

## Comparison of Serogroup W-135 Meningococci Isolated in Sweden during a 23-Year Period and Those Associated with a Recent Hajj Pilgrimage

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**An outbreak of serogroup W-135 meningococcal disease was reported among pilgrims returning from the annual hajj (pilgrimage to Mecca) in mid-March 2000. Molecular characterization was used to investigate the similarity of the hajj-associated W-135 strains with those isolated in Sweden during a 23-year period (1978 to 2000). The same hajj-associated genosubtype, genosubtype P1.5,2,36b, has been documented in Sweden since 1979, while pulsed-field gel electrophoresis and the sulfadiazine resistance of the W-135 isolates indicated that the outbreak was probably due to a new clone of W-135 meningococci.**

*Neisseria meningitidis* is a major cause of meningitis and septicemia in areas throughout the world where they are endemic and epidemic, with high rates of morbidity and mortality. On the basis of differences in their capsular polysaccharides, the epidemiology of meningococcal disease (MCD) is usually investigated by serogrouping *N. meningitidis* strains that have been isolated (1). Among the 13 known serogroups, serogroups A, B, and C account for about 90% of all cases (14), and serogroups Y and W-135 account for the majority of the remaining 10% of the cases. Individual *N. meningitidis* isolates can be further characterized, e.g., by serosubtyping of the PorA outer membrane protein (6) and, more recently, by genosubtyping of the *porA* gene (9, 19). This subtyping system primarily offers a useful epidemiological marker. For a more complete means of strain identification, fingerprinting of the whole chromosome, e.g., by pulsed-field gel electrophoresis (PFGE), is useful for investigation of the clonal relationships of isolates within meningococcal disease outbreaks (2, 11, 16).

Even though meningococci of serogroups B and C have caused several epidemics, serogroup A is the leading cause of outbreaks, particularly in the so-called meningitis belt (14). The annual hajj pilgrimage to Mecca, which attracts more than a million Muslim pilgrims from around the world, has played a role in the global spread of MCD. In 1987, an *N. meningitidis* serogroup A clone, previously isolated in East Asia and Europe, occurred among the pilgrims and spread globally when the pilgrims returned to their home countries (10, 12, 17). Saudi authorities now demand evidence that the pilgrims have been immunized with a serogroup A meningococcal vaccine.

An outbreak of serogroup W-135 MCD occurred in mid-March 2000 among Muslim pilgrims returning from the pilgrimage to Mecca and their contacts, with more than 340 cases being reported from around the world (World Health Organization, <http://www.who.int/emc/outbreak-news/n2000/may/>

12may2000.html). All the serosubtyped strains reported to the World Health Organization were serosubtype P1.5,2. A few of them were also genosubtyped as genosubtype P1.5,2 by sequencing of variable regions 1 and 2 (VR1 and VR2, respectively) of the *porA* gene (5). Two individuals with invasive MCD caused by W-135, as well as two healthy carriers colonized with the same serogroup, all of whom were contacts of returning Muslim pilgrims, were also reported from Sweden in April 2000. The isolates were genosubtyped as genosubtype P1.5,2,36b by sequencing of VR1, VR2, and VR3 of the *porA* gene (9).

*N. meningitidis* serogroup W-135, previously known to cause MCD sporadically, particularly in those with complement deficiencies (4), has not been described to cause epidemics until the hajj-associated outbreak. The aim of the present study was to gain more insight into the evolving epidemiology of W-135 strains. By *porA* gene sequencing and PFGE, we have characterized invasive and carrier isolates of *N. meningitidis* W-135 collected in Sweden during a 23-year period (1978 to 2000) and compared the findings with those for reference isolates from pilgrims to the hajj.

In all, 46 *N. meningitidis* W-135 invasive and carrier strains isolated in Sweden from 1978 to 2000 and kept at the Swedish Reference Laboratory for Pathogenic Neisseria were included in the study along with two representative W-135 reference strains from pilgrims who attended the 2000 hajj (strains M7089B and M7034, kindly provided by T. Popovic, Centers for Disease Control and Prevention, Atlanta, Ga.) (Table 1). The isolates were cultured on GCSP agar (3% GC medium base [Difco Laboratories, Detroit, Mich.], with 0.4% D-glucose, 0.01% L-glutamine, 0.0001% cocarboxylase, 0.0005% ferric nitrate, and 0.5% IsoVitaleX enrichment [BBL, Becton Dickinson Europe, Meylan, France]) for 18 to 20 h at 37°C in 5% CO<sub>2</sub>.

The strains were serogrouped and serosubtyped (with the available monoclonal antibodies, P1.2, P1.7, P1.15, and P1.16) by coagglutination (13). Antibigrams for the strains were established by the E-test (Biodisc, Solna, Sweden). Sulfadiazine was tested on ISOSP agar (3% Iso-Sensitest agar [Oxoid, Bas-

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TABLE 1. Genosubtypes, PFGE patterns, and sulfadiazine susceptibilities of W-135 meningococci isolated in Sweden between 1978 and 2000 in comparison with those of two 2000 hajj-associated reference strains<sup>a</sup>

Strain designation	Yr of isolation	Site of isolation	Genosubtype (VR1, VR2, VR3) <sup>b</sup>	PFGE type	Sulfadiazine susceptibility
1 (608-9901)	1979	CSF	P1.5,2,36b	A	S
2 (600-101)	1980	Blood	P1.5,2,36b	A	S
3 (900-112)	1987	Nasopharynx	P1.5,2,36b	Unique	S
4 (90-137)	1994	Blood	P1.5,2,36b	Unique	S
5 (90-170)	1995	Joint fluid	P1.5,2,36b	Unique	S
6 (90-66)	1997	Nasopharynx	P1.5,2,36b	B	S
7 (90-68)	1997	Nasopharynx	P1.5,2,36b	B1	S
8 (90-71)	1997	Throat	P1.5,2,36b	B	S
9 (90-7)	1998	Blood	P1.5,2,36b	Unique	R
10 (90-188)	1998	Blood	P1.5,2,36b	Unique	R
11 (90-410)	1998	Joint fluid	P1.5,2,36b	Unique	R
12 (590-26)	2000	CSF	P1.5,2,36b	Unique	S
13 (590-66) <sup>c</sup>	2000	CSF	P1.5,2,36b	C	R
14 (590-75) <sup>c</sup>	2000	CSF	P1.5,2,36b	C	R
15 (590-87) <sup>c</sup>	2000	Throat	P1.5,2,36b	C	R
16 (590-95) <sup>c</sup>	2000	Throat	P1.5,2,36b	C	R
17 (590-121)	2000	Blood	P1.5,2,36b	C	R
Ref 1 (M7089B) <sup>c</sup>	2000	CSF	P1.5,2,36b	C	R
Ref 2 (M7034) <sup>c</sup>	2000	Blood	P1.5,2,36b	C	R
18 (600-773)	1978	Urogenital tract	P1.18a,3,38	Unique	S
19 (606-549)	1978	CSF	P1.18a,3,38	Unique	S
20 (606-396)	1985	Blood	P1.18a,3,38	Unique	R
21 (90-95)	1990	Bronchial	P1.18a,3,38	Unique	S
22 (90-193)	1991	Blood	P1.18a,3,38	Unique	R
23 (90-34)	1993	Blood	P1.18a,3,38	Unique	R
24 (90-142)	1993	Nasopharynx	P1.18a,3,38	Unique	R
25 (90-149)	1995	Nasopharynx	P1.18a,3,38	D	R
26 (90-122)	1996	Blood	P1.18a,3,38	D1	R
27 (90-192)	1998	Throat	P1.18a,3,38	E	R
28 (90-193)	1998	Throat	P1.18a,3,38	E	R
29 (90-184)	1998	Blood	P1.18a,3,38	E1	R
30 (90-272)	1998	Unknown origin	P1.18a,3,38	F	S
31 (90-274)	1998	Unknown origin	P1.18a,3,38	F	S
32 (590-194)	1999	Blood	P1.18a,3,38	G	S
33 (590-200)	1999	Throat	P1.18a,3,38	G1	S
34 (590-49)	2000	Nasopharynx	P1.18a,3,38	Unique	R
35 (900-193)	1987	CSF	P1.5c,10a,36b	H1	R
36 (90-65)	1990	CSF	P1.5c,10a,36b	H	R
37 (90-6)	1996	Blood	P1.5c,10a,36b	H	R
38 (590-133)	2000	Blood	P1.5c,10a,36b	Unique	R
39 (NK 793)	1990	Urogenital	P1.5a,2c,36b	I	S
40 (90-76)	1993	Blood	P1.5a,2c,36b	I	S
41 (90-105) <sup>d</sup>	1996	CSF	P1.5a,2c,36b	J	R
42 (90-121) <sup>d</sup>	1996	CSF	P1.5a,2c,36b	J	R
43 (90-55)	1997	Nasopharynx	P1.5a,10d,36b	Unique	R
44 (90-106) <sup>e</sup>	1995	Throat	P1.7,16,35	Unique	R
45 (590-177)	1999	CSF	P1.7a,4a,37a	Unique	R
46 (90-15) <sup>e</sup>	1991	Nasopharynx	P1.21,26-2,36b <sup>f</sup>	Unique	S

<sup>a</sup> CSF, cerebrospinal fluid; Ref, reference strain; R, resistant; S, sensitive.

<sup>b</sup> Amino acid sequences of VR3: 35 (LIGSGSDQ), 36b (LLGSGSDE), 37a (LIGSATSDE), and 38 (LLGRIGDDDE).

<sup>c</sup> Strains isolated from patients or carriers in Sweden; all were associated with returning Muslim pilgrims returning from the 2000 hajj as well as two reference strains from the 2000 hajj.

<sup>d</sup> Serosubtyped as serosubtype P1.2.

<sup>e</sup> Serosubtyped as serosubtype P1.16.

<sup>f</sup> New VR2 variant found in the present study.

ingstoke, England], 5% defibrinated horse blood [Statens Veterinärmedicinska Anstalt, Stockholm, Sweden]), with MIC breakpoints of  $\geq 12$  mg/liter for resistance and  $\leq 8$  mg/liter for sensitivity. Susceptibility to penicillin G was tested on chocolate Mueller-Hinton agar (8). The strains were also tested for  $\beta$ -lactamase production by the chromogenic cephalosporin method (18) with nitrocefin disks (Biodisc).

Genosubtyping was performed as described previously (9),

with DNA isolated with the Dynabeads DNA DIRECT system I (DynaL, Oslo, Norway). The VR1, VR2, and VR3 DNAs within the *porA* gene were sequenced. The deduced amino acid sequences of VR1 and VR2 were assigned genosubtype names according to the *Neisseria meningitidis* PorA Variable Region Database (<http://outbreak.ceid.ox.ac.uk/porA-vr/>). VR3 was assigned names according to the same system.

PFGE was performed with the GenePath Group 3 Reagent

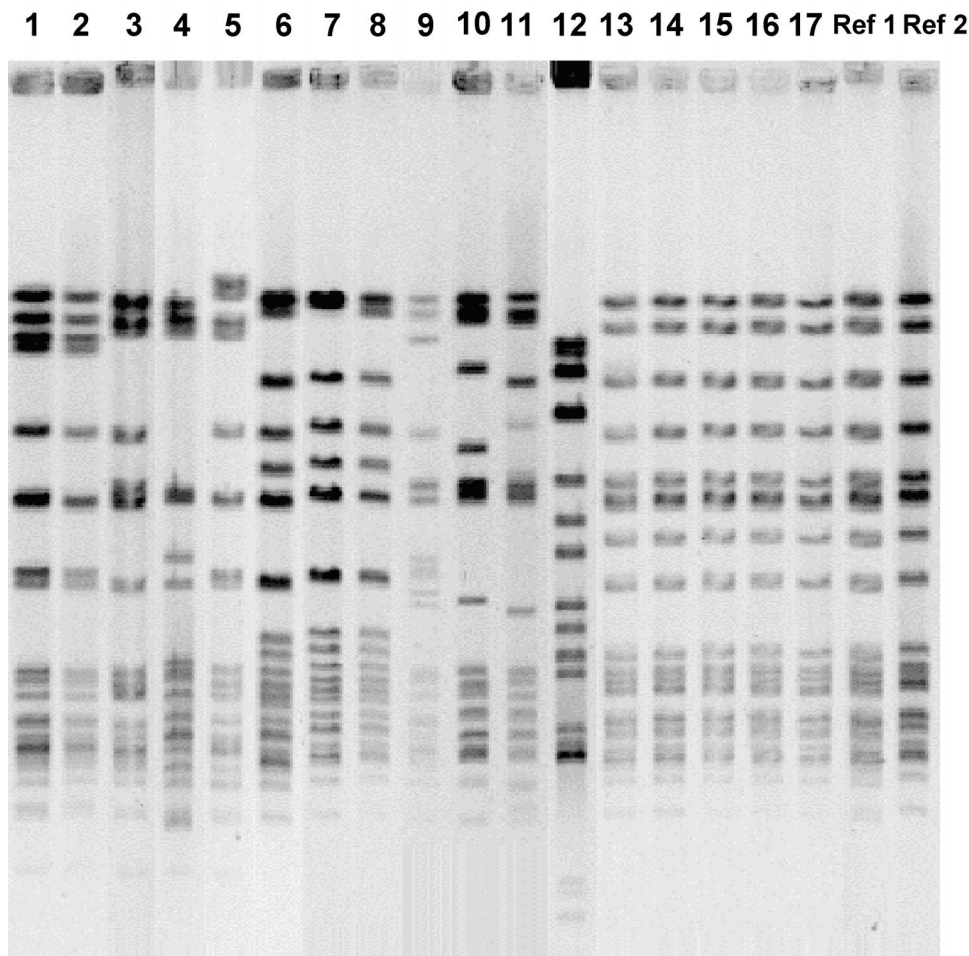


FIG. 1. PFGE fingerprints obtained with the *SpeI* endonuclease for all W-135 meningococci of genosubtype P1.5,2,36b found in Sweden since 1979 (strains 1 to 17) as well as two reference strains (Ref) from the 2000 hajj-associated outbreak (Table 1). Strains 13 to 16 are known to be hajj associated.

kit by using *SpeI* (Bio-Rad Laboratories, Hercules, Calif.). One colony of each *N. meningitidis* W-135 strain, grown overnight on GCSP agar, was inoculated into 3 ml of Mueller-Hinton broth (0.3% beef extract [Difco Laboratories], 1.75% casein hydrolysate [Oxoid], and 0.15% starch [Merck, Darmstadt, Germany]) and grown on a shaker for 18 to 20 h at 37°C in 5% CO<sub>2</sub>. A total of 300 µl from each culture was used for preparation of agarose-embedded DNA according to the instructions of the manufacturer. Gel electrophoresis was run on a Gene Path System (Enb program; Bio-Rad Laboratories), photographed with a Gel Doc 2000 camera, and normalized with Molecular Analyst Fingerprinting software (version 1.6; Bio-Rad Laboratories). Comparative analysis of the DNA fingerprinting patterns between isolates divided the strains into different categories. Clusters of particular PFGE patterns are strains with identical band patterns with the same designation, e.g., type A, B, and C; and strains of, e.g., types B and D showing differences of one to three bands were designated types B1 and D1, respectively. PFGE patterns with more than three band differences compared with the patterns for types A, B, and C, etc., were designated unique for the present study.

The phenotypic characterization showed that only two

strains each were serosubtypeable, and these were serosubtyped as serosubtypes P1.16 and P1.2, respectively. All other strains were nonserosubtypeable with the available monoclonal antibodies. The strains were sensitive to penicillin G (MICs, ≤0.125 mg/liter), and none were β-lactamase positive. Sulfadiazine resistance was found in 27 of the 46 strains (Table 1).

By *porA* gene sequencing all the strains were classified by subtype, with genosubtype P1.5,2,36b being the most prevalent one. It was demonstrated in 11 invasive and 6 carrier strains and in the 2 reference strains from participants in the 2000 hajj. As can be seen from Table 1, the four strains isolated in Sweden in April 2000 from contacts of returning Muslim pilgrims were of this genosubtype, and they made up one cluster with the same PFGE pattern as the reference strains; all six strains were resistant to sulfadiazine. This was also true for another invasive W-135 isolate from May 2000 (strain 17, Table 1); however, no information on contact with pilgrims was available. The remaining 12 strains of the same genosubtype, including the 2 earliest ones from 1979 and 1980 (both invasive isolates), were different from the hajj-associated isolates with regard to their PFGE patterns (Fig. 1). Two clusters, together with seven unique PFGE patterns, were seen for those 12



strains. Moreover, the strains of this genosubtype isolated before 1998, eight in all, were sensitive to sulfadiazine, whereas eight of nine strains isolated in the last 3 years (1998 to 2000) were resistant (Table 1).

Another seven different genosubtypes were demonstrated, with P1.18a,3,38 being the second most frequent genosubtype found among both invasive ( $n = 7$ ) and carrier ( $n = 8$ ) strains (Table 1). Only a few strains of a particular genosubtype had similar PFGE patterns; however, all strains of a PFGE type had the same genosubtype. Within these subtypes, the PFGE patterns revealed seven clusters, and 13 unique strains were found. One genosubtype, genosubtype P1.21,26–2,36b, represented a new VR2 variant not described previously, as indicated in Table 1, footnote *f*.

The present study demonstrated eight different subtypes of the *porA* gene among 46 isolates of W-135 meningococci collected in Sweden during a 23-year period (1978 to 2000). The four W-135 isolates from the patients and the carriers with contact with Muslim pilgrims returning from the 2000 hajj were identical to the hajj-associated reference strains of this W-135 outbreak with regard to genosubtype, PFGE pattern, and sulfadiazine resistance. We also showed that the same genosubtype as that of the hajj-associated isolates had already occurred among W-135 strains in the late 1970s and early 1980s. In fact, it was the most prevalent genosubtype. However, PFGE distinguished them from the hajj-associated isolates, and no tendency for the secondary spread of W-135-related disease has been noted in Sweden. It therefore seems most likely that the hajj-associated outbreak in 2000 was due to a particular clone of this W-135 genosubtype. A recent report by Popovic et al. (T. Popovic, C. T. Sacchi, M. W. Reeves, A. M. Whitney, L. W. Mayer, C. A. Noble, G. W. Ajello, F. Mostashari, N. Bendana, J. Lingappa, R. Hajjeh, and N. E. Rosenstein, Letter, Emerg. Infect. Dis. 6:428–429, 2000), who used multilocus enzyme electrophoresis, showed that the hajj-associated isolates were clonally related to the disease-associated electrophoretic type 37 complex, which gives further support for the assumption that there is a new and particular clone of W-135 that has invasive characteristics.

The sulfadiazine resistance of meningococci is sometimes used as a marker for the epidemiology of *N. meningitidis* outbreaks (7). It was therefore of interest to note that our hajj-associated isolates, as well as the reference strains, were resistant to sulfadiazine. In contrast, all W-135 isolates recovered before 1998 were sensitive, whereas 89% of the isolates recovered in the last 3 years (1998 to 2000) were resistant.

The use of phenotypic markers such as the serosubtype of the PorA protein has proved to be efficient and informative in epidemiological investigations, both of sporadic cases and of outbreaks, particularly those of serogroup B MCD. In the present study, less than 10% of the W-135 isolates could be serosubtyped. In this respect, genosubtyping of the *porA* gene seems to be the method of choice. The stability within this gene was obvious when sequencing of VR3 was also included with sequencing of VR1 and VR2. With the use of PFGE the hajj-associated strains were discriminated from the previous W-135 isolates in Sweden with the same genosubtype.

The reasons for the spread of the hajj-associated W-135 strains remain unclear and cannot be explained by the findings of the present study. It is well known that a high rate of

carriage of particular clones of serogroup A, B, and C meningococci may precede an outbreak (3). However, no data are available from the hajj on the rate of carriage of W-135. After the *N. meningitidis* group A outbreak in 1987, Saudi authorities requested that pilgrims be vaccinated. The use of the bivalent group A and C vaccine might have favored the prevalence of W-135, a corollary to the increased incidence of serogroup B meningococcal disease recently reported from England among first-year university students immunized with the group C vaccine (15). Pilgrims from the United States can be vaccinated with the quadrivalent A/C/Y/W-135 polysaccharide vaccine, which is licensed in the United States. Even though the protection that this vaccine offers against MCD caused by serogroups A and C is well documented, it has not been shown for disease caused by serogroups Y and W-135, and in fact, pilgrims vaccinated with the quadrivalent vaccine also had W-135-associated disease (Popovic et al., Letter).

In conclusion, the genosubtype of the strains associated with the 2000 hajj outbreak of W-135-related MCD was common among *N. meningitidis* strains isolated in Sweden over a 23-year period (1978 to 2000). PFGE indicated that the 2000 hajj meningococcal W-135 P1.5,2,36b genosubtype was newly introduced in Sweden after the pilgrimage. The origin of this clone and the reasons for its spread are unclear; therefore, further investigations are warranted.

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