MOLECULAR AND SEROLOGICAL DIVERSITY OF *NEISSERIA MENINGITIDIS*

CARRIER STRAINS ISOLATED FROM ITALIAN STUDENTS AGED 14-22 YEARS

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Neisseria meningitidis is an obligate human commensal which commonly colonizes the oropharyngeal mucosa. Carriage is age-dependent and very common in young adults. The relationships between carriage and invasive disease are not completely understood. In this work, we performed a longitudinal carrier study in adolescents and young adults (173 subjects). Overall, 32 subjects (18.5%) resulted positive to meningococcal carriage at least at one visit (average monthly carriage rate 12.1%). Only five subjects were positive at all four visits. All meningococcal isolates were characterized by molecular and serological techniques. Multilocus sequence typing, PorA typing and sequencing of the 4CMenB vaccine antigens were used to assess strain diversity. The majority of positive subjects were colonized by Capsule-null (34.4%) and by capsular group B strains (28.1%), accounting for 23.5% and 29.4% of the total isolates, respectively. Fhbp and nhba genes were present in all isolates, while nadA in 5% of the isolates. The genetic variability of the 4CMenB vaccine antigens in this collection was relatively high if compared with other disease-causing strain panels. Indications about the persistence of the carriage state were limited to the time span of the study. All strains isolated from the same subject were identical, or cumulated minor changes overtime. The expression levels and antigenicity of the 4CMenB vaccine antigens in each strain were analyzed by the meningococcal antigen typing system (MATS), and it revealed that expression can change overtime in the same individual. Future analysis of antigen variability and expression in carrier strains after the MenB vaccine introduction will allow to define its impact on naso/oropharyngeal carriage.
INTRODUCTION

*Neisseria meningitidis* has its own unique survival niche in humans (1). The bacterium has developed sophisticated mechanisms to evade the human immune system (2) and, although it can cause serious invasive diseases, it can be considered a normal temporary commensal of the upper respiratory tract of healthy carriers, constituting the reservoir of the microorganism (3,4). The worldwide epidemiology of invasive meningococcal disease (IMD) varies by region and over time. This disease burden mainly affects children under 5 years of age with peaks of incidence in infants under 1 year of age and adolescents (5,6). The relationships between asymptomatic carriage and the development of invasive disease are not still completely understood. Carriage rates are low in infants and school-age children, while increase during adolescence and early adulthood (7,8). The carrier status can be regarded as a natural booster that contributes to the spontaneous acquisition of herd immunity (9). Little knowledge on duration of meningococcal carriage is available, as few studies have been carried out following subjects with repeated throat samples over time (10,11).

Studies performed in some European countries by multilocus sequence typing (MLST) have demonstrated that a limited number of hypervirulent genetic lineages are over-represented in invasive isolates worldwide (12,13). Despite extensive genetic exchange among meningococci, the hypervirulent lineages are stable over time and associated with specific antigenic repertoires. In contrast, meningococci isolated from healthy individuals present extensive genetic diversity (14,15,16). The capsule is the major virulence factor described for *Neisseria meningitidis*. Indeed, carriage meningococcal isolates are often non-capsulated (i.e. “non groupable”) (17,18). This is due to either genetic down regulation of the capsule expression (phase variation) or to the absence of the genes involved in capsule expression. These latter strains are referred to as capsule null locus (cnl) meningococci (19). On the contrary, invasive meningococcal disease is, with rare exceptions, caused by encapsulated strains. Twelve capsular groups have been identified, five of which (A, B,
C, Y and W) are responsible for the majority of worldwide invasive disease (20). Recently, capsular
group X meningococci have also revealed an epidemic potential (21).

Vaccination with polysaccharide conjugate vaccines is highly effective in preventing invasive
disease caused by capsular groups A-C-W-Y (MenA, MenC, MenW and MenY). In Italy, conjugate
vaccines against MenC were introduced in 2005 (http://www.salute.gov.it/imgs/C_17_pubblicazioni_543_allegato.pdf.). The vaccination is usually
offered free of charge to all infants aged 12-13 months. At the moment, two tetravalent-conjugate
vaccines anti-MenACWY (http://www.ema.europa.eu) are available and have been recommended
to adolescents in some Italian Regions from 2012. In contrast, as the development of group B
capsular polysaccharide-based vaccines has not been successful (22), meningococcus B (MenB)
remains one of the major causes of invasive disease in the US and Europe. Recently, 4CMenB
(brand name Bexsero®), a multicomponent recombinant protein vaccine targeting MenB strains has
been licensed in Europe, Australia and Canada (23,24,25,26, http://www.hc-sc.gc.ca,
http://www.tga.gov.au). 4CMenB includes outer membrane vesicles (OMV) from the New Zealand
epidemic isolate NZ98/254 (27), and three major protein antigens: factor H-binding protein main
variant 1 sub-variant 1 (fHbp-1.1) (28), Neisserial Heparin-Binding Antigen sub-variant or peptide
2 (NHBA-2) (29) and Neisseria adhesin A variant 3 (NadA-3) (30).

Serum bactericidal assay (SBA) is the only accepted correlate of protection to estimate the
effectiveness of a meningococcal vaccine against meningococcal disease (5,6). Assessment of the
potential coverage of a multicomponent MenB vaccine would require performing SBA against
many isolates due to both sequence and expression variability of the vaccine antigens. For this
reason, a rapid and reproducible meningococcal antigen typing system (MATS), was developed,
which allowed to estimate vaccine coverage by determining for each strain the expression level and
cross-reactivity to the 4CMenB vaccine variants of fHbp, NHBA and NadA (31).
This work is a longitudinal carriage study on Italian adolescents and young adults aged 14-22 years aiming at the characterization of the carriage of *Neisseria meningitidis*, five years after the introduction of the MenC-conjugate vaccine. In order to assess the permanence of the same isolate at different visits of the same individuals, meningococcal carrier isolates were analysed for capsular group, sequence type (ST) and, presence and sequence variability of the genes coding for PorA, fHbp, NHBA and NadA. All strains were also analysed by MATS in order to monitor the 4CMenB antigen expression level of the same isolate over time.

**MATERIALS AND METHODS**

The Ethics Committee of San Martino Hospital (Genoa, Italy) approved the study protocol (n°117/09) (date 29/10/2009), which conformed to the ethical guidelines of the 1975 Declaration of Helsinki and good clinical practice.

The study was carried out from January 2011 to October 2012 and the collection of the samples was performed from February – May 2011.

**Study population and recruitment.** Subjects aged 14-22 years attending two secondary technical schools in Genoa were enrolled in the study. The first 200 subjects who accepted to participate to the study and met the inclusion criteria (consecutive set of subjects) were enrolled. Individuals who had taken antibiotics within one week before the collection of pharyngeal swabs were excluded.

A clear complete written informed consent form was signed by each participant; consent was also obtained from parents/guardians if the subject was aged less than 18 years. The aims and procedures of the study were explained to each volunteer by medical staff. Before the first sample collection, the volunteers answered a structured questionnaire (Supplementary Table S1) that included information on risk factors for *Neisseria meningitidis* carriage and meningococcal C vaccination. Meningococcal C vaccination status was also checked by referring to the vaccination registers of the Local Health Unit (LHU). Previous vaccination with MenC vaccine was not
considered as an exclusion criterion. A trained physician took a posterior pharyngeal swab (by using Eswab 480CE, COPAN, Italy) from behind the uvula, from the posterior wall of the oropharynx and from both tonsils. As one of the aims of the study was to evaluate the duration of carrier status, three further swabs were taken at intervals of 1 month (total: 4 visits February – May 2011).

**Isolation of Neisseria meningitidis strains.** Swabs were immediately plated on site on selective medium (Martin Lewis Modified Agar BD – Agar chocolate II + amphotericin B + vancomycin and 1% IsoVitaleX). Both the plates and the swabs were transported to the microbiology laboratory of the Department of Health Sciences (University of Genoa) in insulated containers to ensure a constant temperature of about 20-25°C. The time from sample collection to arrival at the microbiology laboratory never exceeded 2 hours.

Plates were then incubated at 35-37°C for 36/48 hours in a 5-10% CO₂ atmosphere. Subsequently, morphological evaluation of bacterial colonies was performed. All colonies recognized as possible colonies of *Neisseria meningitidis* were plated on Chocolate Agar (GC II Agar + IsoVitaleX - BD), and were then incubated at 35-37°C for 24 hours in a 5-10% CO₂ atmosphere. If necessary, colony sowing was repeated in order to obtain pure colonies at the end of the procedure.

**Genomic DNA extraction.** DNA was extracted from isolates by emulsifying colonies in 500 μl of 0.85% saline to obtain a standardized 0.5 McFarland suspension. The homogenized suspension was extracted by means of a QIAamp DNA mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, after a pre-incubation step of 1 h in 180 μl of pre-lysis buffer (ATL supplemented with 20 μl of proteinase K) at 56°C.

**CrgA and ctrA PCR amplification.** To confirm *Neisseria meningitidis* species identity, all isolates were assayed by PCR amplification for the presence of the conserved genes *crgA* and *ctrA* (32). All confirmed isolated were frozen at -80°C in Muller Hilton broth + 10-20% of glycerol and sent to the Research Centre of Novartis Vaccines and Diagnostics for typing at controlled temperature.
Capsular and PorA typing. Serogroups were assessed by standard bacterial latex agglutination test (Difco and Oxoid) according to manufacturer’s instructions. In addition, molecular detection of capsular groups A, B, C, W, X, Y, Z and E was accomplished by PCR (33,34). Detection of the capsule null locus by PCR amplification and sequencing was performed according to methods described by Claus et al. (19). Amplification of the porA gene was performed following the protocol of Molling et al. (35). DNA was sequenced as indicated by Clarke et al. (36) and VR1 and VR2 were assigned according to the Neisseria MLST website (http://pubmlst.org/neisseria/PorA/).

MLST and 16S rRNA gene typing. MLST was performed according to the method described by Maiden et al. (13), and sequence types (STs) were assigned according to information available at the Neisseria MLST website (http://pubmlst.org/neisseria). 16S rRNA gene typing was carried out as previously described (37).

fHbp, nhba and nadA sequence typing. PCR and gene sequencing of fHbp, nhba and nadA were evaluated as previously described (16,28,30,38,39). Alleles and corresponding protein variants were assigned by using the meningococcal typing database (http://pubmlst.org/neisseria).

MATS. MATS consists of the combination of an ELISA assay performed on bacterial lysates (for NHBA, fHbp and NadA) and the sequencing of PorA-VR2 variable region, as described by Donnelly et al. (31). Briefly, bacteria were grown on chocolate agar plates and then resuspended in Mueller-Hinton broth until OD_{600} = 0.4. Following the addition of a detergent, bacterial extracts were serially diluted in ELISA plates coated with an antigen-specific capture antibody. The binding of the antigen was then detected by means of a biotin labeled secondary antibody and a streptavidin–HRP conjugate. The plates were read at 492 nm in an ELISA reader. For each strain the MATS ELISA reactivity of each of the three tested antigens was obtained mathematically comparing the serial dilution curve of each Neisseria meningitidis strain to that obtained using a reference MenB strain (Relative Potency (RP)).
Statistical analysis. Quantitative variables are expressed as means and standard deviations (SD), and qualitative variables as frequencies and percentages with 95% Confidence Intervals (95% CI). Fisher exact test was used to evaluate the association between the single risk condition (active or passive smoking and cohabitation with 4 or more persons) and outcome (to be meningococcal carrier). The level of significance adopted was $p < 0.05$. Gender and nationality were not considered as risk condition.

RESULTS

Carriage rates. A total of 200 students aged 14-22 years were invited to participate to the study, 23 of whom declined (response rate of 88.5% [95% CI: 84.1-93.0]). The reasons for refusal were not further investigated. Overall, 177 subjects were recruited. Four subjects (2 females and 2 males) withdrew their consent before the first sampling. As shown in Table 1, 173 subjects were studied, 66.5% of whom were male (33.5% female).

32 (18.5%) subjects were found to be carriers of *Neisseria meningitidis* on at least one sample. Figure 1 shows the percentages of carriers at different ages. The highest percentage of carriage was observed at 17 and 18 years (25.0% and 25.5%, respectively). However, comparing the 95% CI, the difference between ages was never statistically significant. No statistically significant difference was found considering gender as a variable.

Table 2 reports some characteristics and risk conditions of the studied population. In detail, 59.4% of the carriers were exposed to active or passive smoking while 57.9% of the non-carriers was exposed ($p=0.52$). In addition, 71.9% and 55.2% of carriers and non-carriers, respectively, cohabited with 4 or more persons ($p=0.06$).

A total of 145 pharyngeal swabs were collected at visit 1, 17 of which were positive for *Neisseria meningitidis* (11.7%). After one month (visit 2) 157 pharyngeal swabs were obtained (20 positives, 12.7%). At visit 3, 136 swabs were obtained (15 positives, 11.0%). Finally, at visit 4, 16 positives
(13.0%) from 123 total swabs were found (Figure 2). In total, 68 isolates of *Neisseria meningitidis* were identified. The average carriage rate was 12.1%. Twenty (62.5%) of the 32 carrier subjects were positive more than once, while 12/32 (37.5%) were positive only once. Figure 2 reports all 68 positive swabs, consecutive and/or not consecutive.

**Capsular groups and molecular typing.** Capsular groups were determined both by molecular typing, sequencing of group-specific capsule genes to assess the presence and integrity of the capsule operon, and serologically, by slide agglutination to evaluate the expression of the capsular polysaccharide (Figure 2 and Supplementary Table S2). The isolates which lacked the capsule operon genes were indicated as capsule null (cln), while the isolates, which did not react with any of the serological reagents, were indicated as serologically non-groupable (NG). By molecular procedures we assessed that the majority of NG isolates showed an intact group B capsule operon. 11 of the 32 positive subjects (34.4%) were colonized by cln strains, 9/32 (28.1%) by capsular group B, 18.7%, 6.2%, 6.2%, 3.1%, 3.1% were colonized by MenY, MenE, MenW, MenZ and MenX, respectively. No subject was colonized by MenA and MenC.

Capsule null and capsular group B isolates accounted for 23.5% and 29.4% of the total isolates (n=68), respectively. All cln strains were found devoid of the *ctrA* gene, as described (19).

Overall, 79.4% (54/68) of the isolates fell into 14 already known MLST clonal complexes (cc). Twenty three different sequence types (STs) were identified, 2 of which were new (ST-10178 and ST-10179). Clonal complex 22 (cc22) was the most frequent (11.7%), followed by cc53 (10.3%), cc60 (8.8%) and cc865 (8.8%). The most frequent STs were ST-184 (cc22), ST-5436 (NA), ST-3327 (cc865) and ST-53 (cc53) (Supplementary Table S2). Figure 3A shows the distribution of the *Neisseria meningitidis* isolates by capsular group and clonal complex. The majority of serologically non-groupable (NG, based on slide agglutination) isolates, had intact capsule operons capable of coding for the capsular group B type, as assessed by capsular operon sequencing, and were associated with cc865, cc162, cc35 and cc269. The majority of sero-groupable isolates belonged to
serogroups Y (cc23), W (cc22) and E (cc60). Interestingly, as shown in Figure 3B, the clonal complex associated to more subjects was cc53 (4 subjects), while the 8 cc22 strains identified were W and were associated only to two subjects.

**4CMenB sequence typing and MATS analysis.** To verify the distribution and variation of 4CMenB vaccine antigens, all isolates were analysed for antigen sequence typing and MATS. Figure 4 shows the fHbp, NHBA, PorA-VR2 and NadA genetic characterisation for all isolates. The fHbp gene was present in all isolates. All three fHbp main variants were identified. Variant 2 (fHbp-2) was the most prevalent (73.5%), followed by variant 1 (fHbp-1, 19.1%) and variant 3 (fHbp-3, 7.4%). Fifteen fHbp sub-variants were found, the most frequent was fHbp-2.16 (38.2%), mostly associated with cc22 (ST-184), followed by fHbp-2.102 (10.3%) and 2.119 (10.3%). Two not previously assigned fHbp sub-variants (fHbp-1.932 and fHbp-1.933) were found. fHbp-1.1, which is present in the 4CMenB vaccine and predominant among invasive isolates, was not found in the carrier isolates of this study.

The nhbA gene was found in all isolates. Fifteen different sub-variants or peptides were identified. NHBA peptide 20 (NHBA-20), associated with cc22 (ST-184) and cc162 (ST-162) was the most frequently found (30.9%), followed by NHBA-24 (22.0%, mostly associated with cc865), by NHBA-21 (7.3%) and by NHBA-58 (7.3%). One peptide, NHBA-550, was first described in the present study. NHBA-2, which is present in the 4CMenB vaccine and predominant in invasive isolates, was found in 3 isolates (cc41\44) from the same subject (S118).

The nadA gene was present in only 5 isolates (7.3%), 1 belonging to cc32 and 4, isolated from the same subject not assigned to any cc. NadA variant 3, present in the 4CMenB vaccine, was found in the 4 isolates from the same subject (S121).

As of PorA, we were able to identify 12 different VR1 variants and 20 different VR2 variants, according to the nomenclature available at the web-accessible database (http://pubmlst.org/neisseria/PorA/). The most common were VR1 variant 18-1 (26.5%) and VR2...
variant 3 (19.1%) and 10-1 (16.2%) (Supplementary Table S2). Furthermore, two new PorA-VR2 variants (25-53 and 25-54) were identified. The VR2 variant 4 present in 4CMenB was found only in 2 consecutively isolated cc162 strains from subject S099.

All MenB isolates were analysed for the antigenic distance from the 4CMenB antigens and expression level by MATS. The strains carrying fHbp-1 sub-variants, isolated from five subjects (15.6%), showed detectable expression levels of the antigen. As expected, expression of fHbp-2 or fHbp-3 sub-variants was not detectable by MATS (Supplementary Table S2). All the isolates showed detectable expression levels of NHBA (Supplementary Table S2). The NadA expression was detected by MATS only in the isolates from subject S121, which have been molecularly assessed as nadA(+) (Supplementary Table S2).

In subjects with consecutive positive swabs (S054, S081, S115, S118, S121, S142, S143) fHbp, NHBA and NadA expression levels of isolates, detected by MATS, were comparable (Figure 5). Notably, in the isolates from subject S121 the pattern of NadA expression showed a remarkable higher expression level in the second swab with respect to all the others (Figure 5C).

Isolate similarities. The complete molecular characterisation of the 68 isolates allowed the identification of those that were identical or very similar. In general, when isolated from the same subject, the strains were the same (based on the genetic parameters studied), even though sometimes associated to different levels of capsule expression (Figure 2 and Supplementary Table S2). In detail: the 4 isolates from subject S121 differed in the expression of the capsule and in the MLST allelic profile at the adK gene level (visit 4); the three isolates from subject S041 were identical apart from PorA-VR2; the two isolates from subject S094 differed in PorA-VR2 (Supplementary Table S2). Of note, in the few cases where we evidenced slight differences in isolates from the same subjects at different time periods, the positive swabs were not consecutive. Interestingly, in 6 cases (Figure 6) identical or similar strains were isolated in more than 1 subject.
The subjects carrying identical or very similar strains most frequently attended the same class or school or were friends (Figure 6).

**DISCUSSION**

The understanding of *Neisseria meningitidis* carriage is fundamental to figure out meningococcal pathogenesis (40). A better understanding of the carriage state could provide key information to comprehend the connection between carriage and disease.

For the last 10 years, conjugate vaccines against meningococci of serogroups other than B have been used worldwide (41,42). Recently, a multicomponent protein based vaccine against meningococcal group B (4CMenB) was licensed (23). To monitor vaccine induced changes in meningococcal strains, extensive typing of invasive and carrier isolates will be necessary.

In this study, the overall carriage prevalence (18.5%), with a peak (25.5%) observed in subjects of 18 years of age, was comparable to that observed in previous studies. Indeed, Ronne et al. reported a prevalence of carriage of 20.4% in subjects aged between 16 and 20 years in a Danish school in November 1983, and 19.8% in the following March (43). Moreover, Fraser et al. reported a prevalence of carriage of 25.7% in a cohort of young people aged 15-16 years attending a naval school in April 1972 and 75.8% nine months later (44).

Few studies have analyzed the duration of meningococcal carriage (10,11). The duration of carriage may actually depend on the ability of meningococcal strains to establish a long-term commensal relationship with the host, and on the host’s immune status and genetic properties, as well. To better understand this point all carrier isolates were fully characterized to determine their presence at successive swabs from the same individuals, also their spreading in the community.

Some subjects were carriers for the entire period of our survey, while others were positive at one swab of four. We cannot exclude that negative samples, in particular in subjects with not consecutive positive swabs, might result from low sensitivity of the swabbing method. It is
interesting to note that of 12 carriage episodes at one swab only, 7 were associated to cnl isolates (58.3%). This could suggest that cnl strains might be associated with shorter carriage. However, in some cases, the same subject was not present at all the four visits. For this reason, while prolonged carrier status was associated with encapsulated strains, a complete association of cnl with sporadic carriage cannot be assumed. Indications about the persistence of the carriage state were obtained, limited to the time span of the study.

MacLennan and colleagues conducted a study on the risk factors that may affect carrier status in British teenagers (45). They found that the main risk conditions were frequenting pubs/clubs, intimate kissing and exposure to active and passive smoking. In our study, there was no statistically significant difference between carriers and non-carriers with regard to exposure to active and/or passive smoking. Furthermore, our results showed that the cohabitation with four or more persons could potentially condition the carrier status, even though the association was only indicative and not significant.

Several isolates, although bearing intact capsule operons, were non groupable by serological methods. Interestingly, the majority of serologically non groupable (NG) isolates belonged to capsular group B by sequence analysis. Besides cnl and MenB strains, MenY and MenW were the most frequently found. MenY, which increased in the US and United Kingdom during the past decades, has the potential to become important also in Italy, (www.simi.iss.it/meningite_batterica.htm). None of the MenW isolates found in this study was related to the Hajj outbreak (46). Also, one MenX isolate was identified. To date, only one case of invasive disease caused by this capsular group has been reported in Italy (47, wwwwnc.cdc.gov/eid/article/16/2/09-1553.htm). We did not detect any MenC isolate, although none of the subjects had been vaccinated with the MenC conjugate vaccine. Provided that capsular group C meningococci have a much lower propensity to be carried by healthy individuals (14) this finding may also suggests that the vaccination coverage required to hinder the circulation of MenC strains.
could be limited. In the Region of the study vaccination coverage in infants is currently about 70%, while it is lower in teenagers (20%). This finding is consistent with the results obtained by Germinario et al. (48) and with British and European data (49,50). No MenA strains were isolated. This is in line with the fact that capsular group A meningococci only sporadically caused invasive disease in Europe and are rare in healthy individuals (51).

A few isolates analysed in this study were associated to meningococcal hypervirulent clonal complexes, compatibly with the quite different outline of the carrier population. The most frequently identified clonal complexes were cc53 and cc198, which are typically associated with cnl strains (52). Moreover, the isolates belonging to the hypervirulent complexes were often unencapsulated.

The association between certain serogroups and particular clonal complexes (51) was confirmed. cc41/44, cc269, cc162 and cc213 strains were all associated with capsulated or unencapsulated Men B strains. Instead, cc23 was associated with capsular group Y, and cc22 with MenW isolates. Unexpectedly, we found two cnl isolates belonging to cc32 and cc35, respectively.

We observed that all subjects positive at multiple swabs have been carrying the same strains for the entire period of the survey, or cumulated very little differences over time. In most cases, close contacts carried identical or very similar strains.

With regard to the 4CMenB antigens all three variants of fHbp were present in the strains analysed. fHbp variant 2 clearly prevailed over fHbp-1, as expected from other carriage studies (53,54). fHbp-1 has indeed been described to be predominant in invasive isolates (16,31,55,56,57). Thirteen of the fifteen fHbp sub-variants were also detected in invasive isolates, and only two were new. However, the diversity of fHbp in this strain panel, was relatively high if compared with strain panels composed of disease-causing isolates (58).

Thirteen of the fourteen NHBA peptides identified in this study were already found in invasive isolates, and only peptide was new. The nadA gene was found only in 5 carrier isolates.
(corresponding to two carriage episodes). Previous studies have demonstrated that NadA is underrepresented in carrier isolates and is associated to Neisseria meningitidis pathogenicity (59). In this strain panel, the gene was present in one cc32-cnl strain, and in one MenZ strain.

Due to the non-random association with clonal complexes (16), we found fHbp-1.4, fHbp-3.94 and NHBA-10 associated with cnl isolates belonging to cc198, whereas fHbp-2.102 and NHBA-58 were associated with cnl isolates belonging to cc53, as expected from recent studies (60).

MATS assay was performed in order to evaluate expression levels and antigen cross-reactivity toward 4CMenB components in all isolates. All data from the phenotypic approach (MATS) were consistent with the genotypic (sequencing) analysis. Moreover, MATS revealed the possibility for a strain to express different amounts of NadA protein at different isolations from the same subject, demonstrating that the level of expression can vary over time. However, it is important to highlight that the positive bactericidal threshold (PBT) has been defined only for MenB strains, so far. In the case of unencapsulated strains, the relation between MATS and SBA could be difficult to define because of the intrinsic susceptibility of unencapsulated strains to complement-mediated killing.

Since MenB strains accounted only for 29.4% of the total isolates, any prediction of coverage would have been hardly significant.

In conclusion, in our study two main groups of carrier isolates could be distinguished. On one side, we found isolates genetically unable to express a functional capsule, because missing the capsule operon (60). These isolates are defined cnl and are typically found associated with meningococcal harmless carriage. On the other side, we also found encapsulated isolates and/or isolates capable of expressing the capsule, with the same molecular features of meningococci recovered from patients, that is to say potentially capable to cause the meningococcal disease. Anyway, all carrier isolates in this study showed a higher genetic variability with respect to invasive isolates, as already reported (40).
The purpose of this study was an overview of the overall serological and molecular diversity of carrier strains isolated from individuals sharing defined age group and the same living spaces, to compare with previous analogous studies. The study was based on the characterisation of strain heterogeneity by standard molecular and serological methods, applied to the antigens included in the new vaccine 4CMenB. A future assessment of antigen variability and expression, possibly performed after the vaccine introduction and also in different age groups, will be of great help to monitor the ability of 4CMenB vaccine to impact naso/oropharyngeal carriage.

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REFERENCES


FIGURE LEGENDS

Figure 1 Distribution of the percentage with 95% Confidence Intervals of carriers by age.

Figure 2 Representation of *Neisseria meningitidis* carriage positivity across the four visits for the 32 carrier subjects.

Subjects are ordered based on the number of positive visits and by numerical order. Boxes corresponding to positive visits are coloured. Colour coding is indicated in the legend. Serogroups are reported (weak expression of capsule is indicated by an asterisk). For Non-Groupable (NG) isolates, capsular groups are reported into brackets. Negative isolations are indicated by minus marks, n.a. (not assessed) indicates the absence of the subject at a given visit, (+) indicates PCR positivity of unavailable isolates. The carriage rates are calculated from the number of individual swabs at each visit.

Figure 3 Association of serogroups and MLST clonal complexes identified in this study.

A) Histograms are built taking into account the total number of *Neisseria meningitidis* isolates which are stratified based on the combination of the cc and serogroup determined by PCR. Within bars the different shading corresponds to different capsule expression determined by slide agglutination. B) Histograms showing the correspondence serogroup/cc are built assuming that for each subject identical strains have been isolated at different visits (each of the 32 positive subjects corresponds to one strain).

Figure 4 Genetic characterisation of the 4CMenB vaccine antigens in the 68 *Neisseria meningitidis* strains isolated in this study.

A) fHbp main variants 1, 2 and 3 and sub-variants numeric identifiers. B) Prevalence of NHBA peptide. C) Prevalence of VR2 PorA subtype. D) *nadA* gene presence.

Figure 5 4CMenB antigen expression trends determined by MATS on strains isolated from subjects showing detectable differential expression at different visits.
A) The histogram shows the RP trend for fHbp in subject S115; B) The histogram shows the RP trends for NHBA in subjects S054, S081, S115, S118, S121, S142 and S143; C) The histogram shows the RP trend for NadA in subject S121.

Figure 6. Relationships among subjects carrying strains with identical or very similar features.

Six identical or similar strains were isolated in more than 1 subject. The different subjects carrying identical or very similar strains most frequently attended the same class or school or were friends. Colour coding of the boxes is the same reported in Figure 2. The differences identified through the molecular characterization are highlighted.
Table 1 – Number of subjects (N) within studied population stratified by age and gender.

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<th>Age (years)</th>
<th>Males (N)</th>
<th>Females (N)</th>
<th>Total (N)</th>
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<tr>
<td>Total (N)</td>
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<td>58</td>
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Table 2 – Characteristics and risk conditions of the studied population.

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<tr>
<th>Carrier condition</th>
<th>Sex</th>
<th>Nationality</th>
<th>Active smoking</th>
<th>Exposed to passive smoking</th>
<th>Cohabitant</th>
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<td>F</td>
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<td>Foreign</td>
<td>&lt; 4</td>
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<tr>
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<td>90</td>
<td>51</td>
<td>126</td>
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</table>

* = 7 subjects not responder
° = 8 subjects not responder
# = 7 subjects not responder
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<th>Visit 3</th>
<th>Visit 4</th>
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<td>NG (B)</td>
<td>NG (B)</td>
</tr>
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<td>NG (cni)</td>
<td>NG (cni)</td>
<td>-</td>
<td>NG (cni)</td>
</tr>
<tr>
<td>S078</td>
<td>NG (Y)</td>
<td>Y*</td>
<td>+</td>
<td>NG (Y)</td>
</tr>
<tr>
<td>S132</td>
<td>Y</td>
<td>-</td>
<td>Y</td>
<td>NG (Y)</td>
</tr>
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<td>E</td>
<td>E</td>
<td>n.a.</td>
</tr>
<tr>
<td>S099</td>
<td>B</td>
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<td>-</td>
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<td>NG (cni)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>NG (B)</td>
<td>n.a.</td>
</tr>
<tr>
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<td>-</td>
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<td>+</td>
<td>NG (B)</td>
</tr>
<tr>
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<td>NG (cni)</td>
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</tr>
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<td>-</td>
<td>-</td>
<td>B'</td>
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<tr>
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<td>12.74% (20/157)</td>
<td>11.03% (15/136)</td>
<td>13.00% (16/123)</td>
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Legend:
- Blue: 1 positive swab
- Purple: 3 non-consecutive swabs
- Green: 2 non-consecutive swabs
- Yellow: 3 consecutive swabs
- Orange: 2 consecutive swabs
- Red: 4 consecutive swabs

* Weak positive
cni = capsul null
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<th>Subject</th>
<th>Capsular group (PCR)</th>
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<th>adk</th>
<th>aroE</th>
<th>fumC</th>
<th>gdh</th>
<th>pdhC</th>
<th>pgm</th>
<th>CC</th>
<th>PorA VR1</th>
<th>PorA VR2</th>
<th>fhbp variant</th>
<th>fhbp oxford</th>
<th>NHBA peptide</th>
<th>NadA gene presence</th>
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</tbody>
</table>

*1 different nucleotide in the fumC gene  **1 different aa (1 different nucleotide)  NA= not assigned