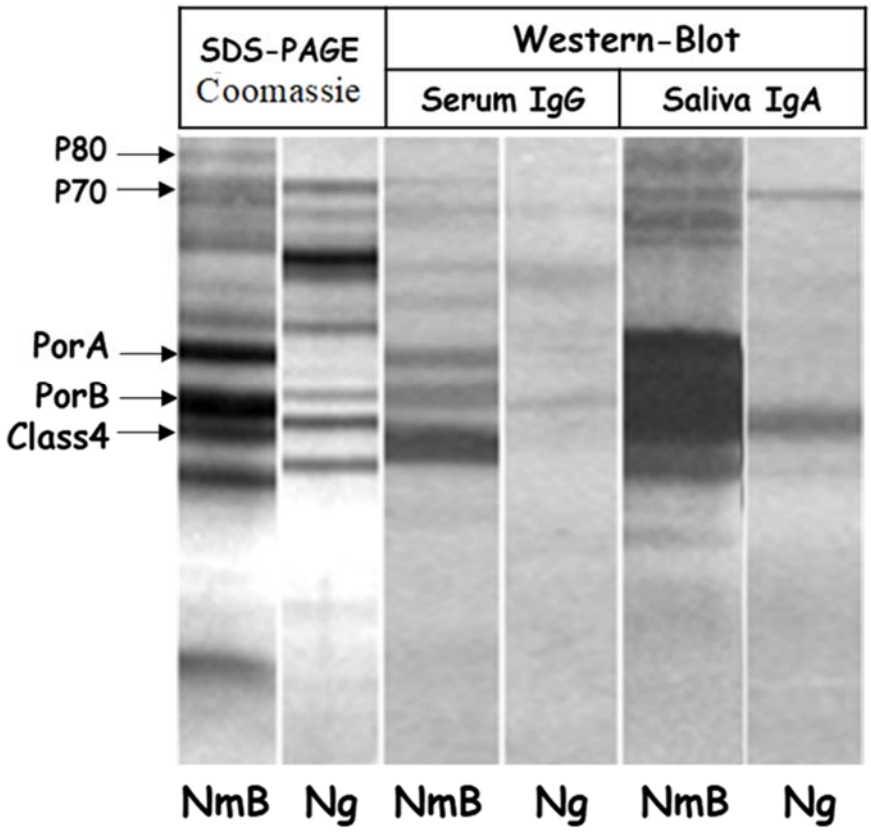
SDS-PAGE
Coomassie
Western-Blot
Serum IgG
Saliva IgA
P80
P70
PorA
PorB
Class4
NmB
Ng
NmB
Ng
NmB
Ng