JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2001, p. 1235–1240 Vol. 39, No. 4
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.4.1235–1240.2001

Phenotypic and Genotypic Approaches to Characterization of Isolates of *Neisseria meningitidis* from Patients and Their Close Family Contacts

G. TZANAKAKI,[1] R. URWIN,[2] M. MUSILEK,[3] P. KRIZ,[3] J. KREMASTINOU,[1] A. PANGALIS,[4] C. C. BLACKWELL,[4] AND M. C. J. MAIDEN[2]*

National Meningococcal Reference Laboratory, National School of Public Health,[1] and Agia Sophia Children's Hospital,[4] Athens, Greece; Wellcome Trust Centre for the Epidemiology of Infectious Disease, Department of Zoology, University of Oxford, Oxford, England[2]; National Reference Laboratory for Meningococcal Infections, NIPH, Prague, Czech Republic[3]; and Department of Medical Microbiology, University of Edinburgh, Edinburgh, Scotland[5]

Received 27 October 2000/Returned for modification 1 December 2000/Accepted 12 January 2001

Characterization of isolates of *Neisseria meningitidis* obtained from patients with meningococcal disease or from pharyngeal swabs of asymptomatic carriers can be achieved by several approaches which provide different levels of discrimination. A total of 45 gram negative, oxidase-positive diplococcus strains isolated from 15 individuals with meningococcal disease and 30 of their family contacts were examined by three approaches: serological typing, multilocus enzyme electrophoresis (MLEE), and multilocus sequence typing (MLST). For 10 of the 15 patient and contact groups, all of the isolates were confirmed as meningococci, and the bacteria obtained from the patients and contacts, including their mother or principal caregiver in the case of children, were indistinguishable by all three methods. In the remaining five groups the isolates from the patients were distinct from those recovered from the contacts, and in three examples, in two separate groups, the contacts were shown by MLST to be carrying strains of *Neisseria lactamica*. The data obtained from the three techniques were consistent, although complete serological typing was possible for only a minority of isolates. Both MLEE and MLST established the genetic relationships of the isolates and identified members of known hypervirulent lineages, but MLST was faster than MLEE and had the additional advantages that it could be performed on noninfective material distributed by mail and that the results from different laboratories could be compared via the internet (http://mlst.zoo.ox.ac.uk).

For many years, epidemiological investigations of outbreaks of disease caused by *Neisseria meningitidis* have relied on the serological characterization of isolates (14). This culminated in the development of a scheme that included the capsular polysaccharide (serogroup) and two of the major outer membrane protein antigens PorB (serotype) and PorA (serosubtype) (15). This scheme was of great assistance in numerous epidemiological investigations of meningococcal disease, and the development and distribution of commercial kits for serogroup determination (37) and monoclonal antibodies for serotype and serosubtype identification (1, 2) resulted in the wide acceptance of this technique.

Despite these advantages, serological typing exhibits several flaws (24). Serogroup remains an essential characteristic since only meningococci expressing 5 of the 13 recognized serogroups cause most disease (38). Given that most isolates from diseased patients in Europe and the Americas are either serogroup B or C, this characteristic is insufficiently discriminating for epidemiological investigations. Greater discrimination is provided by the determination of the serotype and serosubtype, but many isolates remain nontypeable or not subtypeable (28, 35), particularly those recovered from asymptomatic carriers (9).

There are a number of reasons why serotyping and serosubtyping fail, including the high diversity of the PorA and PorB antigens and limitations in the panel of monoclonal antibodies available. Indeed, it is likely that the rate of evolution of these antigens is such that it may not be possible to generate and maintain a comprehensive panel of these reagents (13). Further, some meningococci downregulate expression of the PorA gene (36), while in others expression of this gene is disrupted by an insertion sequence (3), precluding phenotypic characterization. Performance of the assays and variations in experimental techniques can also lead to no result or an incorrect result (27).

Additional difficulties are caused by strong diversifying selection, presumably imposed by the host immune response, acting on meningococcal surface antigens and the propensity of these bacteria to exchange DNA by transformation (22). These factors result in serological characterization being an unreliable indicator of genetic relatedness. This is problematic since only a few lineages of this diverse species are regularly involved in outbreaks of disease, and it is often not possible to associate an outbreak with a particular lineage unambiguously by serological characterization alone (11). Meningococci never associated with outbreaks may have serological characteristics common among those that are and vice versa. The application of multilocus enzyme electrophoresis (MLEE) to meningococ-

* Corresponding author. Mailing address: Wellcome Trust Centre for the Epidemiology of Infectious Disease, Department of Zoology, University of Oxford, South Parks Rd., Oxford OX1 3FY, United Kingdom. Phone: 44-1865-271284. Fax: 44-1865-271284. E-mail: martin.maiden@zoo.ox.ac.uk.

cal isolate collections (30) has established the existence of particular meningococcal lineages associated with invasive disease and played a seminal role in elucidating the epidemiology and population biology of the meningococcus (7); however, for largely practical reasons this technique has not been widely adopted by reference laboratories and has rarely been employed during outbreak investigations.

The advent of high-throughput nucleotide sequence determination technologies, including multilocus sequence typing (MLST), provides additional approaches to isolate characterization (23). The MLST approach is equivalent to MLEE in that genetic relationships are established by the analysis of housekeeping genes, but it differs in that nucleotide sequence determination is employed to identify the alleles. This approach has a number of advantages. Nucleotide sequences are ultimately discriminating in that all genetic variation is identified, and consequently the number of loci examined can be reduced from ca. 15 to 20 down to 7 without loss of resolution. Nucleotide sequence determination is a generic technology that is portable among laboratories, and nucleotide sequence data are ideal for electronic transfer via the internet. A further advantage is that killed cell suspensions or purified DNA can be used for MLST, thus eliminating the need to transfer live meningococci among laboratories.

Studies done in central and southern Europe have established that many of the isolates obtained from patients and asymptomatic carriers did not react with the serological reagent panel, despite the introduction of new reagents (20, 34). The present study compared isolate characterization by MLST with serological typing and MLEE on a set of meningococcal strains obtained from patients with invasive meningococcal disease and their contacts in Greece.

MATERIALS AND METHODS

Bacterial isolates. A total of 15 isolates from patients with meningococcal disease and 30 from oropharangeal swabs of their family contacts or caregivers (between one and four for each patient isolate; contact groups 1 to 15 in Table 1) were included in the study. The patients were aged between 2 months and 63 years, and all had presented at pediatric or infectious disease hospitals in Greece in the period from 1996 to 1998. The patient isolates were grown from clinical specimens on chocolate agar (Columbia agar supplemented with 5% blood). The carrier isolates were grown from swabs on modified New York City Medium. In each case suspected meningococcal colonies were characterized by Gram stain, oxidase test, and rapid carbohydrate utilization test (Gallerie Pasteur; Pasteur-Merieux).

Serological typing. Serogroups were determined by slide agglutination with polyclonal antisera to serogroups A, B, C, W-135, X, Y, and Z (Wellcome Diagnostics). Serotypes and subtypes were determined by whole-cell enzyme-linked immunoassay with monoclonal antibody reagents supplied by the Rijksinstituut voor Volksgezondheid en Milieu (Bilthoven, The Netherlands) and the National Institute for Biological Standards and Control (Potters Bar, United Kingdom) (1, 2).

MLEE. The following enzymes were assayed: malic enzyme (EC 1.1.1.40), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), leucine aminopeptidase (EC 3.4.11.1), isocitrate dehydrogenase (EC 1.1.1.42), aconitase (EC 4.2.1.3), glutamate dehydrogenase (NADP dependent) (GD1; EC 1.4.1.4), glutamate dehydrogenase (NAD dependent) (GD2; EC 1.4.1.2), alcohol dehydrogenase (EC 1.1.1.1), fumarase (EC 4.2.1.2), alkaline phosphate (EC 3.1.3.1), superoxide dismutase (EC 1.15.1.1), and adenylate kinase (EC 2.7.4.3). Preparation of enzyme extracts, horizontal starch gel electrophoresis of fumarase, and enzyme-staining procedures were performed as described previously (30) with the exception of the substitution of 0.2 M Tris-maleate (pH 6) for 0.2 M Tris-Cl (pH 8) in PEP-staining solution and of direct superoxide dismutase pattern determination on gels stained for ACO. Other isozymes were separated by polyacrylamide gel electrophoresis on 10% gels with 3% stacking gels, both containing 0.2 M Tris-Cl (pH 8.3) at 500 V at 4°C for 2.5 h (12 h for PEP, GD1, and GD2 assays) in standard Tris-glycine buffer and in Protean II (Bio-Rad). Enzyme extracts were diluted 1:4 in 40% glycerol-water solution and loaded on to the gels as 10-μl samples. This setting provided better isozyme discrimination than starch gel electrophoresis. Data management was performed with the Statistica package (StatSoft).

MLST. Isolates were sequence typed from boiled cell suspensions using the procedures described previously (16, 23). The killed-cell suspensions were prepared in Greece and sent by regular mail to the United Kingdom for MLST. Briefly, MLST loci were amplified by the PCR and purified by precipitation with 20% polyethylene glycol 8000–2.5 M Nacl (12), and their nucleotide sequences were determined at least once on each DNA strand with BigDye Ready Reaction Mix (Applied Biosystems) used in accordance with the manufacturer's instructions. Unincorporated dye terminators were removed by precipitation of the termination products with 95% ethanol, and the reaction products were separated and detected with an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Sequences were assembled from the resultant chromatograms with the STADEN suite of computer programs (31).

Isolates were assigned sequence types (STs) by reference to the MLST website (http://mlst.zoo.ox.ac.uk), and membership of STs to lineages was established with the computer program BURST (written by E. J. Feil and M. S. Chan). BURST defined lineages as groups of isolates where each member shared at least four alleles with at least one other member of the lineage. Lineages were named after the central ST, as defined by BURST, followed by the word "complex," e.g., ST-11 complex. If the lineage had been identified previously and was present in the MLST database (http://mlst.zoo.ox.ac.uk), then the previously associated ST was used for the name of that lineage. For example, ST-153 was a member of the ST-8 complex (cluster A4) complex, although no examples of ST-8 were present in the sample described here. For clarity both the original nomenclature for clusters, based on MLEE designations, and the designations based on the ST assignments are used throughout this work. The BURST program is available interactively on the MLST website (http://mlst.zoo.ox.ac.uk/).

RESULTS

There was good agreement among serological characterization, electrophoretic type, and sequence type for all 45 isolates examined (Table 1). Three isolates from patient contacts (contact group 11, isolates BM35a and BM35b, and contact group 12, isolate BM68a) were serologically nontypeable and had anomalous electrophoretic types. In each of these cases the nucleotide sequences of the housekeeping alleles determined for MLST analysis were consistent with *Neisseria lactamica* sequence types: these isolates were subsequently confirmed by further microbiological analyses to be *N. lactamica*. The 42 meningococcal strains comprised 1 serogroup A, 33 serogroup B, 7 serogroup C, and 1 nongroupable isolates. Serological characterization was mostly incomplete: 4 isolates were fully characterized with one serotype and two serosubtypes, 27 isolates were characterized for serotype and one serosubtype, 1 isolate was characterized only for serotype, 7 isolates were characterized for only one serosubtype, and 3 isolates were not characterized for serotype or subtype. The isolates were resolved into 13 electrophoretic types and 14 STs by MLEE and MLST, respectively.

In 10 of the contact groups (1 to 10, Table 1), the patient isolates were indistinguishable from those obtained from the patient contacts by all criteria. These isolates included seven contact groups from which the mother or principal caregiver of a child with meningococcal disease had also been sampled. For the remaining five sets of patient-contact isolates, the invasive isolate had phenotypic and genotypic characteristics that were distinct from those carried by the contacts.

The MLEE and MLST analyses established that 9 of the 15 patient isolates belonged to known hyperinvasive lineages: three ET-37 (ST-11) complex isolates, one lineage III (ST-41

TABLE 1. Serological and molecular characterization of isolates from patients and their close contacts

Contact group (yr)	Isolate	Source (age or relationship to patient)	Serological characteristics[a]	MLEE classification	MLST classification
1 (1996)	BM34	Patient (7 yr)	B:NT:NST	O	ST-161 (ST-432 complex)
	BM34a	Carrier (caregiver)	B:NT:NST	O	ST-161 (ST-432 complex)
2 (1996)	BM45	Patient (8 yr)	C:2a:P1.5	ET-15 (ET-37 complex)	ST-11 (ST-11 complex)
	BM45a	Carrier (mother)	C:2a:P1.5	ET-15	ST-11 (ST-11 complex)
3 (1996)	BM48	Patient (5 yr)	C:2a:P1.2	ET-15	ST-11 (ST-11 complex)
	BM48a	Carrier (mother)	C:2a:P1.2	ET-15	ST-11 (ST-11 complex)
4 (1996)	BM49	Patient (7 yr)	B:4:P1.14	I	ST-160 (ST-35 complex)
	BM49a	Carrier (sister)	B:4:P1.14	I	ST-160 (ST-35 complex)
	BM49b	Carrier (mother)	B:4:P1.14	I	ST-160 (ST-35 complex)
5 (1996)	BM55	Patient	B:4:P1.9	Lineage IV	ST-163
	BM55a	Carrier (father)	B:4:P1.9	Lineage IV	ST-163
6 (1996)	BM59	Patient (4 yr Dutch)	B:4:P1.4	Lineage III	ST-41 (ST-41 complex)
	BM59a	Carrier (sister)	B:4:P1.4	Lineage III	ST-41 (ST-41 complex)
	BM59b	Carrier (mother)	B:4:P1.4	Lineage III	ST-41 (ST-41 complex)
	BM59c	Carrier (father)	B:4:P1.4	Lineage III	ST-41 (ST-41 complex)
7 (1997)	BM65	Patient (15 mo)	B:4:P1.6	Lineage IV	ST-164
	BM65a	Carrier (father)	B:4:P1.6	Lineage IV	ST-164
	BM65b	Carrier (brother)	B:4:P1.6	Lineage IV	ST-164
	BM65c	Carrier (mother)	B:4:P1.6	Lineage IV	ST-164
8 (1997)	W72	Patient (63 yr)	C:2a:P1.2,5	ET-37 complex	ST-211 (ST-11 complex)
	W72a	Carrier (wife)	C:2a:P1.2,5	ET-37 complex	ST-211 (ST-11 complex)
	W72b	Carrier (son)	C:2a:P1.2,5	ET-37 complex	ST-211 (ST-11 complex)
9 (1998)	BM95	Patient (2 mo)	B:15:P1.13	S	ST-187
	BM95a	Carrier (father)	B:15:P1.13	S	ST-187
	BM95b	Carrier (mother)	B:15:P1.13	S	ST-187
10 (1998)	W134	Patient	B:NT:P1.14	D	ST-162
	W135	Carrier (mother)	B:NT:P1.14	D	ST-162
	W134b	Carrier (sister)	B:NT:P1.14	D	ST-162
11 (1996)	BM35	Patient (2 yr)	B:NT:P1.14	D	ST-162
	BM35a	Carrier (father)			*N. lactamica*
	BM35b	Carrier (mother)			*N. lactamica*
12 (1997)	BM68	Patient (5 yr)	B:NT:P1.14	D	ST-162
	BM68a	Carrier (father)			*N. lactamica*
	BM68b	Carrier (aunt)	NG:NT:P1.2	R	ST-196
13 (1997)	BM80	Patient (14 yr)	B:2b:P1.10	A4 cluster	ST-153 (ST-8 complex)
	BM80a	Carrier (mother)	B:NT:P1.14	D	ST-162
14 (1998)	W138	Patient (4 mo)	B:2a:P1.2,5	ET-37 complex	ST-11 (ST-11 complex)
	W138a	Carrier (aunt)	A:4:NST	T	ST-103
	W138b	Carrier (grandfather)	B:NT:NST	U	ST-197
15 (1996)	BM33	Patient (7 yr)	B:4:P1.15	ET-5 complex	ST-33 (ST-32 complex)
	BM33a	Carrier (mother)	B:4:P1.14	I	ST-160 (ST-35 complex)
	BM33b	Carrier (sister)	B:4:P1.14	I	ST-160 (ST-35 complex)
	BM33c	Carrier (grandfather)	B:4:PI.14	I	ST-160 (ST-35 complex)
	BM33d	Carrier (cousin)	B:4:P1.14	I	ST-160 (ST-35 complex)
	BM33e	Carrier (cousin)	B:4:P1.14	I	ST-160 (ST-35 complex)

[a] NG, not groupable; NT, not typeable; NST, not subtypeable.

complex) isolate, two lineage IV isolates, one A4 cluster (ST-8 complex) isolate, and one ET-5 (ST-32) complex isolate. The members of the ET-37 complex exhibited two STs (ST-11 and ST-211), but ET-15 variants of this complex, which were distinguished by the MLEE, were indistinguishable from other ST-11 isolates by MLST. The ET-37 complex isolates all shared similar, but not identical, serological characteristics. The two lineage IV isolates were distinguished by their STs (ST-163 and ST-164) and by their serological properties, but not by the MLEE analysis. The four lineage III (ST-41 complex) isolates were uniform for all characteristics examined. Of the remaining six isolates obtained from patients with invasive disease, three were ST-162 meningococci, which may represent a previously undescribed hyperinvasive lineage. These bacteria were also isolated from four carriers and were uniform by MLEE analysis and for serological characteristics. One case of meningococcal disease was caused by a meningococcus belonging to the ST-432 complex, with sequence type ST-161. Most members of this complex in the MLST database were isolated from healthy carriers rather than from patients with invasive meningococcal disease. A further case of disease was caused by a novel variant of the ST-35 complex, ST-160. There was one case caused by a novel ST, ST-187.

DISCUSSION

The meningococcus is primarily a harmless inhabitant of the nasopharynx of adult humans (5). The organism is, however, capable of causing devastating meningococcal disease, which is manifested in four distinct epidemiological forms: endemic, hyperendemic, localized outbreak, and epidemic or pandemic outbreak (25). Since different lineages of meningococci are

associated with each of these epidemiologies, assignment of disease-associated isolates to lineages is important for appropriate public health management. With the exception of the subgroups of serogroup A (32), which are responsible for large-scale epidemic or pandemic outbreaks in Africa and Asia, meningococcal lineages cannot be unambiguously identified by serological means, as a consequence of antigenic diversity or lack of reagents. Therefore, genetic characterization techniques such as MLEE or MLST are important for disease surveillance and for informing public health policy and management.

In Europe and the Americas, meningococcal disease is a sporadic endemic infection, associated mainly with infants and, to a lesser extent, young adults (17). Many meningococcal genotypes can be responsible for sporadic infections, but occasionally a lineage with a particular propensity to cause disease may arise and spread within and among countries, leading to the elevated levels of infection known as hyperendemic disease, as has been documented for the ET-5 (ST-32) complex (8) and lineage 3 (ST-41 complex) (29). In addition, certain meningococcal lineages, especially the ET-37 (ST-11) complex, are particularly likely to cause localized outbreaks of disease, which are often associated with closed or semi-closed communities such as schools and military recruit camps (40).

The data from each of the characterization methods employed in the present study were consistent but provided different levels of discrimination. As has been noted in previous studies, serological typing provided partial information which, while being potentially useful in short-term outbreak investigation, did not allow unambiguous assignment of meningococcal isolates to lineages (11). Both MLEE and MLST analyses enabled lineages to be identified with minor variation in levels of discrimination. For example, MLEE was able to discriminate ET-15 meningococci from other members of the ET-37 complex, which MLST did not, although one variant of the ET-37 (ST-11) complex (contact group A, patient, ST-211) which was not distinguished by MLEE was discriminated by MLST. This is because the difference defining these two variants by MLEE is a single nonsynonymous nucleotide polymorphism in the *fumC* gene at base position 640 which results in a glutamate-to-lysine change in the protein, thereby altering the electrophoretic mobility of the FumC protein. Although the *fumC* locus is included in the MLST system, this base position is not used in allele assignment for MLST purposes. In this particular case the nucleotide present at this base position could be determined to identify an ET-15 variant of the ET-37 complex (39). On the other hand, MLST discriminated two related strains which were both assigned as lineage IV by MLEE. Since no genotyping method exhaustively samples the variation present among isolates, such minor discrepancies are to be expected with different approaches and can never be completely eliminated.

In all cases of sporadic meningococcal disease, and often in localized epidemic or hyperendemic outbreaks of meningococcal disease, analyzing solely disease-associated isolates provides little information on the spread of the disease-associated meningococcus. This is because the majority of infections of humans with meningococci, even those belonging to hyperinvasive lineages, do not result in disease. Consequently, studies of asymptomatic carriage are essential in order to understand the spread of the meningococcus. Such studies have shown that small children do not carry the meningococcus and therefore must acquire meningococci from older people, while the next most vulnerable age group, young adults, experience extremely high carriage rates, ranging from 20 to 40% (6, 10). In seven of the contact groups reported here, all of the data indicated that family members or caregivers, including seven mothers or principal caregivers of children, carried meningococci indistinguishable from those isolated from the patient. In each of these contact groups all of the meningococci recovered were indistinguishable. In the remaining five groups, the disease-causing genotype was not recovered from contacts, although meningococcal or *N. lactamica* isolates were identified in each group. In one example (contact group 15) five family members of an individual with meningococcal disease caused by a meningococcus belonging to the ST-32 (ET-5) complex were carrying meningococci that belonged to a different lineage. Since sampling of contacts occurs after a case has been identified and since meningococcal carriage is transitory, in each of these examples it is possible that some family members had carried the disease-causing meningococcus but were undetected. Further, sampling by throat swab cannot be considered to be one 100% sensitive and, without the isolation of multiple meningococci from each sample, it is impossible to rule out multiple carriage with the disease-associated meningococcus unidentified. It is also feasible that the disease-causing meningococcus had been acquired from a carrier outside the family.

The data provided a number of insights relevant to the current epidemiology of meningococcal disease in Europe. Meningococci expressing subtype P1.14 have been prevalent in Greece for several years (33) in two distinct phenotypes: B:4:P1.14, isolated in 1996 and 1997, and B:NT:P1.14 strains isolated from 1996 to 1998. These phenotypes correlated with distinct lineages, the B:4:P1.14 phenotype associated with ST-160 (ST-35 complex) and the B:NT:P1.14 associated with ST-62, which was unrelated to the ST-35 complex. Given the problems with the distinguishing phenotypic character, serotype 4 (35), this distinction highlights the need for genotypic characterization to complement serotyping. Consistent with experience in other countries in Europe and elsewhere, there was an increased number of cases of disease caused by serogroup C meningococci reported in Greece from 1996 to 1999 (18). These meningococci exhibited the phenotype C:2a:P1.2,5 and were particularly associated with disease outbreaks. A number of such meningococci were represented in the present study: two isolates from 1996 were identified as belonging to the ET-15 variant of the ET-37 (ST-11) complex and were identified by MLST as ST-11, whereas in 1997 non-ET-15 members of the ET-37 (ST-11) complex were identified. In 1998, an ET-15 (ST-11) meningococcus was isolated, expressing the serogroup B capsule (19): there would be no protection against this meningococcus provided by the serogroup C conjugate capsular polysaccharide vaccine currently being used to combat ET-37 disease in the United Kingdom. Finally, there was one example of a case caused by a lineage III (ST-41 complex) meningococcus, which was present in a Dutch family visiting Greece: The Netherlands was experiencing hyperendemic meningococcal disease caused by lineage III meningococci at this time (4).

These data indicate that the results obtained by phenotyping

are generally consistent with genotyping methods but that it is necessary to carry out genotyping to fully characterize isolates and to place them in a broader epidemiological context. The findings also highlight the need for studies of carriage in the definition of meningococcal outbreaks. The advantages of the MLST approach were that the typing was done remotely on boiled cell suspensions without the transport of live meningococci, it did not require a collection of reference isolates, and the data were readily comparable to other data by means of the MLST website. While sequencing has long been one of the more difficult and expensive techniques available for the characterization of bacterial isolates (26), due to recent technical developments this is no longer true (21). Commercial provision of sequencing reaction mixes and the widespread and the increasing availability of high-throughput sequencing equipment continue to increase speed and reduce costs. In this study the determination of MLST sequence types (seven loci on both strands) was achieved with commercial reagents at a cost of approximately $31 per isolate. Further, the setting up of sequencing reactions and the interpretation and analysis of nucleotide sequence data are especially amenable to automation, substantially reducing, and perhaps in time virtually eliminating, labor costs.

ACKNOWLEDGMENTS

We acknowledge the support of the Greek Ministry of Health, the families who participated in the study, the Meningitis Association of Scotland, The Grant Agency of the Czech Republic (grant 310/96/K102), and the Wellcome Trust. M.C.J.M. is a Wellcome Trust Senior Research Fellow.

This study made use of the Multi Locus Sequence Typing website (http://mlst.zoo.ox.ac.uk) developed by M.S. Chan and located at the Wellcome Trust Centre for the Epidemiology of Infectious Disease, University of Oxford. The development of this site is funded by the Wellcome Trust.

REFERENCES

1. **Abdillahi, H., and J. T. Poolman.** 1988. Definition of meningococcal class 1 OMP subtyping antigens by monoclonal antibodies. FEMS Microbiol. Immunol. **1:**139–144.
2. **Abdillahi, H., and J. T. Poolman.** 1987. Whole-cell ELISA for typing *Neisseria meningitidis* with monoclonal antibodies. FEMS Microbiol. Lett. **48:** 367–371.
3. **Arhin, F. F., F. Moreau, J. W. Coulton, and E. L. Mills.** 1998. Sequencing of *porA* from clinical isolates of *Neisseria meningitidis* defines a subtyping scheme and its genetic regulation. Can. J. Microbiol. **44:**56–63.
4. **Bart, A., J. Dankert, and A. van der Ende.** 1999. Antigenic variation of the class 1 outer membrane protein in hyperendemic *Neisseria meningitidis* strains in The Netherlands. Infect. Immun. **67:**3842–3846.
5. **Broome, C. V.** 1986. The carrier state: *Neisseria meningitidis*. J. Antimicrob. Chemother. **18**(Suppl. A)**:**25–34.
6. **Cartwright, K. A. V., J. M. Stuart, D. M. Jones, and N. D. Noah.** 1987. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol. Infect. **99:**591–601.
7. **Caugant, D. A., L. O. Froholm, K. Bovre, E. Holten, C. E. Frasch, L. F. Mocca, W. D. Zollinger, and R. K. Selander.** 1986. Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. Proc. Natl. Acad. Sci. USA **83:**4927–4931.
8. **Caugant, D. A., L. O. Froholm, K. Bovre, E. Holten, C. E. Frasch, L. F. Mocca, W. D. Zollinger, and R. K. Selander.** 1987. Intercontinental spread of *Neisseria meningitidis* clones of the ET-5 complex. Antonie Leeuwenhoek J. Microbiol. **53:**389–394.
9. **Caugant, D. A., E. A. Hoiby, P. Magnus, O. Scheel, T. Hoel, G. Bjune, E. Wedege, J. Eng, and L. O. Froholm.** 1994. Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. J. Clin. Microbiol. **32:**323–330.
10. **Caugant, D. A., B. E. Kristiansen, L. O. Froholm, K. Bovre, and R. K. Selander.** 1988. Clonal diversity of *Neisseria meningitidis* from a population of asymptomatic carriers. Infect. Immun. **56:**2060–2068.
11. **Caugant, D. A., L. F. Mocca, C. E. Frasch, L. O. Froholm, W. D. Zollinger, and R. K. Selander.** 1987. Genetic structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer membrane protein pattern. J. Bacteriol. **169:**2781–2792.
12. **Embley, T. M.** 1991. The linear PCR reaction: a simple and robust method for sequencing amplified rRNA genes. Lett. Appl. Microbiol. **13:**171–174.
13. **Feavers, I. M., A. J. Fox, S. Gray, D. M. Jones, and M. C. J. Maiden.** 1996. Antigenic diversity of meningococcal outer membrane protein PorA has implications for epidemiological analysis and vaccine design. Clin. Diagn. Lab. Immunol. **3:**444–450.
14. **Frasch, C. E.** 1987. Development of meningococcal serotyping, p. 39–55. *In* N. A. Vedros (ed.), Evolution of menigococcal disease, vol. II. CRC Press Inc., Boca Raton, Fla.
15. **Frasch, C. E., W. D. Zollinger, and J. T. Poolman.** 1985. Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. Rev. Infect. Dis. **7:**504–510.
16. **Jolley, K. A., J. Kalmusova, E. J. Feil, S. Gupta, M. Musilek, P. Kriz, and M. C. J. Maiden.** 2000. Carried meningococci in the Czech Republic: a diverse recombining population. J. Clin. Microbiol. **38:**4492–4498.
17. **Jones, D. M.** 1995. Epidemiology of meningococcal disease in Europe and the USA, p. 147–157. *In* K. A. V. Cartwright (ed.), Meningococcal disease. John Wiley & Sons, Ltd., Chichester, England.
18. **Kremastinou, J., G. Tzanakaki, A. Kansouzidou, A. Pagalis, V. Danielides, G. Kouppari, E. Lada, P. Kriz, M. Musilek, D. M. Weir, and C. C. Blackwell.** 1999. Recent emergence of serogroup C meningococcal disease in Greece. FEMS Immunol. Med. Microbiol. **23:**49–55.
19. **Kriz, P., B. Kriz, E. Svandova, and M. Musilek.** 1999. Antimeningococcal herd immunity in the Czech Republic—influence of an emerging clone, *Neisseria meningitidis* ET-15/37. Epidemiol. Infect. **123:**193–200.
20. **Krizova, P., M. Musilek, V. Danielova, and J. Holubova.** 1996. New serotype candidate of *Neisseria meningitidis*. Cental Eur. J. Public Health **4:**169–172.
21. **Maiden, M. C. J.** 2000. High-throughput sequencing in the population analysis of bacterial pathogens. Int. J. Med. Microbiol. **290:**183–190.
22. **Maiden, M. C. J.** 1993. Population genetics of a transformable bacterium: the influence of horizontal genetical exchange on the biology of *Neisseria meningitidis*. FEMS Microbiol. Lett. **112:**243–250.
23. **Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA **95:**3140–3145.
24. **Maiden, M. C. J., and I. M. Feavers.** 1994. Meningococcal typing. J. Med. Microbiol. **40:**157–158.
25. **Maiden, M. C. J., and I. M. Feavers.** 1995. Population genetics and global epidemiology of the human pathogen *Neisseria meningitidis*, p. 269–293. *In* S. Baumberg, J. P. W. Young, E. M. H. Wellington, and J. R. Saunders (ed.), Population genetics of bacteria. Cambridge University Press, Cambridge, England.
26. **Olive, D. M., and P. Bean.** 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. **37:**1661–1669.
27. **Poolman, J. T., P. Kriz-Kuzemenska, F. Ashton, W. Bibb, J. Dankert, A. Demina, L. O. Frøholm, M. Hassan-King, D. M. Jones, I. Lind, K. Prakash, and H. Xujing.** 1995. Serotypes and subtypes of *Neisseria meningitidis*: results of an international study comparing sensitivities and specificities of monoclonal antibodies. Clin. Diagn. Lab. Immunol. **2:**69–72.
28. **Scholten, R. J., H. A. Bijlmer, J. T. Poolman, B. Kuipers, D. A. Caugant, L. van Alphen, J. Dankert, and H. A. Valkenburg.** 1993. Meningococcal disease in The Netherlands, 1958–1990: a steady increase in the incidence since 1982 partially caused by new serotypes and subtypes of *Neisseria meningitidis*. Clin. Infect. Dis. **16:**237–246.
29. **Scholten, R. J. P. M., J. T. Poolman, H. A. Valkenburg, H. A. Bijlmer, J. Dankert, and D. A. Caugant.** 1994. Phenotypic and genotypic changes in a new clone complex of *Neisseria meningitidis* causing disease in The Netherlands, 1958–1990. J. Infect. Dis. **169:**673–676.
30. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51:**837–884.
31. **Staden, R.** 1996. The Staden sequence analysis package. Mol. Biotechnol. **5:**233–241.
32. **Suker, J., I. M. Feavers, M. Achtman, G. Morelli, J.-F. Wang, and M. C. J. Maiden.** 1994. The *porA* gene in serogroup A meningococci: evolutionary stability and mechanism of genetic variation. Mol. Microbiol. **12:**253–265.
33. **Tzanakaki, G., C. C. Blackwell, J. Kremastinou, D. M. Weir, A. Mentis, and R. J. Fallon.** 1993. Serogroups, serotypes and subtypes of *Neisseria meningitidis* isolated from patients and carriers in Greece. J. Med. Microbiol. **38:** 19–22.
34. **Tzanakaki, G., P. Kriz, J. Kremastinou, M. Musilek, L. E. Smart, and C. C. Blackwell.** 1997. Reactivity of the new monoclonal antibody '22' with meningococcal strains isolated from patients and carriers in Greece. FEMS Immunol. Med. Microbiol. **19:**1–5.
35. **Urwin, R., I. M. Feavers, D. M. Jones, M. C. J. Maiden, and A. J. Fox.** 1998. Molecular variation of meningococcal serotype 4 antigen genes. Epidemiol. Infect. **121:**95– 101.

36. **van der Ende, A., C. T. P. Hopman, S. Zaat, B. B. Oude Essink, B. Berkhout, and J. Dankert.** 1995. Variable expression of class I outer membrane protein in *Neisseria meningitidis* is caused by variation in the spacing between the −10 and −35 regions of the promoter. J. Bacteriol. **177:**2475–2480.
37. **van der Ende, A., I. G. Schuurman, C. T. Hopman, C. A. Fijen, and J. Dankert.** 1995. Comparison of commercial diagnostic tests for identification of serogroup antigens of *Neisseria meningitidis*. J. Clin. Microbiol. **33:**3326–3327.
38. **Vedros, N. A.** 1987. Development of meningococcal serogroups, p. 33–37. *In* N. A. Vedros (ed.), Evolution of meningococcal disease, vol. II. CRC Press, Inc., Boca Raton, Fla.
39. **Vogel, U., H. Claus, M. Frosch, and D. A. Caugant.** 2000. Molecular basis for distinction of the ET-15 clone within the ET-37 complex of *Neisseria meningitidis*. J. Clin. Microbiol. **38:**941–942.
40. **Wang, J.-F., D. A. Caugant, G. Morelli, B. Koumaré, and M. Achtman.** 1993. Antigenic and epidemiological properties of the ET-37 complex of *Neisseria meningitidis*. J. Infect. Dis. **167:**1320–1329.