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Characterization of *Neisseria meningitidis* isolates collected from 1974 to 2003 in Japan by multilocus sequence typing

Hideyuki Takahashi,[1] Toshiro Kuroki,[2] Yuko Watanabe,[2] Hiroshi Tanaka,[3] Hiroo Inouye,[3] Shiro Yamai[2] and Haruo Watanabe[1]

[1]Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan

[2]Department of Microbiology, Kanagawa Prefectural Public Health Laboratory, Chigasaki, Japan

[3]Department of Bacteriology, Ehime Prefectural Institute of Public Health and Environmental Science, Matsuyama, Japan

Correspondence
Haruo Watanabe
haruwata@nih.go.jp

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Analysis of 182 *Neisseria meningitidis* strains isolated over the past 30 years in Japan by serogroup typing and multilocus sequence typing (MLST) was performed. The serogroups of the 182 Japanese isolates were B (103 isolates), Y (39), W135 (1) and non-groupable (39). By MLST analysis, 65 different sequence types (ST) were identified, 42 of which were not found in the MLST database as of January 2004 and seemed to be unique to Japan. Statistical analysis of the MLST results revealed that, although the Japanese isolates seemed to be genetically divergent, they were classified into six major clonal complexes and other minor complexes. Among these isolates, well-documented ST complexes found worldwide were present, such as ST-23 complex (49 isolates), ST-44 complex (41 isolates) and ST-32 complex (8 isolates). On the other hand, a new clonal complex designated ST-2046 complex (28 isolates), which has not been identified in other countries, was also found, suggesting that this clone was indigenous to Japan. Taken together, it was speculated that meningococcal isolates in Japan comprised heterogeneous clones, which were derived both from clones identified in other countries and clones unique to Japan.

INTRODUCTION

Neisseria meningitidis frequently colonizes the nasopharyngeal mucosal membranes of humans and occasionally causes life-threatening diseases such as meningitis or septicaemia, with an estimated 500 000 cases annually worldwide (WHO, 1998). The annual incidence of the disease differs in each geographical area, ranging from 1 case per 100 000 in an endemic area to as much as 1 % of the population in epidemic areas (WHO, 1998). Outbreaks caused by *N. meningitidis* occur around the world, especially in the African 'meningitis belt', and sporadic cases sometimes occur in industrialized countries (WHO, 1998). In Japan, over 4000 cases of meningococcal infection were reported annually before World War II. However, with the introduction of a meningococcal vaccine, the number of cases has rapidly decreased, and at present stands at only approximately ten per year for meningococcal meningitis. Meningococcal infection is recognized as a rare infectious disease in Japan, so reports on the epidemiology or characterization of Japanese *N. meningitidis* isolates are rare.

Several molecular typing methods for *N. meningitidis* have been developed and used for epidemiological investigation of meningococcal outbreaks. In addition to serotyping or subserotyping (Caugant *et al.*, 1987; Sacchi *et al.*, 1998, 2000, 2001; Wedege *et al.*, 1990), multilocus enzyme electrophoresis has long been used as one of the most effective techniques for analysis of long-term trends in the epidemiology of meningococcal isolates and for the identification of several major epidemic-prone clones as electrophoretic types (ET) (Selander *et al.*, 1986). For instance, the ET-5 complex was identified as a prevalent clone of meningococcal outbreaks in several European countries including Norway, Belgium and the UK (Caugant *et al.*, 1986; Poolman *et al.*, 1986) and in Cuba, Chile and Brazil in the late 1970s to 1980s (Cruz *et al.*, 1990; Sacchi *et al.*, 1992). A single clonal complex designated group III-1 was identified as a causative agent for epidemics in China, Nepal, Saudi Arabia and Chad in the 1980s (Moore *et al.*, 1989; Zhu *et al.*, 2001). A single serogroup W135 clone of the ET-37 complex was spread in 2000 among Hajj pilgrims, who returned to their home countries and spread it to their relatives (Mayer *et al.*, 2002;

Abbreviations: ET, electrophoretic type; MLST, multilocus sequence typing; ST, sequence type; UPGMA, unweighted pair grouping with mathematical averaging.

Popovic *et al.*, 2000). Multilocus sequence typing (MLST), which classifies meningococcal strains as sequence types (ST) by comparing the DNA sequences of seven housekeeping genes, has also been used widely for molecular epidemiological analysis (Enright & Spratt, 1999; Maiden *et al.*, 1998).

Although several genetic types of *N. meningitidis* are known to be involved in outbreaks that occur around the world, to our knowledge, there is no such report on serological and genetic characterization of *N. meningitidis* isolates in Japan. In this study, in order to study the epidemiology of *N. meningitidis* in Japan, we analysed all meningococcal isolates in our collections from the past 30 years.

METHODS

Meningococcal isolates and growth conditions. *N. meningitidis* strains (182 isolates) were non-systematically isolated from throughout Japan from 1974 to 2003. The isolates were stored in gelatin disks at −80 °C (Yamai *et al.*, 1979). *N. meningitidis* strains (serogroup A, NCTC 10025^T; serogroup B, ATCC 13090; serogroup C, ATCC 13102) were used as reference strains for serogroup typing. The stocked strains were routinely grown on GC agar plates at 37 °C in 5 % CO_2 for 18 h as described previously (Takahashi & Watanabe, 2002).

Serogroup typing. Serogroup was determined by slide agglutination with polyclonal antisera to serogroups A, B, C, D, X, Y, Z, 29E and W135 (Difco).

MLST. MLST was performed as described by Maiden *et al.* (1998) with some modifications. Briefly, MLST loci were amplified with Ex *Taq*

Table 1. Distribution of STs and allelic profiles among 182 Japanese human isolates

An ST complex is defined as described in Methods. STs shown in bold indicate clones that were not registered in the MLST database as of January 2004. Underlined alleles indicate a different allele from that of the standard ST clone within the complex.

ST	No. of isolates (%)	Source			Allele							Year of isolation	
		Patients	Healthy	Unknown	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	Earliest	Latest
ST-23 complex (Cluster A3)													
23	46	28	15	3	10	5	18	9	11	9	17	1974	2003
2039	1	1			6	5	18	9	11	9	17	1984	–
2163	1		1		10	5	18	199	11	9	17	1975	–
2038	1	1			10	5	12	9	11	198	17	1984	–
Subtotal	49 (27)	30	16	3									
ST-41/44 complex (lineage III)													
44	2	1	1		9	6	9	9	9	6	9	1974	2000
43	1	1			12	6	9	9	9	6	9	1984	–
2055	1	1			46	6	9	9	9	6	9	1995	–
2134	1	1			164	6	9	9	9	6	9	1990	–
2136	1	1			10	6	9	9	9	6	9	1994	–
2327	1		1		164	5	9	9	9	6	9	1979	–
41	1	1			3	6	9	5	9	6	9	1990	–
180	1	1			9	6	9	9	9	6	2	1997	–
437	3	2		1	9	6	9	17	9	6	9	1995	2002
687	14	6	8		9	3	9	9	9	6	9	1981	2003
1475	1	1			3	6	108	5	9	6	9	2002	–
2034	3		3		9	6	9	9	202	6	9	1979	1979
2036	1	1			9	6	9	9	9	192	9	1980	–
2042	1		1		2	6	9	9	5	6	9	1974	–
2044	1	1			2	3	9	9	203	6	9	1988	–
2045	3		3		9	3	9	9	13	6	9	2002	2003
2150	1	1			9	6	9	9	63	6	9	1997	–
2162	1	1			10	3	9	9	9	6	9	1974	–
2320	1	1			9	6	9	3	9	6	12	1978	–
2331	1		1		9	6	9	7	9	6	9	1977	–
2341	1	1			183	6	227	9	6	6	9	1982	–
Subtotal	41 (23)	22	18	1									
ST-2046 complex													
2046	19	3	16		35	4	205	199	14	2	12	1976	2001
2033[*]	5		5		35	4	17	199	14	2	12	1979	1983

Table 1. cont.

ST	No. of isolates (%)	Source			Allele							Year of isolation	
		Patients	Healthy	Unknown	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	Earliest	Latest
2325	1			1	35	4	205	199	144	2	199	1978	–
2330	1		1		35	4	18	199	14	2	12	1979	–
2332	1	1			35	4	204	199	14	63	201	1986	–
2340	1	1			35	4	204	199	14	2	202	1987	–
Subtotal	28 (15)	5	22	1									
ST-198 complex													
198	8	1	7		5	4	17	15	14	7	12	1990	2003
39	1			1	5	4	17	15	14	7	16	2001	–
2043	1		1		5	10	17	4	6	7	12	2002	–
2146	1		1		5	4	17	17	14	7	12	2001	–
2033*					35	4	17	199	14	2	12		
Subtotal	11 (6)	1	9	1									
ST-32 complex (ET-5 complex)													
32	4	3	1		4	10	5	4	6	3	8	1979	2001
33	1	1			8	10	5	4	6	3	8	1999	–
803	1	1			4	10	5	17	6	3	8	2002	–
2145	1		1		4	10	4	4	6	3	8	2001	–
2338	1	1			4	10	79	4	6	3	8	1984	–
Subtotal	8 (4)	6	2	0									
ST-254 complex													
254	4	1	3		2	16	12	11	3	60	7	1979	2002
2041	1	1			2	16	12	11	3	26	7	1987	–
Subtotal	5 (3)	2	3	0									
Others													
11	2	1		1	2	3	4	3	8	4	6	1983	2002
37	1		1		12	2	15	5	13	21	10	1975	–
175	1		1		6	7	4	56	26	18	8	1979	–
185	3		3		12	5	34	17	5	38	17	1978	2001
269	1	1			4	10	15	9	8	11	9	2002	–
1060	1	1			6	5	34	13	73	24	17	2000	–
1418	2	2			8	3	6	17	29	18	9	2001	2003
2032	13	10	2	1	7	16	55	198	3	56	46	1982	2003
2035	1		1		46	5	4	25	7	18	8	1979	–
2037	1		1		4	10	34	15	1	138	12	1979	–
2040	1	1			12	29	2	26	26	65	17	1988	–
2057	1	1			8	5	13	11	6	2	2	2002	–
2058	1	1			10	3	4	142	3	4	6	2002	–
2135	2	1	1		8	3	13	53	205	41	77	1976	1990
2137	1	1			4	10	208	15	206	138	2	1987	–
2138	1		1		4	123	2	110	58	138	188	1974	–
2149	1		1		8	3	13	7	6	9	17	2001	–
2165	1		1		171	16	72	203	211	197	46	1974	–
2263	1	1			12	2	4	219	13	8	16	2002	–
2339	1		1		2	3	4	200	8	202	20	1995	–
2348	1	1			8	3	13	7	6	2	17	1995	–
3316	1	1			4	10	2	16	5	11	20	1987	–
3317	1		1		4	4	17	4	30	7	8	2000	–
Subtotal	40 (22)	23	15	2									
Total	182 (100)	89	85	8									

*ST-2033 was overlapped between the ST-2046 complex and ST-198. In this table, ST-2033 was counted as an ST-2046 complex.

DNA polymerase (Takara Bio) and GeneAmp PCR System 9600 (PE Biosystems). The amplified fragments were purified with the High Pure PCR product purification kit (Roche) and sequenced with the Big Dye terminator cycle sequencing ready reaction kit v2.0 (PE Biosystems) using an ABI PRISM 3100 Genetic Analyzer (PE Biosystems) according to the supplier's protocol.

Sequence data from each allele were analysed using the software DNASIS (Hitachi) and then referred to the MLST database (http://neisseria.org/nm/typing/mlst/) to determine the allele number. The ST of each strain was determined from the profiles of the seven alleles in the MLST database. In the current study, STs that could not be found in the MLST database as of January 2004 were defined as unique to Japan.

Statistical analysis of results by MLST. A clonal complex was defined as described by Jolley *et al.* (2000); a clonal complex is a group containing more than five isolates, of which four or more alleles are coincident against the alleles of the central standard strain of the complex. Statistical analysis of the results by MLST were performed using the software START (Jolley *et al.*, 2001) with the UPGMA method. A relationship among the clones belonging to ST-2046 and ST-198 complexes was also analysed by constructing a distance matrix of allelic mismatches with START (Jolley *et al.*, 2001) and visualized by split decomposition analysis with the program SPLITSTREE, version 3.2 (Huson, 1998).

RESULTS AND DISCUSSION

Origin of the meningococcal isolates

All of the 182 strains were isolated from independent persons who live(d) in Japan (only one isolate was chosen from family cases). The *N. meningitidis* isolates comprised 85 (47 %) throat swab samples from healthy persons, 89 (49 %) samples of blood, cerebrospinal fluid or sputum from patients and 8 (4 %) samples with an unknown source (Table 1).

Serogroup typing of the Japanese isolates

Serogroups of the *N. meningitidis* isolates were classified as follows: serogroup B, 103 isolates (57 %); Y, 39 (21 %); W135, 1 (1 %); non-groupable, 39 (21 %). The result suggested that serogroups B and Y were dominant in Japan. This trend of serogroups is one of the characteristics of the Japanese *N. meningitidis* isolates, compared with that in other industrialized countries; serogroups B, C and Y are predominant in the USA (Jackson & Wenger, 1993) and serogroups B and C are predominant in Europe (Connolly & Noah, 1999). The absence of serogroups A and C in our collection suggests that meningococcal strains prevalent in other countries have not frequently been introduced into Japan or do not persist in Japan. Moreover, the emergence of serogroups A and C meningococci in Japan could be considered as a sign of the entry of new meningococci into Japan.

Genetic relatedness among the Japanese isolates

By MLST analysis, 65 STs were identified among the Japanese isolates, which could be classified into six major clonal complexes, plus other minor clonal complexes (Table 1). Forty-two of the 65 STs identified in this study have not been previously found in the database (shown in bold in Table 1 and Fig. 1) and appear to be unique to Japan. The UPGMA dendrogram of 30 typical ST clones (Fig. 1) suggested the genetic diversity of the Japanese isolates (Fig. 1). Overall, the STs of Japanese isolates seem to be largely classified into the following three types: (i) global STs that are also identified in other countries (e.g. ST-11, 23, 43, 44, 198, 254, 687); (ii) Japan-specific STs that are genetically different from the above types and have not been found outside Japan (e.g. ST-2046 complex, ST-2149 and ST-2032) and (iii) Japan-specific derivatives of global STs that have not been identified outside Japan but are genetically related to global STs (e.g. ST-2038, 2055 and 2145, which are the derivatives of ST-23, ST- 44 and ST-32, respectively). Some of the Japanese isolates have evolved and diverged independently of the isolates in other countries.

In the respective STs or clonal complexes, strains of ST-23 were found most frequently (49/182; 27 %) in our collection (Table 1), suggesting that ST-23 was dominant in Japan. The fact that only three derivatives of the ST-23 clone were found in the ST-23 complex suggested that the ST-23 clones have evolved homogeneously in Japan. In contrast, the ST-44 complex (41 isolates; 23 %) included 20 derivatives of ST-44, 13 of which have not been found outside Japan (Table 1). These results suggested that some ST clones in the ST-44 complex have diverged within the domestic area after the introduction of their ancestral clone to Japan. ST-2046 was isolated second most frequently (19 isolates) and formed a clonal complex with four derivatives (Table 1), designated the ST-2046 complex in this study. None of the related STs have been found outside Japan (Table 1), and ST-2046 and its relatives (ST-2033, 2325 and 2330) were isolated first in Japan in the late 1970s (Table 1), suggesting that the ST-2046 complex is indigenous to Japan. On the other hand, the ST-2046 complex was suspected to diverge from the ST-198 complex (Fig. 2) because ST-2033 in the ST-2046 complex was also classified in the ST-198 complex (Table 1 and Fig. 1) and because the allele profiles of clones in ST-2046 complex also matched those of clones in the ST-198 complex at two or three loci (Table 1). Taken together, it was speculated that the clones of ST-2046 complex derived from those of ST-198 complex many years ago and then originally diverged within Japan (Fig. 2). The geographical features of Japan, which is isolated by the sea, might have helped the genetic expansion of the original Japanese meningococcal clones within Japan.

Relationship of the Japanese isolates to those from other countries

Strains of the ST-44 complex (lineage III), ST-32 complex (ET-5 complex) and ST-11 (ET-37 complex), which are termed 'hypervirulent lineages' (Maiden *et al.*, 1998), were found in the Japanese isolates (Table 1). Strains of lineage III are known as causative agents for the increased incidence of meningococcal disease in The Netherlands and other European countries after 1982 (Caugant *et al.*, 1990; Scholten *et*

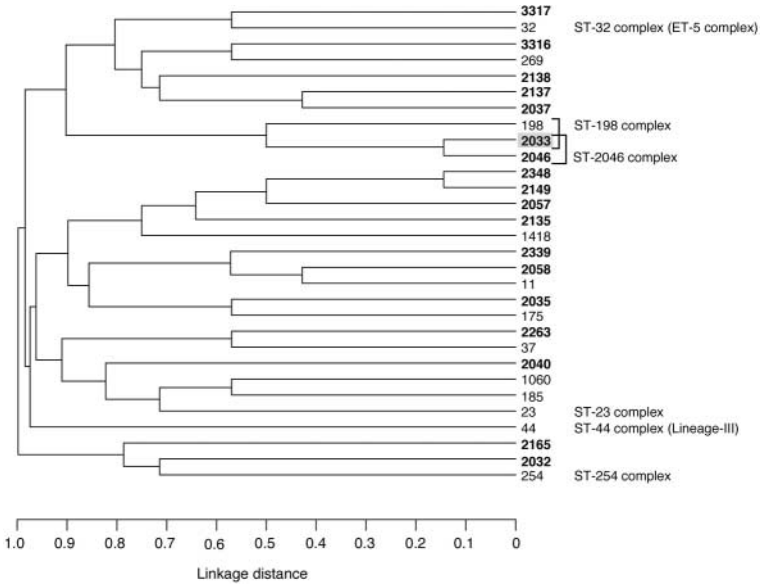

Fig. 1. Dendrogram showing the relationship of 30 typical STs of Japanese isolates by UPGMA analysis. The 30 STs included the central standard strains of each complex, and non-clustered STs that were classified as 'others' in Table 1. ST-2033 was classified into both ST-2046 complex and ST-198 complex (see Table 1). STs that were not found in the MLST database as of January 2004 are shown in bold as new STs.

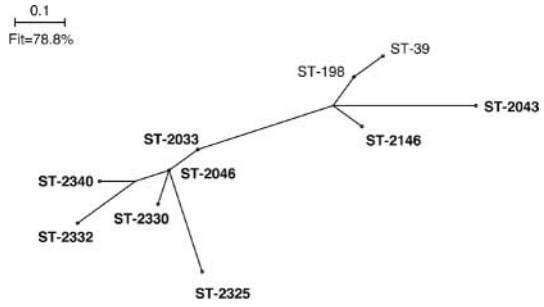

Fig. 2. A splits graph showing the relationship between ST clones belonging to the ST-2046 and ST-198 complexes. STs shown in bold indicate clones that are indigenous to Japan. The scale bar represents uncorrected distances, and a fit parameter is shown.

al., 1994). Our results suggest that such 'hypervirulent lineage' strains exist even in Japan, where no meningococcal outbreaks have been detected for a long time.

Carriage of the 'hypervirulent lineage' clones does not always result in meningococcal disease, but meningococcal outbreaks could occur in Japan because a large number of strains of the ST-44 complex (lineage III), one of the 'hypervirulent lineage' (Maiden *et al.*, 1998), were found among Japanese isolates (Table 1). A meningococcal outbreak has not been reported in Japan since World War II (see above). Approximately 10 cases of meningococcal meningitis have been reported annually since 1990 in Japan (National Institute of Infectious Diseases, unpublished), by which the recent incidence rate in Japan is estimated at around 0·01/100 000. This seems to be much lower than that of other industrialized countries such as the USA, Canada and the countries of Europe, where the incidence is approximately 1/100 000 (Connolly & Noah, 1999; Jackson & Wenger, 1993; Rosenstein *et al.*, 1999; Whalen *et al.*, 1995). We investigated meningococcal carriage in Japan from 2000 to 2002 and found that only 22 out of 3419 healthy Japanese people carried *N. meningitidis*, most of whom were college students around two cities, Yokohama and Matsuyama (unpublished data). The carriage rate for meningococci in the investigation was calculated as 0·64 % (22/3149), which also seems to be significantly lower than in other countries, such as 16·7 % in the UK (Maiden & Stuart, 2002), 9·6 % in Norway (Caugant *et al.*, 1994) and 16 % in Israel (Block *et al.*, 1999). We do not know the exact reasons for the low meningococcal carriage rate, but it is likely to be related to the low incidence of meningococcal diseases in Japan.

It is also interesting that strains of ST-44 (lineage III) and ST-32 (ET-5 complex) were isolated in Japan from a healthy carrier in 1974 and from a female patient in 1979, respectively (Table 1). This seems to suggest that the same ST clones that caused meningococcal disease outbreaks in Europe spread to Japan during the same period (the 1970s) as the outbreaks occurred in Europe (Table 1). Although further investigations would be required to characterize this phenomenon in detail, these data should help to understand the historical spread of meningococcal clones in the world.

To our knowledge, this is the first report to analyse Japanese meningococcal isolates by molecular epidemiological methods. Since the meningococcal isolates used in this study were not collected systematically from the whole of Japan, the present data on the Japanese meningococcal isolates may not reflect the full picture in Japan. However, at least, the results in this study clearly showed the presence of indigenous clones to Japan and the genetically diverse profiles of meningococcal isolates in Japan. Further studies will give us more information about meningococcal diseases in Japan.

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REFERENCES

Block, C., Gdalevich, M., Buber, R., Ashkenazi, I., Ashkenazi, S. & Keller, N. (1999). Factors associated with pharyngeal carriage of *Neisseria meningitidis* among Israel Defense Force personnel at the end of their compulsory service. *Epidemiol Infect* **122**, 51–57.

Caugant, D. A., Froholm, L. O., Bovre, K., Holten, E., Frasch, C. E., Mocca, L. F., Zollinger, W. D. & Selander, R. K. (1986). Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. *Proc Natl Acad Sci U S A* **83**, 4927–4931.

Caugant, D. A., Mocca, L. F., Frasch, C. E., Froholm, L. O., Zollinger, W. D. & Selander, R. K. (1987). Genetic structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer membrane protein pattern. *J Bacteriol* **169**, 2781–2792.

Caugant, D. A., Bol, P., Hoiby, E. A., Zanen, H. C. & Froholm, L. O. (1990). Clones of serogroup B *Neisseria meningitidis* causing systemic disease in the Netherlands, 1958–1986. *J Infect Dis* **162**, 867–874.

Caugant, D. A., Hoiby, E. A., Magnus, P., Scheel, O., Hoel, T., Bjune, G., Wedege, E., Eng, J. & Froholm, L. O. (1994). Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. *J Clin Microbiol* **32**, 323–330.

Connolly, M. & Noah, N. (1999). Is group C meningococcal disease increasing in Europe? A report of surveillance of meningococcal infection in Europe 1993–6. European Meningitis Surveillance Group. *Epidemiol Infect* **122**, 41–49.

Cruz, C., Pavez, G., Aguilar, E. & 12 other authors (1990). Serotype-specific outbreak of group B meningococcal disease in Iquique, Chile. *Epidemiol Infect* **105**, 119–126.

Enright, M. C. & Spratt, B. G. (1999). Multilocus sequence typing. *Trends Microbiol* **7**, 482–487.

Huson, D. H. (1998). SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14**, 68–73.

Jackson, L. A. & Wenger, J. D. (1993). Laboratory-based surveillance for meningococcal disease in selected areas, United States, 1989–1991. *Morbid Mortal Wkly Rep CDC Surveill Summ* **42** (SS-2), 21–30.

Jolley, K. A., Kalmusova, J., Feil, E. J., Gupta, S., Musilek, M., Kriz, P. & Maiden, M. C. (2000). Carried meningococci in the Czech Republic: a diverse recombining population. *J Clin Microbiol* **38**, 4492–4498.

Jolley, K. A., Feil, E. J., Chan, M. S. & Maiden, M. C. (2001). Sequence type analysis and recombinational tests (START). *Bioinformatics* **17**, 1230–1231.

Maiden, M. C. & Stuart, J. M. (2002). Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination. *Lancet* **359**, 1829–1831.

Maiden, M. C., Bygraves, J. A., Feil, E. & 10 other authors (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**, 3140–3145.

Mayer, L. W., Reeves, M. W., Al-Hamdan, N. T. & 15 other authors (2002). Outbreak of W135 meningococcal disease in 2000: not emergence of a new W135 strain but clonal expansion within the electophoretic type-37 complex. *J Infect Dis* **185**, 1596–1605.

Moore, P. S., Reeves, M. W., Schwartz, B., Gellin, B. G. & Broome, C. V. (1989). Intercontinental spread of an epidemic group A *Neisseria meningitidis* strain. *Lancet* ii, 260–263.

Poolman, J. T., Lind, I., Jonsdottir, K., Froholm, L. O., Jones, D. M. & Zanen, H. C. (1986). Meningococcal serotypes and serogroup B disease in north-west Europe. *Lancet* ii, 555–558.

Popovic, T., Sacchi, C. T., Reeves, M. W. & 9 other authors (2000). *Neisseria meningitidis* serogroup W135 isolates associated with the ET-37 complex. *Emerg Infect Dis* **6**, 428–429.

Rosenstein, N. E., Perkins, B. A., Stephens, D. S. & 9 other authors (1999). The changing epidemiology of meningococcal disease in the United States, 1992–1996. *J Infect Dis* **180**, 1894–1901.

Sacchi, C. T., Pessoa, L. L., Ramos, S. R., Milagres, L. G., Camargo, M. C., Hidalgo, N. T., Melles, C. E., Caugant, D. A. & Frasch, C. E. (1992). Ongoing group B *Neisseria meningitidis* epidemic in São Paulo, Brazil, due to increased prevalence of a single clone of the ET-5 complex. *J Clin Microbiol* **30**, 1734–1738.

Sacchi, C. T., Lemos, A. P., Brandt, M. E., Whitney, A. M., Melles, C. E., Solari, C. A., Frasch, C. E. & Mayer, L. W. (1998). Proposed standardization of *Neisseria meningitidis* PorA variable-region typing nomenclature. *Clin Diagn Lab Immunol* **5**, 845–855.

Sacchi, C. T., Whitney, A. M., Popovic, T. & 7 other authors (2000). Diversity and prevalence of PorA types in *Neisseria meningitidis* serogroup B in the United States, 1992–1998. *J Infect Dis* **182**, 1169–1176.

Sacchi, C. T., Lemos, A. P., Popovic, T. & 8 other authors (2001). Serosubtypes and PorA types of *Neisseria meningitidis* serogroup B isolated in Brazil during 1997–1998: overview and implications for vaccine development. *J Clin Microbiol* **39**, 2897–2903.

Scholten, R. J., Poolman, J. T., Valkenburg, H. A., Bijlmer, H. A., Dankert, J. & Caugant, D. A. (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.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.

Takahashi, H. & Watanabe, H. (2002). A broad-host-range vector of incompatibility group Q can work as a plasmid vector in *Neisseria meningitidis*: a new genetical tool. *Microbiology* **148**, 229–236.

Wedege, E., Hoiby, E. A., Rosenqvist, E. & Froholm, L. O. (1990). Serotyping and subtyping of *Neisseria meningitidis* isolates by co-agglutination, dot-blotting and ELISA. *J Med Microbiol* **31**, 195–201.

Whalen, C. M., Hockin, J. C., Ryan, A. & Ashton, F. (1995). The changing epidemiology of invasive meningococcal disease in Canada, 1985 through 1992. Emergence of a virulent clone of *Neisseria meningitidis*. *JAMA* **273**, 390–394.

WHO (1998). *Control of Epidemic Meningococcal Disease*. WHO Practical Guidelines, 2nd edn. Geneva: World Health Organization.

Yamai, S., Obara, Y., Nikkawa, T., Shimoda, Y. & Miyamoto, Y. (1979). Preservation of *Neisseria gonorrhoeae* by the gelatin-disc method. *Br J Vener Dis* **55**, 90–93.

Zhu, P., van der Ende, A., Falush, D. & 13 other authors (2001). Fit genotypes and escape variants of subgroup III *Neisseria meningitidis* during three pandemics of epidemic meningitis. *Proc Natl Acad Sci U S A* **98**, 5234–5239.