Lessons from meningococcal carriage studies

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Abstract

Neisseria meningitidis, an obligate commensal of humans, normally colonizes the mucosa of the upper respiratory tract without affecting the host, a phenomenon known as carriage. In Europe, as much as 35% of young adults are carriers at a given time. Recent studies using molecular methods for clone identification have demonstrated the extensive genetic diversity of the strains isolated from carriers, in comparison with a limited number of hypervirulent strains associated with invasive disease. Published studies and new data generated through the framework of the EU-MenNet clearly indicated significant differences in pathogenicity between meningococcal clones and in the distribution of multilocus sequence types among isolates from asymptomatic carriers among European countries; simultaneous carriage of more than one meningococcal strain in the throat is rare, but occasionally occurs; and the commensal association of particular clones with a host is a long-term relationship, often lasting several months. Further investigations of the carrier state are warranted to improve our understanding of the epidemiology and pathogenesis of meningococcal disease, as well as to support the introduction and to measure the impact of mass vaccination.

Meningococcal disease is a life-threatening illness with annual incidence rates varying from 1 to 1000 per 100 000 in different parts of the world. The organism responsible for the disease, *Neisseria meningitidis*, is a strict human commensal which commonly colonizes the oropharyngeal mucosa of the man, its sole ecological niche, without causing any detectable symptoms. Asymptomatic carriage is an age-dependent phenomenon, with point prevalence carriage rates usually ranging from 10% to 35% in young adults (Cartwright *et al.*, 1987; Caugant *et al.*, 1994; Stephens, 1999; Claus *et al.*, 2005). The majority of individuals will, at one time or another throughout life, harbour the bacterium asymptomatically in the throat. Thus, invasive disease is a very rare outcome of infection, usually occurring shortly after acquisition of the bacterium by a susceptible host (Edwards *et al.*, 1977).

Causing disease is of no importance for the spread of meningococci and the population biology of *N. meningitidis* (Maiden, 2004); most patients with meningococcal disease have not been in contact with another case. The bacterium is transmitted between individuals when respiratory droplets from an infected – but symptom-less – person are spread to other individuals through close contact (deVoe, 1982). In closed and semi-closed populations, such as military recruits and university students, transmission increases and carriage rates may then reach close to 100% (Olcen *et al.*, 1981; Caugant *et al.*, 1992). Meningococci are particularly susceptible to drying; thus, transmission likely requires that the bacterium is protected by a polysaccharide capsule (Lipsitch & Moxon, 1997; Weber *et al.*, 2006). Colonization of a new host necessitates, in addition, that the meningococci are piliated to permit adhesion to the epithelial cell surfaces (Stephens & McGee, 1981).

Studies performed in Europe have shown that carriage rates are very low in the first years of life, then sharply increase in teenagers and reach a peak in 20–24-year olds. Carriage rates decrease to less than 10% in older ages (Cartwright *et al.*, 1987; Blackwell *et al.*, 1990; Caugant *et al.*, 1994; Claus *et al.*, 2005).

In addition to age, a number of risk factors have been shown to be associated with meningococcal carriage (Cartwright, 1995; Krizova & Kriz, 1999). There are slightly more carriers in males than in females. Individuals with respiratory tract infections, of viral or bacterial origin, may be at high risk to become carriers (Stephens, 1999). Smoking, active as well as passive, is one of the strongest risk factors

for becoming a meningococcal carrier (Stuart *et al.*, 1989; Blackwell *et al.*, 1992; Kremastinou *et al.*, 1994). Low socioeconomic status in some populations appears to increase the risk for carriage (Davies *et al.*, 1996). These risk factors have been found to be independently associated with carriage in multivariate analyses, but in a number of recent studies it appears that the number and closeness of social contacts might be the most important elements (McLennan *et al.*, 2006).

Exposure to meningococci through asymptomatic carriage has been shown to elicit an immune response. Mucosal immunity in carriers can be detected by increasing concentration of IgA in saliva (Robinson *et al.*, 2002; Horton *et al.*, 2005). Although not sufficient to prevent further colonization of the oropharynx, development of mucosal immunity may play an important role in prevention of the invasion of epithelial cells (Griffiss, 1995). Carriage of *N. meningitidis* also results in a humoral response, as demonstrated by increased serum bactericidal antibody activity (Jones *et al.*, 1998; Kriz *et al.*, 1999; Jordens *et al.*, 2004; Raghunathan *et al.*, 2006). Although the response is mainly strain-specific, some degree of cross-reactivity with heterologous strains is developed (Jordens *et al.*, 2004).

Despite the importance of the carrier state in the biology of the meningococcus, the relationships between carriage and disease have not been fully elucidated. Noncarriers are considered a high-risk group for meningococcal disease as their capability to maintain a commensal relationship with an acquired strain is not known (Griffiss, 1995). On the other hand, development of serum bactericidal activity against a range of strains was demonstrated in a longitudinal study in individuals in whom no meningococcal carriage was detected (Jordens *et al.*, 2004).

Most carrier studies are performed as cross-sectional analyses of a target population at a single point in time – snapshot studies. In those cases, a positive sample will not tell when the individual acquired the strain and a negative sample might simply result from low sensitivity of the swabbing method. A few studies have attempted to measure duration of meningococcal carriage by following subjects with repeated throat samples over time. These studies have shown that the meningococcal carrier state may be chronic, last for several months, be intermittent or transient (Kuzemenska *et al.*, 1978; Broome, 1986). In most cases, strain characterization, to determine whether the same carried strain is kept, has either not been performed or the methods employed were relatively coarse. Duration of carriage may be dependent on the properties of the colonizing strain, not all meningococcal strains having the same propensity to establish a long-term commensal relationship with the host. This kind of investigation requires methods such as multilocus enzyme electrophoresis (MLEE) (Selander *et al.*, 1986) or multilocus sequence typing (MLST) (Maiden *et al.*, 1998) that can identify specific strains with a high degree of precision by assessing the diversity of randomly chosen, multiple genes encoding essential metabolic functions. With MLEE, genetic variation is assessed indirectly by examination of the gene products (normally metabolic enzymes) after starch gel electrophoresis and specific histochemical staining. With MLST, the DNA sequencing technology is used to measure the variation of genes directly and accurately. With both methods, genetic variants at multiple loci are combined into an allelic profile, designated as an electrophoretic type (ET) for MLEE or as sequence type (ST) for MLST.

Molecular characterization of the organisms colonizing the throat is essential to our understanding of the dynamics of transmission of *N. meningitidis* in the human population and, thus, the epidemiology of the disease. Because the meningococcus is both highly variable and naturally competent for transformation, it may readily change its genome by incorporating DNA from other meningococcal strains or from other closely related bacterial species, adapting to its environment, the human oropharyngeal mucosa. This phenomenon occurs during the carriage stage, when multiple strains of meningococci may colonize the same site or meningococci may share the same ecological niche with a variety of other commensal or pathogenic bacteria. Although it is well known that multiple strains of *Streptococcus pneumoniae* may be detected from a single nasopharyngeal sample (Brondson *et al.*, 2004), attempts to determine such occurrences with *N. meningitidis* are basically nonexisting.

Much information regarding the meningococcal strains causing disease throughout the world has become available in the past two decades and this has been used to develop models for the population structure of the species (Maynard Smith *et al.*, 1993). However, it is still very unclear to what extent meningococcal populations from healthy carriers are structured. For example, is the species thoroughly panmictic, if highly virulent strains are disregarded, or are some clones successful commensals? Are populations of carrier strains highly differentiated in function according to their geographic distribution or to characteristics of their host? To answer these questions and fully understand the epidemiology of meningococcal disease, the unequivocal genetic characterization of large collections of disease-associated and carried strains is indispensable.

Methodological considerations

Swabbing and cultivation

The swabbing technique and culture conditions are factors greatly influencing the detection of meningococcal carriage in healthy individuals. The site of sampling should include

the posterior pharyngeal wall behind the uvula, and possibly the tonsils (Cartwright, 2001). Charcoal-impregnated swabs are recommended. The use of selective antibiotics, such as a combination of colimycin, lincomycin, amphotericin, and trimethoprim in media, is an excellent choice as it inhibits the growth of nonmeningococcal bacteria and thus significantly increases the yield of meningococci from the samples (Caugant *et al.*, 1992).

Swabs may be directly plated onto solid medium on site or placed in an appropriate transport medium, for example Stuart medium, before plating later on in the laboratory. A comparison of these two methods using samples from high school students in the UK (Cunningham *et al.*, 2001) showed a statistically significant higher carriage rate with direct plating (11.6% vs. 6.2%), whereas earlier investigations had not revealed such differences. In our hands, direct plating was not significantly better than the use of Stuart's transport media, at least when the plating in the laboratory was performed within 4 h of taking the samples (Table 1), but a somewhat lower carriage rate was obtained when the samples were kept in the transport medium for up to 24 h before plating (27.4% vs. 23.2%). Consequently, if plating can be performed within a few hours, the use of Stuart's medium is recommended as it clearly facilitates the manipulation, when performing a large carriage study.

Carriage studies may need to be performed at sites lacking personnel with sufficient microbiological experience for differential identification of meningococci in a complex sample as represented by the throat flora. After 24–48 h incubation of a sample on a selective plate, the whole bacterial growth may then be collected with a sterile loop, suspended in Greaves solution (Craven *et al.*, 1978) and then frozen immediately at or below $-20\,^{\circ}C$. Frozen samples can then be transported and taken up for analysis by trained personnel when convenient (Caugant *et al.*, 2006). Meningococci have been recovered after 14 months from all the tested samples treated that way, even when as few as 10 meningococcal colonies were seen on the primary plate.

Table 1. Comparison of Stuart's transport medium for 4 or 24 h before plating and direct plating for identification of meningococcal carriers among 164 18–22-year-old military recruits, Norway 2003

Method	Meningococcal growth						No. of carriers (%)
Direct plating	+	+	–	+(7)	+(1)	–	45 (27.4)
Stuart (4 h)	+	+	+ (1–2)	–	–	–	46 (28.0)
Stuart (24 h)	+	–	–	+ (1)	–	–	38 (23.2)
Number of samples	37	6	3	1	1	116	48 (29.3)

+Growth of meningococci; –, no growth of meningococci. In positive samples, the number of colonies is given in brackets if < 20.

PCR-based methods for rapid discrimination of meningococcal clones within single throat samples

Alternative methods, including immunohistochemistry (Sim *et al.*, 2000) and specific real-time PCR assays based on the *porA* gene (Jordens & Heckels, 2005), have been tested to detect meningococcal carriage and appear to be at least as sensitive as culture of throat swabs. PCR-based methods can also provide a mean of screening multiple colonies from single throat samples for genetic variants of *N. meningitidis*.

To study the genetic diversity of meningococcal colonies from a single throat sample, as part of new carriage studies in the course of the EU-MenNet project, we selected and subcultured usually up to 20 meningococcal colonies per plate after primary cultivation on selective media, for storage and further investigation. The genetic homogeneity of the colonies within each sample was screened by either randomly amplified polymorphic DNA (RAPD) analysis (Bart *et al.*, 1998) using various primers (1281, 1254 and meningococcal USS) or by targeting DNA motifs with potential variable-number tandem repeats (VNTR) in the genome of *N. meningitidis* (Yazdankhah *et al.*, 2005). Development of the VNTR method for meningococci showed that meningococcal patient isolates with similar MLST or MLEE types recovered from epidemiologically linked cases in a defined geographical area often presented similar VNTR patterns, whereas isolates of the same MLST or MLEE types without an obvious epidemiological link showed variable VNTR patterns (Yazdankhah *et al.*, 2005). Thus, VNTR analysis appeared suitable for rapid differentiation between genetic variants within a throat sample.

Differentiation of carrier vs. patient strains

Phenotypic differences

The capsular polysaccharide is the outermost antigen on the meningococcal surface, a major virulence factor and the prime target for mucosal and humoral immunity. On the basis of antigenic variation of the capsule, *N. meningitidis* has been divided into 12 serogroups. Patient strains are encapsulated and five of these serogroups (A, B, C, W135, and Y) cause more than 90% of the invasive disease worldwide (Pollard, 2004). In contrast, *c.* 50% of the strains isolated from carriers lack capsule and are therefore serologically not serogroupable (Caugant *et al.*, 1988; Ala'Aldeen *et al.*, 2000). There are two essential reasons for the lack of capsule – the deletion of the capsule locus (the capsule nul or *cnl* meningococci) or down-regulation of the expression of the capsule either temporarily or permanently by one of a number of genetic mechanisms (Claus *et al.*, 2002). Loss of capsule enhances the capability of meningococci to colonize the

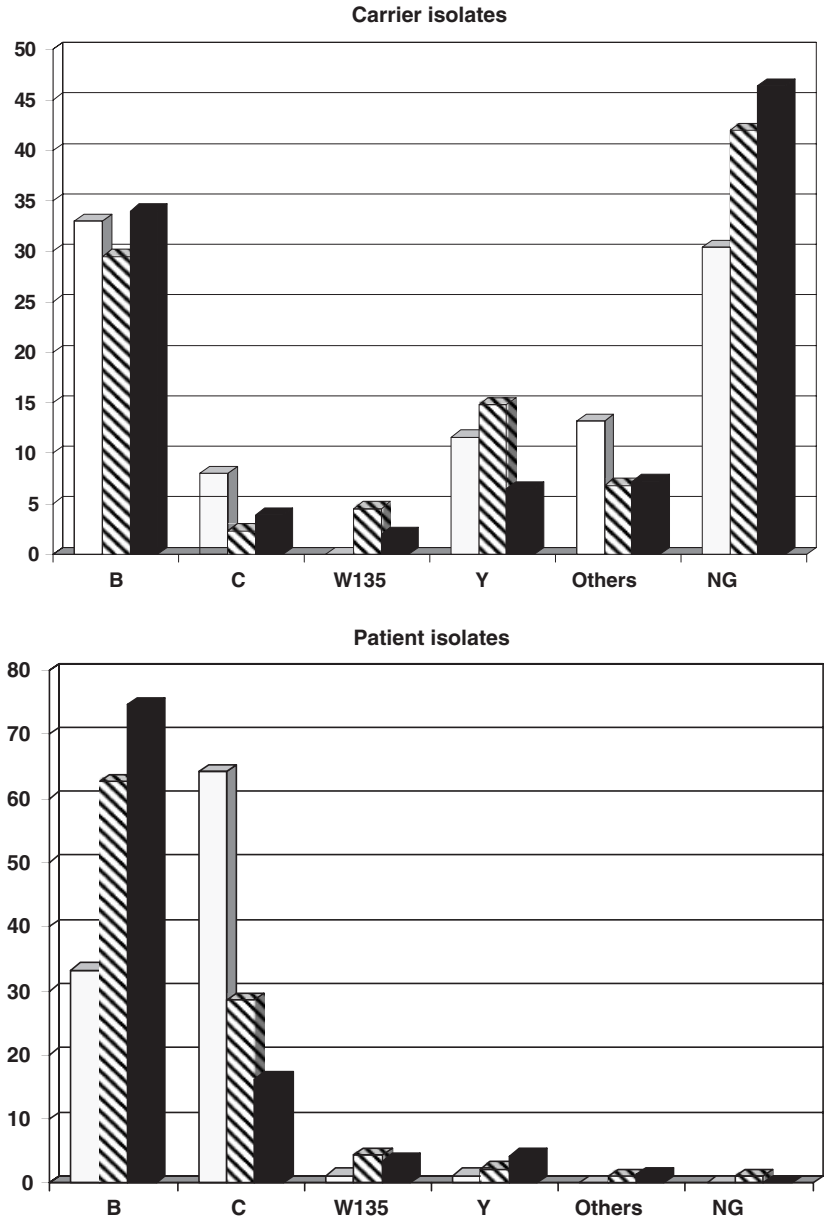

Fig. 1. Distribution of serogroups (%) among patient and carrier isolates of *Neisseria meningitidis* in three European countries, 1991–2000. White, Czech Republic; striped, Greece; black, Norway (adapted from Yazdankhah *et al.*, 2004).

human nasopharynx and to avoid the human defence systems (Hammerschmidt *et al.*, 1994), whereas capsulation is likely to be important to protect the bacterium during transmission, explaining why this property is preserved by the species and full commensalism not achieved (Lipsitch & Moxon, 1997).

To better understand the relationship between carrier and disease-causing strains, we performed a comparative study of the expressed capsular antigens in a collection of 667 meningococci isolated, within a 10-year period, from patients and carriers in three European countries, the Czech Republic, Greece, and Norway, representing the Eastern, Southern and Northern parts of Europe. Strains from all but one patient were serogroupable, whereas 30–45% of the carrier isolates were nonserogroupable (Fig. 1). Serogroup B strains predominated among both patient and carrier isolates: about 30% in each country among carriers, and 30–75% among patients. Although marked differences between countries were observed in the serogroup distribution of patient isolates, the serogroup distribution of the carrier isolates was similar between countries, with a predominance of serogroup B among isolates expressing a capsular polysaccharide, followed by serogroup Y. A highly significant association of serogroup B, C, and W135 capsule expression with disease was evidenced, with serogroup C estimated to

be 14 times more strongly associated with disease than serogroup B; the association with disease was less strong for serogroup Y (Yazdankhah *et al.*, 2004).

There is also some evidence that particular variants of the components of the meningococcal cell membrane are associated with invasive disease. PorA and PorB, the two major porins in *N. meningitidis*, are stable during chronic carriage (Jones *et al.*, 1998), but isolates of the same clone within individual carriers may vary from being typable to nontypable using monoclonal antibodies, suggesting changes in the level of expression of the PorA and PorB antigens during carriage (Caugant *et al.*, 1992). The lipooligosaccharide is another major virulence factor of *N. meningitidis* (Jones *et al.*, 1992) inducing the production of pro-inflammatory mediators that may lead to septic shock (Brandtzæg & van Deuren, 2002). The lipooligosaccharide types identified in carrier strains differed from those expressed by patient isolates (Poolman *et al.*, 1995).

Another structure that significantly differs between disease-causing strains and carrier strains is NadA, a novel surface protein involved in adhesion to host tissues. NadA had been demonstrated in *c.* 50% of the clinical isolates, especially in most of the so-called hypervirulent lineages, as defined by MLEE and MLST. However, most strains isolated from healthy people either lack the *nadA* gene or possess a quite distinct form of the gene (Commanducci *et al.*, 2004).

Genotypic differences

When compared to collections of meningococci recovered from patients, the isolates obtained from the throat of healthy individuals are typically much more diverse. Whereas most of the cases of disease worldwide are caused by a limited number of hypervirulent lineages, which appear to have an increased propensity to cause invasive disease, carrier isolates are genetically extremely heterogeneous, with only a low proportion of carrier strains representing the hyperinvasive clones (Caugant, 1998; Jolley *et al.*, 2000). This finding, originally based on analyses of *N. meningitidis* using MLEE, was generalized through the investigation of a large collection of isolates from patients and carriers in the Czech Republic, Greece, and Norway using the MLST method (Yazdankhah *et al.*, 2004). The frequency distribution of clone complexes differed between disease and carrier isolate collections (Fig. 2). Whereas 20% of the disease isolates had STs that were not assigned to a known clonal

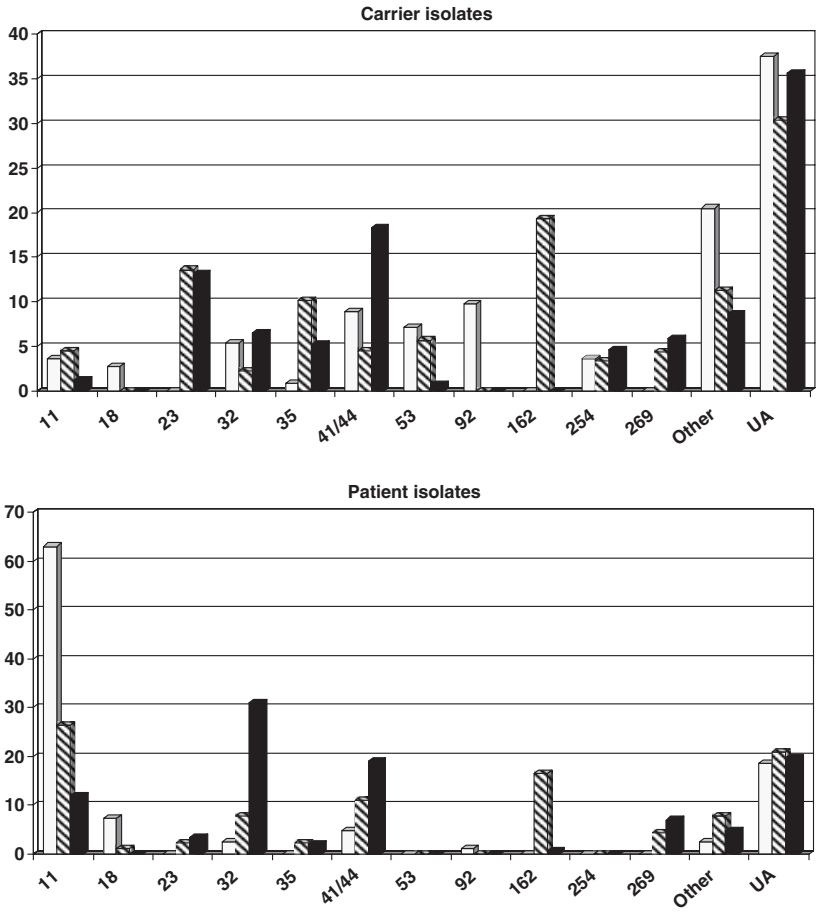

Fig. 2. Distribution of ST-complexes (%) among patient and carrier isolates of *Neisseria meningitidis* in three European countries, 1991–2000. White, Czech Republic; striped, Greece; black, Norway (adapted from Yazdankhah *et al.*, 2004).

Table 2. Association of the predominant clone-complexes with carriage and disease in Europe, 1991–2000 (adapted from Yazdankhah *et al.*, 2004)

ST-complex	Number of patient isolates	Number of carrier isolates	Ratio
11	93	10	9.3
23	7	32	0.2
32	54	18	3.0
35	6	18	0.3
41/44	44	42	1.0
162	16	17	0.9
269	15	7	2.1
Other STs	79	209	0.4

complex, over one third of the carrier isolates fell into that 'not assigned' category. The association of clone-complexes with carriage or disease measured by the ratio of patient to carrier strains for the various lineages represented in the strain collection showed that the ST-11, ST-32 and ST-269 complexes were positively associated with disease, whereas the ST-23 and ST-35 complexes were positively associated with carriage (Table 2). When adjusted for year of isolation and geographical source, the associations of ST-11 with disease and of ST-23 with carriage were still highly significant (Yazdankhah *et al.*, 2004). These data demonstrate that carrier populations are not panmictic and provide further evidence that important differences in pathogenicity exist between meningococcal clone complexes.

Geographic structuring and temporal evolution of carrier populations in Europe

In the same way that clone-complexes of *N. meningitidis* recovered from disease cases are not uniformly distributed throughout the world, clone-complexes of *N. meningitidis* circulating in the asymptomatic population differ among countries. Members of the newly identified ST-162 complex, which has been associated with recent outbreaks in the Athens region (Tzanakaki *et al.*, 2006), were present solely in the carrier isolate collection from Greece (Fig. 2). The clone-complex distributions were fairly similar in Norway and Greece. In contrast, the distribution in the Czech Republic was very divergent; the ST-23 and ST-269 complexes were absent but the ST-18 and ST-92 complexes well represented. Comparisons of the allele sequence frequencies for the seven housekeeping loci by analysis of molecular variance (Excoffier *et al.*, 1992) demonstrated highly significant country-to-country differentiation. Although lower than that evidenced in comparing disease isolates, the genetic differentiation among countries of the carrier isolates was significant at all loci, with F_{ST} values ranging from 0.024 (*abcZ* locus) to 0.071 (*aroE* locus).

In an attempt to look at temporal changes in the meningococcal carrier population, we compared the clone-complex distribution in three collections of carrier isolates obtained over a 12-year period in Norway. Few changes over time were observed. This clonal stability of the carried population was in strong contrast to the variation in the frequency of the disease-causing clones over the same period. For example, the carriage rate of the ST-32 complex remained at around 5% for the whole time, but it was responsible for about 70% of the cases in 1991 and only 15% in 2003. Additionally, in that period the incidence of meningococcal disease decreased by a factor of 4. The reduction in disease rate, in spite of the stability of the prevalence of ST-32 among health carriers, may be the result of acquired immunity of the population toward that clone and/or alterations of the virulence properties of ST-32 strains over time. Therefore, it seems that, just as carriage rates cannot be used to predict changes in the epidemiology of meningococcal disease, carriage rates of hypervirulent clones cannot be directly related to disease incidence rates.

However, in a comparison of two carrier strain collections in the Czech Republic (81 strains in 1994–96 and 35 strains in 2002–2003), an increase in the representation of the ST-22, ST-32 and ST-41/44 complexes was seen over the 10-year period. This increase was associated with a decline of ST-92, a clone associated with carriage in the Czech Republic but rarely recovered elsewhere. It can be speculated that this temporal change in the carried meningococcal population in the Czech Republic may be a consequence of the increase in communication with Western and Southern Europe that has occurred since the early 1990s.

Measurement of carriage of multiple lineages by individuals

Recombination is the main force driving the evolution of the meningococcal genome (Feil *et al.*, 1999; Jolley *et al.*, 2005). Horizontal genetic exchange between strains of *N. meningitidis* must then occur during the carriage stage in the throat of healthy individuals colonized by multiple clones. In an attempt to provide evidence for multiple colonization and to determine the frequency of this occurrence, multiple colonies were isolated and characterized from throat samples of healthy carriers in the Czech Republic, Greece and Norway. Usually up to 20 meningococcal colonies per throat sample were picked and stored. The genetic homogeneity of the colonies in each sample was screened by RAPD or by the VNTR method. From each individual carrier, colonies with distinct RAPD or VNTR patterns were further characterized by serogrouping, serotyping, antibiotic susceptibility testing and MLST. Minor variation among colonies within a sample was more frequently detected using RAPD than the VNTR method. However, in several cases the RAPD variation was

not confirmed by MLST analyses, showing that the minor variation obtained with RAPD reflected instability in experimental conditions rather than true genetic differences. Thus, as was also found in a study comparing RAPD and VNTR for analysis of outbreaks, VNTR proved more reliable than RAPD (Tzanakaki *et al.*, 2006).

In the Czech Republic a carriage study was performed in 206 high school students between October 2002 and March 2003. Thirty three (16.0%) carriers were identified. A total of 1242 colonies from 119 positive samples were isolated and tested by RAPD. Twenty two RAPD patterns were distinguished. RAPD variation was detected among colonies in two samples from two different individuals. The presence of distinct clones was confirmed by MLST (ST-254 and ST-255 in one sample and ST-53 and ST-110 in the other one) (Krizova *et al.*, 2004; Table 3). The genetic relationships among the STs of the carrier isolates in the Czech Republic are shown in Fig. 3.

In Norway a carriage study in 126 military recruits was performed between February and July 2003. A total of 78 carriers (carriage rate = 61.9%) were identified. In all, 5902 individual colonies were isolated and preserved. Nineteen of

Table 3. STs (number of colonies) of *Neisseria meningitidis* colonizing individual carriers at a single swabbing

Carrier	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
CR131	ST-53 (20)	ST-53 (9) ST-110 (1)	ST-110 (11)	NS	ST-110 (16)	ST-110 (6)
CR200	ST-254 (8) ST-255 (2)	ST-254 (20)	NS	NS	ST-254 (47)	NS
N4132	ST-23 (20)	ST-23 (19) ST-32 (1)	NS	ST-23 (3) ST-32 (1)	NS	ST-32 (20)
N4365	ST-2328 (20)	ST-275 (20)	ST-275 (20)	ST-275 (19) ST-2328 (1)	NS	ST-275 (20)

NS, not sampled.

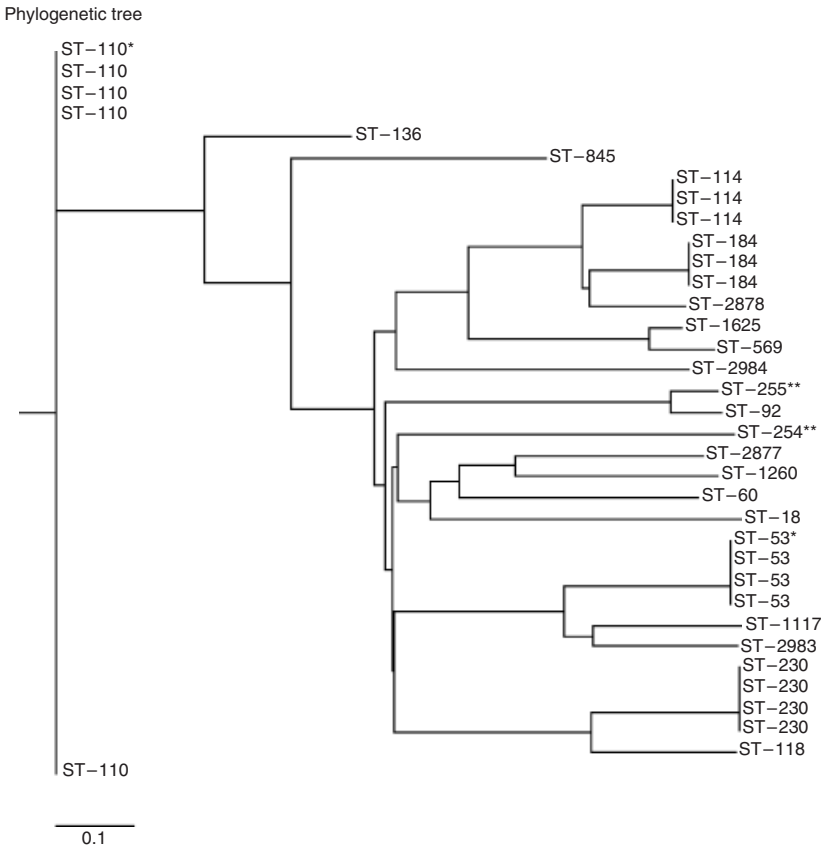

Fig. 3. Genetic relationships among STs of carrier isolates recovered from schoolchildren, Czech Republic, 2003. The tree was constructed with the neighbour-joining method using the program provided at http://pubmlst.org/software/analysis. Isolates of the carrier no. 131 are marked by an asterisk; isolates of the carrier no. 200 are marked by two asterisks.

the 78 carriers were selected for studying multiple carriage; 1897 colonies from 104 positive samples were tested by VNTR. Several VNTR types were seen in some throat samples. The presence of more than one clone, as confirmed by MLST, was detected in only three samples from two carriers (ST-23 and ST-32 in two samples for the same individual and ST-275 and ST-2328 in one sample from another carrier).

In Greece two carriage studies were performed in university students aged 18–25 years old, between February and June 2002 ($n = 102$) and between February and June 2003 ($n = 100$), respectively. Overall, 37 (18%) carriers were identified. About 2000 colonies were isolated from 124 throat samples and tested by RAPD and phenotypic methods (serogroup, serotype/subtype). Minor changes in one of the two primers used were detected within individual samples of three carriers. However, none of these samples harboured more than one clone, as evidenced by MLST.

Overall, about 15 colonies per sample were examined in nearly 350 samples. In five (1.4%) of the samples, more than one clone was detected. In picking 15 colonies, the probability of detecting carriage of more than one clone is about 80% even if the proportion of the less common clone is $< 10\%$ of the colonies. Although rare, colonization of the throat by multiple meningococcal clones was clearly demonstrated. In one individual, colonization of the throat by the same two clones was observed in two samples, 2 months apart, suggesting that the phenomenon can be stable. Meningococcal carriage is a common phenomenon and thousands of genotypes exist. It is thus surprising that in most cases the meningococcal flora is dominated by a single clone. Clonal exclusion may be a consequence of differential production of bacteriocins by various strains, resulting in killing of the susceptible nonproducers. However, bacteriocin production is a very rare property of meningococcal strains (Allunans & Bøvre, 1996). Clonal exclusion may more likely reflect differences in fitness of the colonizing strains, as for example differences in multiplication rate or in resistance to the host defences (Lipsitch & Moxon, 1997). While colonization of healthy individuals by multiple clones in relatively similar abundance is a rare event, it is likely very significant to the biology of *N. meningitidis* and is one of the main reasons for the difficulties in designing an effective universal vaccine against meningococcal disease.

Establishment of the duration of carriage for given lineages

Knowledge regarding the duration of carriage is limited because most longitudinal studies of carriage have used only phenotypic techniques for strain characterization and many carried isolates are both nonserogroupable and nonserotypable. Another difficulty is motivation of the volunteers to repeatedly have a throat sample taken, which, although not dangerous, is not especially pleasant.

Longitudinal studies were performed at monthly intervals over 5 or 6 months. In total, 534 individuals aged 15–25 years old were enrolled in three study sites (Czech Republic, Greece and Norway). Carriage rates fluctuated between 16% and 62% depending on the study site and the population sampled, but the carriage rate showed little variation at each site during the survey. Stable carriers were defined as individuals from whom at least four throat samples had been taken, all of them were positive for meningococci. Overall, 43–69% of the individuals were found to be stable carriers.

Using RAPD or VNTR in combination with phenotypic methods to screen strain heterogeneity, and verification using MLST, the dynamic of meningococcal carriage in the 147 individual hosts was followed. For only one of the schoolchildren (CR-131) was a change of strain evidenced in the course of the study period, whereas eight (10%) of the 77 military recruits experienced at least one strain replacement (Table 4). In several cases, the change of clone was temporary, suggesting that the individuals either were recolonized by the previous colonizing clone from their environment or may be harbouring several different meningococcal clones within a sample, the apparent clone replacement simply being a consequence of the colony picked and analyzed.

These studies demonstrated that the commensal association of particular clones with a host is a long-term relationship, with 90% of the carriers keeping the same clone for at least 5–6 months. Whether some clones are better at establishing a long-term commensal relationship than others still remains to be demonstrated. Despite the rather large size of this study, because of the genetic diversity of the carried meningococcal population the number of carriers of each clone or even of each clone-complex was too limited for analysis. The ST-23 complex seems to be a very good colonizer and readily transmitting among associated individuals. In contrast, as seen in other studies (Caugant *et al.*, 1988; Claus *et al.*, 2005), the ST-11 complex strains (formerly ET-37 complex) were seldom found in healthy carriers; they were absent in the longitudinal studies in Greece and in the Czech Republic, and the two military recruits identified with an ST-11 lost the carriage status in the following samples. These data suggest that strains of the ST-11 complex must have a high transmission rate to balance their short duration of carriage (Moxon & Jansen, 2005). The strong association between the ST-11 complex and expression of a serogroup C capsule (Yazdankhah *et al.*, 2004; Claus *et al.*, 2005) may ensure the fitness of the clonal complex in terms of transmission, as well as its pathogenic

Table 4. Characteristics (ST-complex; serogroup:serotype:serosubtype) of the meningococcal strains in nine healthy carriers experiencing clonal replacement over a 6-month period

Carrier no.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
CR131	53	53	41/44	NS	41/44	41/44
	NG:21:P1.7	NG:21:P1.7	B:4:P1.15		B:4:P1.15	B:4:P1.15
N4132	23	32	NS	32	NS	32
	Y:14:P1.6	NG:15:P1.7		NG:15:P1.7		NG:15:P1.7
N4331	UA	198	UA	NS	198	NS
	29E:16:P1.16	29E:NT:NST	29E:16:P1.16		NG:NT:P1.6	
N4358	UA	UA	UA	UA	23	23
	NG:4,7:P1.7,1	NG:4,7:P1.7,1	NG:4,7:P1.7,1	NG:4,7:P1.7,1	Y:14,19:P1.5,2	Y:14,19:P1.5,2
N4365	23	269	269	269	NS	269
	Y:14,19:P1.6	B:7:P1.9	B:7:P1.9	B:7:P1.9		B:7:P1.9
N5013	269	269	UA	269	269	269
	C:NT:P1.7,1	C:NT:P1.7,1	NG:16:P1.2	C:NT:P1.7,1	C:NT:P1.7	C:NT:P1.7
N5032	UA	198	269	NS	198	UA
	NG:16:P1.5,2	NG:15:P1.6	C:NT:P1.1		NG:15:P1.6	NG:16:P1.5,2
N5036	32	32	32	NS	NS	UA
	NG:15:NST	NG:15:NST	NG:15:NST			NG:7:P1.12,13
N8038	41/44	41/44	NS	NS	269	41/44
	NG:14,19:P1.7	NG:14,19:P1.7			B:7:P1.9	NG:14,19:P1.7

NS, not sampled; NG, nonserogroupable; NT, nonserotypable; NST, nonserosubtypable; UA, unassigned.

behaviour, while counteracting its capability to be carried for a long time.

Carriage and vaccination

A number of studies have been performed to investigate the effect of vaccination on meningococcal carriage, using the polysaccharide vaccines, the polysaccharide conjugate vaccines, and, to a lesser extent, the serogroup B outer membrane vesicle vaccine.

The effect of the polysaccharide vaccines on colonization of the meningococci is, at best, transient, because the level of antibodies declines rapidly to near prevaccination levels and is insufficient to prevent colonization on the mucosa (Jodar *et al.*, 2002). With outer membrane vesicle vaccines against serogroup B disease, a slight reduction in carriage rate among vaccinated individuals has been seen (Rosenqvist *et al.*, 1994). When conjugated to protein antigens, however, polysaccharide vaccines may induce an immune response that is higher than that achieved by the polysaccharide vaccines (Zhang *et al.*, 2000), and leak of antibodies to the mucosa then affects colonization.

An effect on nasopharyngeal carriage was shown by Maiden and colleagues, who demonstrated a 67% reduction from 0.45% to 0.15% in the prevalence of serogroup C carriage in adolescents 1 year after the introduction of the C conjugate polysaccharide vaccine in the UK (Maiden & Stuart, 2002). Reduction of carriage was found to result in a similar reduction in the incidence of meningococcal disease in nonvaccinated groups and contributes to herd immunity (Ramsay *et al.*, 2005). Thus, when introducing new meningococcal vaccines and assessing their effectiveness, it is essential to consider their effect on the dynamics of meningococcal carriage.

Conclusions

Carriage studies are important to improve our understanding of the population structure of *N. meningitidis* and the epidemiology of meningococcal disease. MLST analyses have confirmed the extensive diversity of the carried meningococci, but have also provided evidence that meningococcal populations from healthy carriers comprise a number of successful clones that are geographically widespread. In most cases, the commensal association with a meningococcal strain is a long-term relationship (at least 5–6 months) and clone replacement occurs during long-term carriage, albeit infrequently. Although rare in our experimental conditions, simultaneous colonization by multiple clones was demonstrated, indicating that recombination between them may generate novel genotypes that can be selected within the human host.

Furthermore, it was confirmed that hypervirulent ST-complexes are rare among strains from carriers. Variation in incidence of disease caused by these clonal complexes does not appear to reflect a variation in their carriage rate in the human population. The hypervirulent clones vary greatly in their capability to establish a commensal relationship with their host, the strains of the ST-11 complex being especially poor colonizers. In contrast, some clones, such as the ST-23 complex, appear to be well-adapted to the commensal relationship with the host.

Perspectives

Further investigations of the following aspects of meningococcal carriage are warranted:
(1) As most carriers were stable carriers for a period of 5–6 months, it is clear that to determine the duration of carriage and how it varies for specific clones it will be necessary to follow many individuals for much longer periods.
(2) There is a need for a better assessment of the relation between carried and disease-causing clones and for accurate determination of the relative virulence potential of hypervirulent sequence types and clonal complexes, especially as additional ones are identified.
(3) The geographical component of the diversity of carriage strains should be ascertained further. This might be especially important in relation to the introduction of new meningococcal vaccines and estimation of the herd effect.
(4) Additional carriage studies, including extensive molecular strain characterization, should be performed before and after vaccination in countries where mass vaccination is introduced.
(5) The effect of vaccination in individual carriers should be studied and coupled to serological analyses.

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