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Carriage meningococcal isolates with capsule null locus dominate among high school students in a non-endemic period, Italy, 2012–2013

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ABSTRACT

Meningococcal disease incidence in Italy remains quite low in the overall population except for infants. Within a study on carriage isolates among high school students we aimed to define: i) the prevalence of carriage isolates, ii) the phenotypic and iii) the molecular features of meningococci by Whole Genome Sequencing (WGS).

A total of 1697 pharyngeal samples from undergraduate students (age range 14–19 years) were collected from 2012 to 2013 from six larger cities in Italy. One hundred and twenty culture positive meningococci (7%) were analyzed. Carriage isolates were sent to the National Reference Laboratory for invasive meningococcal disease (IMD) for PCR-based serogroup identification, Multilocus Sequence Typing, PorA and FetA typing. Moreover, factor H binding protein (fHbp), Neisseria Heparin Binding Antigen (NHBA) and Neisserial adhesin A (NadA) were typed. Core genome MLST (cgMLST) was performed on a subsample of 75 carriage isolates.

Capsule null locus (*cnl*) predominated (47%), followed by serogroup B (27%). The antimicrobial susceptibility profile revealed an high prevalence of reduced susceptibility to penicillin G (54%) and a full susceptibility to ceftriaxone, ciprofloxacin and rifampicin. Carriage isolates presented a high genetic diversity: the clonal complexes (cc_s) cc1136, cc198 and cc41/44, were the predominant. An high heterogeneity was also observed for PorA and FetA types. The *fhbp* and *nhba* genes were identified in all the carriage isolates; only 5% of the carriage isolates presented the *nadA* gene. The core genome MLST analysis revealed that the majority of the *cnl* isolates clustered in a distinct group.

The evidence gathered during this study provides the estimate of carriage isolates in high school students in a non-epidemic period in Italy that was lower than expected. Moreover, the highest proportion of carriage isolates were *cnl* and, overall, they were molecular heterogeneous.

1. Introduction

N. meningitidis is often asymptomatically carried at the mucosal surface of the nasopharynx and transmitted through respiratory secretions (Stephens et al., 2007; Soriano-Gabarró et al., 2011). Carriage status plays an important role in the dynamic of transmission of *N. meningitidis* (Soriano-Gabarró et al., 2011; Stefanelli and Rezza, 2016).

Many reports have been described that the microbiological traits of carriage isolates together with the age of the subject play a role for the carriage status (Christensen et al., 2010; Harrison et al., 2015). Most of the studies depicts an high incidence rate in 15 to 24 years old individuals (Christensen et al., 2010). In particular, Rønne et al. (1993) reported a prevalence of carriage from 19.8% to 20.4% in subjects between 16 and 20 years of age; moreover, Fraser et al. (1973) estimated a prevalence of carriage of 25.7% in a cohort of young people, 15 to 16 years of age.

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In Italy, studies on the prevalence of meningococcal carriage isolates have been carried out in the years (Esposito et al., 2013; Gasparini et al., 2014; Germinario et al., 2010; Stroffolini et al., 1990; Tafuri et al., 2012). A study conducted in subjects 14–22 years of age demonstrated a meningococcal carriage peak of 25.5%, in the years 2011 and 2012 (Gasparini et al., 2014).

Few data are available on meningococcal carriage isolates in Europe, in fact, most of them refer to the local meningococcal epidemiology (Soriano-Gabarró et al., 2011).

Overall, the data reported in a number of carriage studies underline the indirect protection mediated by the immunization programs on carriage status as a key element in the dynamic of IMD (Claus et al., 2005; Maiden et al., 2008; Yazdankhah and Caugant, 2004).

In Italy, the quadrivalent meningococcal conjugate vaccination is currently recommended for adolescents aged 12–18 years (http://www.salute.gov.it/imgs/C_17_pubblicazioni_2571_allegato.pdf). Meningococcal B (MenB) and C (MenC) vaccinations are also recommended for primary vaccination (http://www.salute.gov.it/imgs/C_17_pubblicazioni_2571_allegato.pdf).

In 2012–2013, we conducted a carriage study among undergraduate students in six large cities in Italy in a non-epidemic period and prior to widespread availability of MenB vaccines in the country. Furthermore, carriage meningococcal isolates were characterized with the aim to: i) identify the carrier prevalence and the main serogroup of the carriage isolates; ii) determine their susceptibility against 4 antimicrobials; iii) identify, using Whole Genome Sequencing (WGS), or Sanger sequencing, the Sequence Types (ST), the clonal complex (cc), and the antigens comprised in the meningococcal B vaccines.

2. Materials and methods

2.1. Ethical approval

The study protocol was accepted by the Ethics Committee of the Istituto Superiore di Sanità (reference number CE/11/313). A written informed consent form was obtained from parents of participants aged < 18 years old. Participants aged 18 years or above gave their informed consent.

2.2. Study design and population

The collection was carried out from June 2012 to July 2013. A total of 1697 healthy teenagers aged between 14–19 years, attending secondary schools in six different Italian cities, were invited voluntary to participate in this study: Padova (n = 260), Bologna (n = 225), Milano (n = 315), Torino (n = 296), Roma (n = 300) and Napoli (n = 300). Exclusion criteria were signs of respiratory tract infections, asthma, chronic illnesses and/or ongoing antibiotic therapy. Parents of the school children were informed in writing about the purpose and procedure of the study, and were asked to give informed consent for their participation in this study.

2.3. Isolation of N. meningitidis

The cultivation, identification and collection of meningococci, following standard microbiological methods, were carried out in the hospital laboratory at local level. A single posterior pharyngeal swab from behind the uvula was taken from each participant by a nylon flocked swab (eSwab, Copan, Brescia, Italy). The swabs were either plated on site or, using transport medium, plated in the hospital laboratories. Samples were plated on selective Thayer-Martin VCNT agar, containing 3 mg/liter vancomycin, 7.5 mg/liter colistin, 12.5 U/liter nystatin, 5 mg/liter trimethoprim lactate and Vitox supplement) (Becton Dickinson, Sparks, USA). The plates were then incubated at 37 °C for 24 to 48 h in a 5% CO_2 atmosphere. Possible colonies of *N. meningitidis* were identified by conventional methods including Gram-staining, oxidase reaction and by API NH system (BioMérieux, Italy). All *N. meningitidis* isolates were sent to National Reference Laboratory (NRL) for IMD, Istituto Superiore di Sanità (ISS), where the confirmation of bacterial identification and molecular characterization were performed.

2.4. Molecular characterization of meningococcal carriage isolates

DNA was extracted using commercial kit according to the manufacturer's instruction (QIAmp DNA minikit, Qiagen, Hilden, Germany) from the 120 isolates. Serogroup was determined by PCR (Zhu et al., 2012). Those resulted negative by PCR were further analyzed for the presence of the capsul null locus (*cnl*) according to the method described by Claus et al., 2002. Multilocus Sequence Typing (MLST), PorA and FetA typing were performed according to the scheme described on Neisseria website (http://pubmlst.org/neisseria/). PCR and sequencing of *fHbp*, *nhba* and *nadA* genes were carried out as previously described (Bambini et al., 2013). The alleles assignment has been obtained using website http://pubmlst.org/neisseria/.

2.5. Antimicrobial susceptibility testing of meningococcal carriage isolates

Susceptibility to ceftriaxone, ciprofloxacin, penicillin G and rifampicin was determined by MIC Test Strip Method (Liofilchem, Italy) on Mueller-Hinton agar (Oxoid, United Kingdom) supplemented with 5% of sheep blood. The breakpoints were those recommended by the European Committee on Antimicrobial Susceptibility Testing - EUCAST version 8.1, May 16, 2018 (http://www.eucast.org/).

2.6. Whole genome sequencing (WGS)

WGS was performed at the Scientific Department, Army Medical Center in Rome, on a subsample of 75 meningococcal carriage isolates as representatives of the clonal complexes identified.

For each isolate, 1 ng of DNA was used to prepare the sequencing libraries following the Nextera XT DNA protocol, according to the manufacturer's instruction. WGS was performed with the Illumina MiSeq platform (kit v3, 600cycles). An average total of 1.9 million paired-end reads was obtained for each sample. A first quality check of the raw sequence data was performed using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Reads were trimmed to keep high quality bases (Q score > 25) using the software Sickle (https://github.com/najoshi/sickle) and *de novo* assembly was carried out with the ABySS software version 1.5.2 (k parameter = 63), (Simpson et al., 2009). Contigs longer than 500 bp were selected using an *ad hoc* script and kept for further analysis. The final assembly ranged from 143 to 332 contigs/ sample covering the ca 2.2 Mb of the *N. meningitidis* genome. The seventy-five genomes were uploaded to the Neisseria PubMLST website (http://pubmlst.org/neisseria/), which uses the Bacterial Isolate Genome Sequence Database (BIGSdb) platform (Jolley and Maiden, 2010).

2.7. Genomic analysis

All genomes were uploaded into Pub.MLST.org/*Neisseria* and analyzed by means of the BIGSdb Genome Comparator tool. Core genome MLST (cgMLST) was used for phylogenetic analysis (Jolley et al., 2012). Missing or incomplete assembled loci were not included in the pairwise comparison from the distance matrix calculation for the Neighbor-Net graph. Output distance matrices were visualized as Neighbour-net graphs, generated by SplitsTree4 (version 4.13.1), (Huson and Bryant, 2006).

2.8. Statistical analysis

Carriage rate and exact binomial 95% confidence intervals were

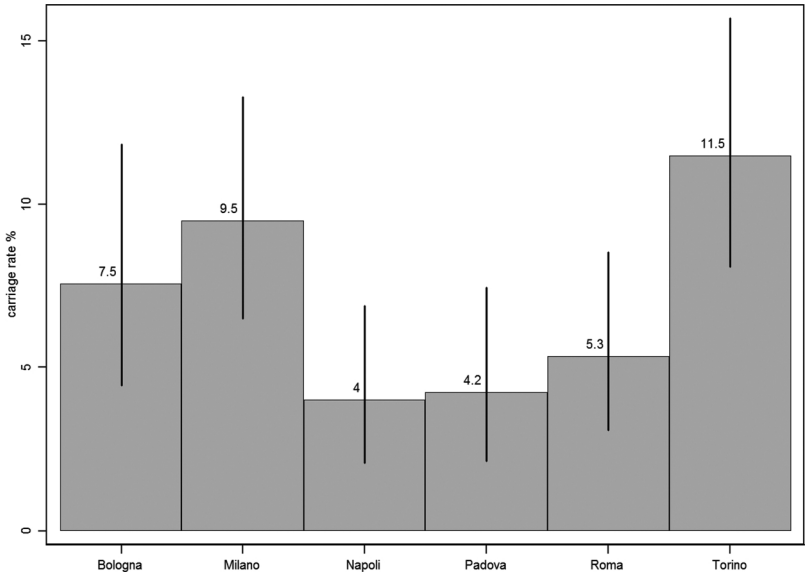

Fig. 1. Meningococcal carriage rates (with 95% confidence intervals) by Italian cities.

calculated using STATA 13 (StataCorp, 2013. Stata Statistical Software: Release 13).

3. Results

3.1. Prevalence of meningococcal carriage isolates

From the 1697 teenagers enrolled, 120 resulted culture positive for *N. meningitidis*. In particular, the overall carriage rate was 7% (95% CI, 5.90–8.40), that varied among the collaborating centers: 4.0% in Napoli (12/300) and 4.2% in Padova (11/260), 5.3% (16/300) in Roma, 7.5% (17/225) in Bologna, 9.5% (31/315) in Milano and 11.5% (34/296) in Torino (Fig. 1). The median age of the carriers was 17.5 years (range 14–19 years). Fig. 2 shows the distribution of the carriers by age. The highest number of meningococcal isolates was observed at 19 years of age (30%, 36/120). Gender information was available for 77% (92/120), of which 61% were male and 39% female. Vaccination status was unavailable.

3.2. Molecular characteristics and antimicrobial susceptibility

Based on capsular serogroup by PCR the 47% (56/120) of carriage isolates were capsule null locus (*cnl*). Moreover, the 27% (32/120) were serogroup B, 8% (9/120) serogroup W, 6% (7/120) serogroup Y, 5% (6/120) serogroup C, 3% (4/120) serogroup E, 2% (2/120) serogroup X and 3% (4/120) non groupable (NG). Forty-five of the 56 *cnl* carriage isolates were further analyzed by WGS to identify the deletion of the regions A–C of the *cps* locus, due to a replacement with a small DNA fragment (data not shown).

Fifty-six Sequence Types (STs), of which 39% (22/56) were new STs, have been identified together with 18 clonal complexes (cc_s). As shown in Fig. 3, among the most common cc_s, the cc198 and the cc1136, were associated exclusively with *cnl* isolates. *Cnl* were also associated with cc53 (N = 8) and cc41/44 (N = 2); cc213, cc32 and cc1117 were identified in one isolate each. Serogroup B was associated with cc41/44 (N = 13) and other 8 cc_s: cc461 (N = 3), cc865 (N = 3), cc213 (N = 2), cc35 (N = 2) and 4 cc_s (cc162, cc32, cc23and cc103) represented by one isolate each. All serogroup C were cc41/44. Serogroup W was mainly associated with cc22 (8/9) and the serogroup Y with cc23 (6/7). Serogroup E and X were associated with cc60 and cc1157, respectively. Nine isolates (5 of serogroup B, 2 NG and 2 *cnl*), were not designated to any known cc and classified as “not assigned” (na).

A total of 58 PorA types and 26 FetA variants were identified. The most frequent PorA types were: P1.18-4, 25 (13/120) and P1.18, 25-14 (10/120). The predominant FetA variant was F4-1 (19/120) and a new FetA variant, F5-168, was identified in 1 isolate. Fifteen carriage isolates (12.5%, 15/120) gave negative PCR results for *fetA* gene.

As shown in Table 1, the fHbp Novartis variant family 2/Pfizer subfamily A (fHbp-2/A) was the most represented (46%, 52/120), followed by Novartis variant family 1/Pfizer subfamily B (fHbp-1/B)

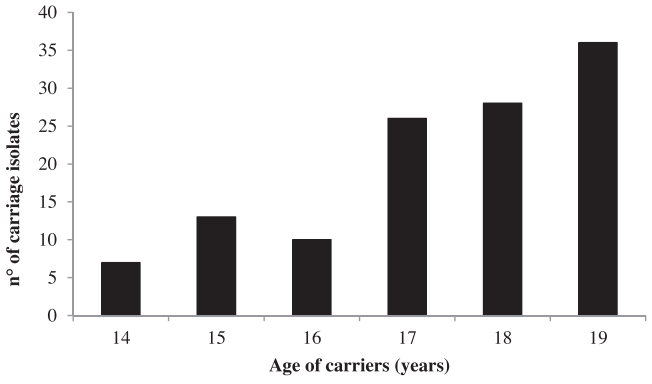

Fig. 2. Distribution of meningococcal carriers by age.

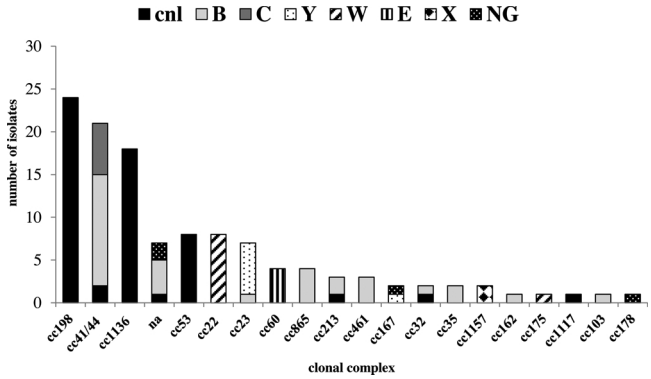

Fig. 3. Distribution of serogroups by clonal complexes in 120 meningococcal carriage isolates.

Table 1
Distribution of FHbp variant, NHBA peptide and NadA variant among 120 carriage meningococci.

Genogroup	fHbp Novartis variant/ Pfizer variant	NHBA peptide	NadA variant	Sequence Type	Clonal complex	Total
cnl	3.94/A103	145	negative	ST-1136	cc1136	12
	3.94/A103	10	negative	ST-1136	cc1136	2
	3.94/A103	118	negative	ST-1136	cc1136	1
	3.94/A103	2	negative	ST-1136	cc1136	1
	3.94/A103	114	negative	ST-1136	cc1136	1
	3.94/A103	10	negative	ST-198	cc198	3
	3.94/A103	3	negative	ST-10775	cc198	1
	3.94/A103	29	negative	ST-11130	cc1136	1
	3.45/A05	603	negative	ST-10353	cc213	1
	1.4/B16	10	negative	ST-823	cc198	15
	1.1/B24	747	negative	ST-823	cc198	1
	1.4/B16	10	negative	ST-11128	cc198	1
	1.4/B16	10	negative	ST-11164	cc198	1
	1.4/B16	10	negative	ST-10349	cc198	1
	1.4/B16	10	negative	ST-11166	cc198	1
	1.1/B24	10	NadA-1	ST-32	cc32	1
	1.14/B03	471	negative	ST-6744	cc41/44	2
	2.102/A73	58	negative	ST-53	cc53	5
	2.102/A73	58	negative	ST-11133	cc53	1
	2.102/A73	602	negative	ST-53	cc53	1
	2	228	negative	ST-1289	na	1
	2.19/A22	26	negative	ST-414	cc41/44	1
	2.21/A07	239	negative	ST-1117	cc1117	1
NG	2.16/A19	601	negative	ST-9908	na	2
	2.23/A26	9	negative	ST-168	cc167	1
	1.12//B06	145	negative	ST-178	cc178	1
B	2.19/A22	2	negative	ST-414	cc41/44	7
	2.19/A22	288	negative	ST-2363	cc41/44	1
	2.19/A22	2	negative	ST-2624	cc23	1
	2.19/A22	236	negative	ST-10099	na	1
	2.119/A95	24	negative	ST-3327	cc865	2
	2.119/A95	24	negative	ST-10852	cc103	1
	2.119/A95	24	negative	ST-6610	na	2
	2.119/A95	10	negative	ST-3327	cc865	1
	2.21/A07	20	negative	ST-162	cc162	1
	2.16/A19	21	negative	ST-35	cc35	2
	2.625/A	116	negative	ST-9898	cc41/44	1
	3.47/A06	118	negative	ST-461	cc461	1
	3.47/A06	6	negative	ST-1946	cc461	1
	3.47/A06	20	negative	ST-1946	cc461	1
	3.184/A66	145	negative	na	cc41/44	1
	3.45/A05	18	negative	ST-3496	cc213	1
	3.45/A05	18	NadA-4/ 5	ST-9922	cc213	1
	1.1/B24	3	NadA-1	ST-6589	cc32	1
	1.90/B79	2	negative	ST-8869	cc41/44	1
	1.13/B09	2	negative	ST-10102	cc41/44	1
	1.14/B03	6	negative	ST-11127	cc41/44	1
	1.14/B03	29	negative	ST-41	cc41/44	1
	1.12/B06	145	negative	ST-11165	na	1
C	2.19/A22	112	negative	ST-9907	cc41/44	5
	2.19/A22	2	negative	ST-6075	cc41/44	1
W	2.16/A19	20	negative	ST-22	cc22	4
	2.16/A19	8	negative	ST-22	cc22	1
	2.16/A19	10	negative	ST-22	cc22	1
	2.16/A19	20	negative	ST-9935	cc22	1
	2.23/A26	9	NadA-2/ 3	ST-2881	cc175	1
	2.807/A	20	negative	ST-11132	cc22	1
Y	2.104/A100	8	negative	ST-23	cc23	3
	2.25/A15	6	negative	ST-10101	cc23	1
	2.23/A26	9	negative	ST-767	cc167	1
	1.692/B	8	negative	ST-10100	cc23	1
	1.251/B68	145	negative	ST-11131	cc23	1
E	1.13/B09	24	negative	ST-9923	cc60	2
	1.13/B09	24	negative	ST-60	cc60	1
	1.13/B09	2	negative	ST-60	cc60	1
X	1.13/B09	114	NadA-2/ 3	ST-1157	cc1157	2

na = not assigned.

(30%, 36/120) and Novartis variant family 3/Pfizer subfamily A (fHbp-3/A) (24%, 29/120). The fHbp-2/A was mostly associated with serogroup B (20/52), while the fHbp-1/B and the fHbp-3/A with *cnl* isolates (23/36 and 23/29, respectively), (Table 1). The fHbp-3.94/A103 and fHbp-1.4/B16 were the predominant and associated with *cnl*/cc1136 and cc198, (Table 1).

As shown in Table 1, 28 NHBA variants were found in all carriage isolates and the NHBA peptide 602 was here identified for the first time. The NHBA peptide 10 was present in 27 isolates, mainly associated with cc198 (22/27), followed by NHBA-145 in 16 isolates cc1136 (12/16); NHBA-2 was detected in 13 isolates, mainly associated with cc41/44 (10/13) (Table 1).

All, except for 6 isolates (5%, 6/120) lacked the *nadA* gene (Table 1). The six NadA variants were: NadA-2/3 in 3 isolates (2 of serogroup X/cc1157 and 1 of serogroup W/ cc175), NadA-1 in 2 isolates (of serogroup B/cc32 and *cnl*/cc32). Finally the NadA-4/5, with a frameshift mutation resulting in a pre–mature stop codon in the gene identified in 1 serogroup B/ cc213 (Table 1).

Eighteen carriage isolates (15%) showed at least one of the gene encoding antigen, as in the multicomponent 4CMenB vaccine. In particular, *nhba* allele 1 (encoding for NHBA peptide 2) in 13 isolates, *fHbp* allele 1 (encoding for fHbp peptide 1) in 3 isolate and *nadA* gene (encoding for NadA peptide 2/3) in 3 isolates.

Only the *fHbp* gene encoding for the variant A05, as in the bivalent rLP2086 vaccine, was found in 3 meningococcal carriage isolates.

Overall, 17.5% (21/120) of the carriage isolates showed the presence of at least one of the vaccines components.

All the examined carriage isolates (n = 113) were susceptible to ceftriaxone (MIC range of < 0.002-0.006 μg/mL), ciprofloxacin (MIC range < 0.002-0.012 μg/mL) and rifampicin (MIC range of < 0.016-0.125 μg/mL). The 54% (61/113), intermediated susceptible to penicillin G (Pen^I) with a MIC range of 0.094-0.25 μg/mL, of which 51% (N = 31), were *cnl*. The remaining isolates were susceptible to penicillin G (MIC range of 0.016-0.064 μg/mL).

3.3. cgMLST

Seventy-five of 120 meningococcal carriage isolates were analyzed by cgMLST for the phylogenetic correlation (Table S1). Carriage isolates mainly clustered by clonal complex, as defined by the analysis of 1605 loci in cgMLST, of which 146 were incomplete and, then, excluded. Only 7 (0.5%) identical genes were identified among all isolates. Fig. 4 shows that all isolates (n = 75) splitted into two clades. The first comprises the majority of those identified as *cnl* (36/46) and belonging to cc198 (18 isolates) and cc1136 (18 isolates). The second clade comprises the encapsulated isolates (serogroup B, C, Y, W, E, X), three NG and *cnl*/cc53 (Fig. 4). Some carriage isolates from students attending the same school, showed highly relationship, they were: P18, P19, P20 and P21 *cnl*/cc1136, with a mean distant of only 6 different loci. The isolates P99 and P100 X/cc1157, differed by 11 loci; P77 and P78 *cnl*/cc1136, for 14 loci; P30 and P44 *cnl*/cc1136, for 21 loci as well as P101 and P105 W/cc22, by 21 loci.

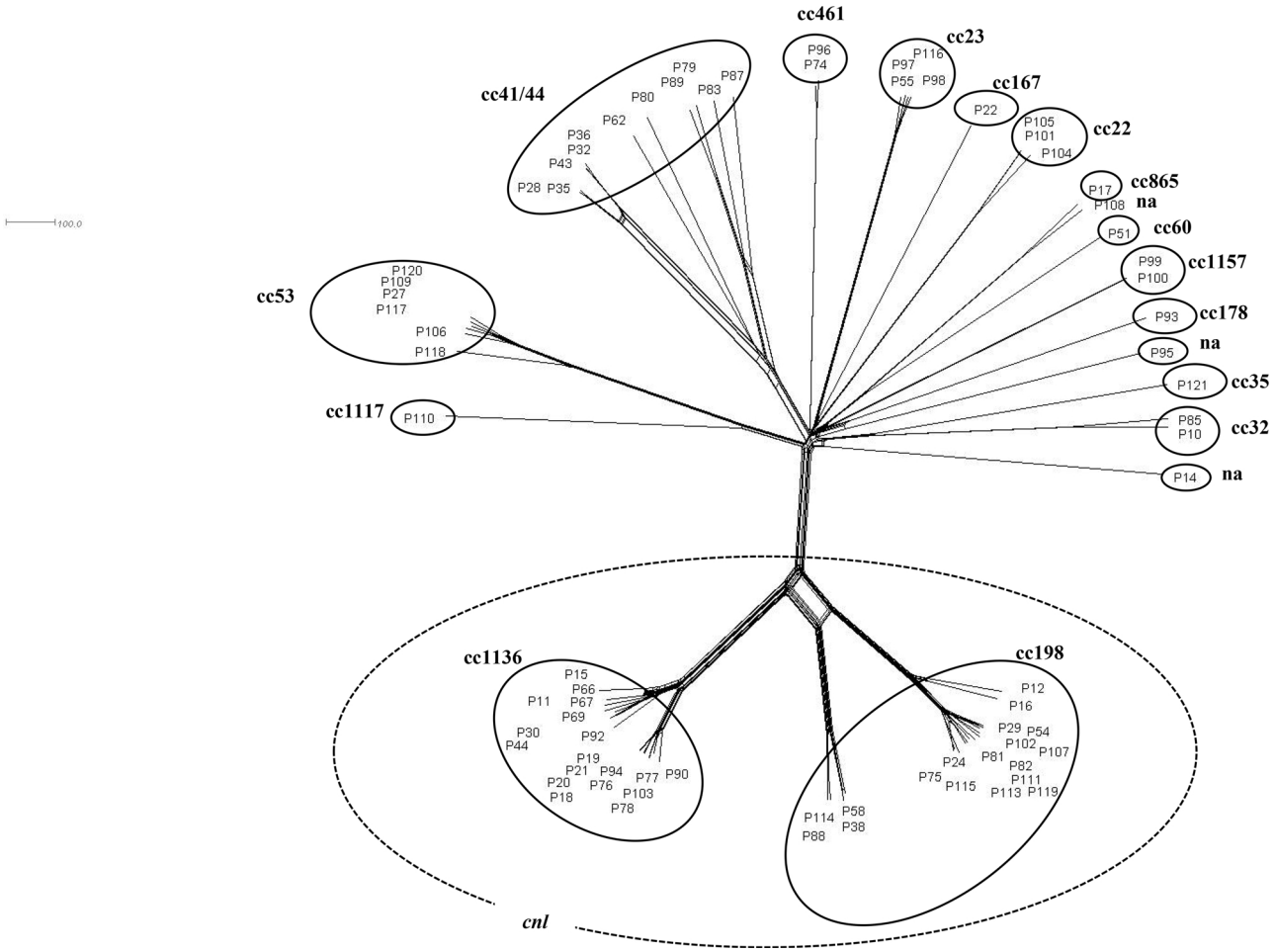

Fig. 4. Phylogenetic analysis by cgMLST of *N. meningitidis* carriage isolates (n = 75). The scale bar represent the number of variant loci. na indicates not assigned to any known clonal complex.

4. Discussion

Although the rate of IMD in Italy is currently low, outbreak and small clusters have been recently reported (Miglietta et al., 2018; Stefanelli et al., 2018). Meningococcal carriage influences the dynamic of the spread of the disease in particular for specific age group (Caugant and Maiden, 2009).

We aimed to evaluate the carriage rate and the molecular characteristics of carriage isolates from high school students prior to the introduction of MenB vaccines in Italy and in a non-epidemic period (Neri et al., 2015).

Previous studies demonstrated a great variety of meningococcal carrier rates, from less than 10% up to more than 50%, in Europe and in United States (Dlawer et al., 2011; Harrison et al., 2015; Maiden et al., 2002; Neal et al., 2000; Oppermann et al., 2006).

This study reported a lower average carrier rate of 7%. Here, the highest number of meningococcal carriage isolates was observed in adolescent aged 19 years, similarly to what reported in other countries (Caugant et al., 1994; Claus et al., 2005).

Prevalence of *cnl* meningococcal carriage isolates was the main result. The loss of the expression of capsular polysaccharide enhances the capability of meningococci to colonize the nasopharynx (Leimkugel et al., 2007). There are two causes determining the lack of the capsule: the deletion of the capsule locus (*cnl*) or the down-regulation of the expression of the capsule genes either temporarily or permanently by different genetic mechanisms (Caugant et al., 2009).

Overall, *cnl* showed a smaller diversity in terms of number of genotypes identified. In fact, the majority of them belonged to cc198, cc1136 and in a small proportion to cc53.

The encapsulated carriage isolates belonged to cc41/44, cc23, cc32, cc162, cc213 mainly associated with invasive meningococci circulating in the country. Thirtly, Pen^I isolates were the majority among the carriage isolates (54%). Thus was already reported with a range from 19% up to 83% in Ethiopia, Spain, Turkey and Greece (Alemayehu et al., 2017; Arreaza et al., 2000; Gazi et al., 2004; Tzanakaki et al., 1992). As described by Vacca et al. (2018), an increase proportion of Pen^I among meningococcal invasive isolates was observed in Italy, thus may explained, at least partially, the high proportion of Pen^I among carriage isolates. Furthermore, commensal *Neisseria* species maybe an additional way to acquire the Pen^I phenotype by meningococcal carriage isolates (Mechergui et al., 2015) due to the horizontal transfer of DNA fragments responsible of *penA* mosaicism (Karch et al., 2015).

Here, the distribution of antigens comprises in both of the MenB vaccines have been also evaluated but further investigations, to measure the vaccine antigens expression among carriage isolates, have to be evaluated.

Some limits of the results were: first of all, the study was a cross-sectional survey that do not permit to investigate the dynamic of the colonization in each student during the time. Secondly, meningococcal vaccination status was not available for the majority of the enrolled students.

A high molecular heterogeneity characterized the meningococcal carriage isolates, and the majority of them were *cnl.* Among those of serogroup B, the clonal complex cc41/44 was one of the most frequently found and associated with invasive meningococcal diseases of serogroup B in Italy.

In our setting, *cnl* were distant from the other meningococcal carriage isolates; all of them clustered by clonal complex. Moreover, cgMLST identified closely related isolates among students attending the same school.

Meningococcal carriage studies permit to better understand the epidemiology of invasive meningococcal disease, at least in a specific setting.

Competing interests

No competing of interest.

Authors' contributions

AN,CF, PV, performed the microbiological analyses on samples. MPL, LR, LD, IS, collected the swabs at local level and identified *Neisseria meningitidis*. AC, AA, FL performed the whole genome sequencing on a subsamples of meningococcal isolates. CF, PV, AN contributed in the analysis of carriage isolates and together with PS revised critically the manuscript. PS conceived the study and together with AN wrote the manuscript. All authors have read and accepted the submitted manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.03.004.

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