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Emergence of a Virulent Clone of *Neisseria meningitidis* Serotype 2a That Is Associated with Meningococcal Group C Disease in Canada

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Multilocus enzyme electrophoresis was used to characterize 378 isolates of *Neisseria meningitidis* serogroup C recovered during a period of an increase in group C meningococcal disease in Canada. Thirty-four enzyme electrophoretic types were found among the isolates, which were predominantly (96.0%) serotype 2a. One clone (ET 15), characterized by a rarely occurring allele for the enzyme fumarase, was responsible for a focal outbreak in Ontario followed by the spread of group C disease across the province. This clone, which occurred infrequently among strains isolated in 1986, accounted for over 65% of group C strains associated with meningococcal disease in Canada in 1990.

Multilocus enzyme electrophoresis (MEE) has demonstrated for meningococci a high degree of genetic variation, which permits differentiation among strains of different serotypes and subtypes of the organism (6, 8, 16). Consequently, MEE has been used to delineate the intercontinental spread of group B disease due to a very closely related set of clones designated the ET 5 complex (5) and the intercontinental spread of group A disease due to a clone designated III-1 (14).

During the winter of 1988 and 1989, a focal outbreak of nine cases of disease due to serogroup C, serotype 2a *Neisseria meningitidis* occurred in Victoria County, Ontario (10). The outbreak has since been followed by the spread of group C disease across a large proportion of Ontario and an increase, in general, of group C disease in Canada. MEE was used to characterize the group C disease isolates. We detected the emergence of a particular clone which is associated with the spread of the disease.

MATERIALS AND METHODS

Bacteria. Three hundred seventy-eight strains of *N. meningitidis* serogroup C, isolated from cerebrospinal fluid and blood of patients with meningococcal disease between 1986 and 1990, were received routinely from provincial laboratories across Canada for laboratory analysis. Of the 378 isolates, 269 were from the province of Ontario and were part of a collection of meningococcal disease isolates of all serogroups which represented about 70% of the cases of meningococcal disease occurring in that province in 1986 and 80 to 90% of the cases occurring between 1987 and 1990. The remaining 109 isolates were from other provinces and represented sporadic cases of group C disease. All isolates were confirmed as *N. meningitidis* (15), and identification of serogroups was done as described previously (3).

Serotyping and subtyping. Typing was carried out with whole-cell meningococci as coating antigens in solid-phase enzyme-linked immunosorbent assays. Antigens were prepared as described by Abdillahi and Poolman (1). Monoclonal antibodies with serotype specificities 1, 2a, 2b, 2c, 4, and 15 and subtype specificities P1.1, P1.2, P1.3, P1.4, P1.6, P1.7, P1.9, P1.10, P1.12, P1.14, P1.15, and P1.16 were used to type strains with rabbit anti-mouse immunoglobulin G conjugated to urease (Sigma Chemical Co.) as described previously (2). The monoclonal antibodies were kindly supplied by J. T. Poolman, National Institute of Public Health and Environment, Bilthoven, The Netherlands, and W. D. Zollinger, Walter Reed Army Institute of Research, Washington, D.C.

Preparation of enzyme extracts. Isolates were grown on GC medium (11) for 18 h in the presence of 5% CO_2. The growth from one petri dish (150 by 15 mm) was harvested and suspended in 2.5 ml of 10 mM Tris–1 mM EDTA–0.5 mM NADP (pH 6.8) with 1.0 ml of powdered glass beads (75 to 100 μm; G-2381; Sigma). Each mixture, which was kept in an ice bath during most of the procedure, was vortexed vigorously for 3 min and centrifuged, and the supernatant was filtered through a Millex-GV filter (0.22-μm-pore size; Millipore). The filtrates were dispensed into aliquots of 0.5 ml and stored at −70°C.

Electrophoretic enzyme typing. Electrophoretic analysis of enzymes and subsequent staining procedures were carried out as described by Selander et al. (18) and Caugant et al. (7). Starch was purchased from Connaught Laboratories Ltd., Willowdale, Ontario, Canada. The following enzymes were assayed: malic enzyme (MAE), glucose-6-phosphate dehydrogenase (G6P), peptidase (PEP), isocitrate dehydrogenase (IDH), aconitase (ACO), NADP-linked glutamate dehydrogenase (GD1), NAD-linked glutamate dehydrogenase (GD2), fumarase (FUM), alkaline phosphatase (ALP), indophenol oxidase (IPO), adenylate kinase (ADK), phosphoglucomutase (PGM), and glutamic oxaloacetic transaminase (GOT).

Electromorphs (alloenzymes) of each enzyme were numbered in order of decreasing anodal mobility and were equated with alleles at the corresponding structural gene locus. Distinctive combinations of alleles over the 13 en-

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TABLE 1. Allele profiles and serotypes of meningococcal isolates belonging to 34 ETs of *N. meningitidis* serogroup C

ET	No. of isolates	Electrophoretic position of allele at the indicated enzyme locus:													Serotype[a]
		MAE	G6P	PEP	IDH	ACO	GD1	GD2	FUM	ALP	IPO	ADK	PGM	GOT	
1	25	4	3	5	5	2	1	4	1	8	2	2	5	4	2a
2	6	3	3	5	5	2	1	4	1	8	2	2	5	4	2a
3	1	4	3	3	5	2	1	4	1	8	2	2	5	4	2a
4	4	4	3	4	5	2	1	4	1	8	2	2	9	4	2a
5	52	4	3	4	5	2	1	4	1	8	2	2	5	4	2a
6	1	4	3	4	7	2	1	4	1	8	2	2	5	4	NT
7	2	4	3	4	5	4	1	4	1	8	2	2	5	4	2a
8	1	4	3	4	5	2	2	4	1	8	2	2	5	4	2a
9	17	4	3	4	5	2	1	3	1	8	2	2	5	4	2a
10	1	4	3	5	5	2	1	3	1	8	2	2	5	4	2a
11	1	4	3	4	5	2	1	4	1	8	2	2	2	4	2a
12	1	4	5	4	5	2	1	4	1	8	2	2	2	4	2a
13	1	4	3	4	8	2	1	4	2	8	2	2	5	4	2a
14	1	4	3	4	8	2	1	4	1	8	2	2	5	4	2a
15	201	4	3	4	5	2	1	4	2	8	2	2	5	4	2a and NT (n = 5)
16	1	1	3	4	5	2	1	4	2	8	2	2	5	4	2a
17	1	4	3	4	5	2	1	2	2	8	2	2	5	4	2a
18	1	4	3	4	5	2	1	4	2	8	2	4	5	4	2a
19	5	4	3	4	5	2	1	4	1	8	2	2	5	2	2a
20	1	1	3	4	5	2	1	4	1	8	2	2	5	2	2a
21	37	4	3	4	5	2	1	2	3	8	2	2	5	4	2a
22	1	4	3	4	10	2	1	2	3	8	2	2	5	4	2a
23	5	4	3	5	5	4	1	4	1	8	2	2	5	4	2a
24	1	4	3	5	5	4	1	4	1	3	2	2	5	4	2a
25	1	1	3	4	3	4	1	2	1	8	2	2	5	2	2a
26	1	1	3	4	3	4	1	2	1	8	2	2	5	10	NT
27	1	1	3	5	7	4	1	3	1	8	2	2	5	10	NT
28	1	1	1	7	7	4	1	3	1	8	2	2	1	9	NT
29	1	4	1	3	9	1	1	3	1	8	2	2	5	2	15
30	1	3	4	4	7	2	1	2	1	3	2	3	7	10	NT
31	1	2	4	4	9	2	1	1	1	2	2	3	5	10	NT
32	1	3	3	5	7	3	1	2	1	3	2	2	5	4	NT
33	1	3	1	5	7	4	1	1	1	3	2	2	1	4	4
34	1	3	1	5	8	4	1	3	1	3	2	1	9	10	4

[a] NT, nontypeable.

zyme loci (multilocus genotypes) were designated electrophoretic types (ETs). ET designations are independent of those described previously (7).

Statistical analysis. Genetic diversity at an enzyme locus among either ETs or isolates was calculated from the allele frequencies among ETs or isolates as $h = (1 - \Sigma x_i^2)(n/n - 1)$, where x_i is the frequency of the ith allele and n is the number of ETs or isolates (18). Mean genetic diversity (H) is the arithmetic average of the h values for all of the loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred (mismatches), and clustering of ETs was performed from a matrix of genetic distances by the average-linkage method (19).

RESULTS

In the collection of 378 serogroup C isolates, 269 were from the province of Ontario and 109 were from other provinces in Canada. Of the Ontario isolates, 258 (95.9%) were serotype 2a, 1 (0.4%) was serotype 4, 1 (0.4%) was serotype 15, and 9 (3.3%) were nontypeable. Of the remainder of the isolates, 105 (96.3%) were serotype 2a, 1 (0.9%) was serotype 4, and 3 (2.8%) were nontypeable. Overall, 363 (96.0%) of the isolates were serotype 2a.

Twelve of the 13 enzymes assayed were polymorphic for a range of two to six alleles encoding electrophoretically detectable variants, and 1 (IPO) was monomorphic. The average number of alleles per locus for all isolates was 3.7.

Comparison of the enzyme profiles of the 378 isolates allowed the identification of 34 ETs, for which the serotypes and individual allelic profiles are shown in Table 1. Cluster analysis demonstrated the presence of a group of very closely related ETs (ETs 1 to 24), among which the genetic distance was less than 0.25 (Fig. 1). A total of 368 (97.4%) of the 378 isolates examined had genotypes in the cluster formed by ETs 1 to 24. The remaining 10 ETs (ETs 25 to 34) diverged from the former cluster at a genetic distance of nearly 0.50. Each of ETs 25 to 34 was represented by a single isolate.

With the exception of the one isolate of ET 25, all the serotype 2a isolates were in the cluster of ETs 1 to 24 which, in addition, included six nontypeable isolates (ET 15, five isolates; ET 6, one isolate). These six nontypeable isolates carried the epitope P1.2, which was the major subtype associated with serotype 2a isolates. ET 15 was represented by the largest number of isolates (n = 201), followed by ET 5 (n = 52), ET 21 (n = 37), and ET 1 (n = 25). For the 13 enzymes assayed, ET 5 exhibited the same enzyme profile as the modal ET of the clonal complex associated with most serotype 2a isolates described by Caugant et al. (7).

The nine non-serotype 2a isolates of ETs 25 to 34 were serotype 4 (two isolates), serotype 15 (one isolate), and nontypeable (six isolates). Six of these 10 ETs were repre-

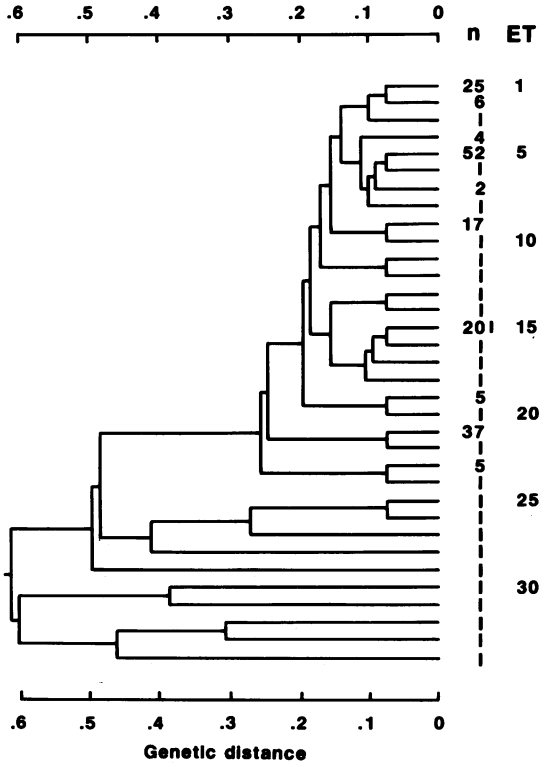

FIG. 1. Genetic relationships among 34 ETs of *N. meningitidis* serogroup C strains isolated in Canada. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of genetic distance between pairs of ETs, based on 13 enzyme loci. ETs are numbered sequentially from top to bottom in order of their listing in Table 1. The number of isolates (n) is indicated for each ET.

sented by single strains isolated from diverse geographical areas in Ontario in 1989 and 1990. The other four ETs were represented by single strains isolated in the provinces of Nova Scotia and New Brunswick and two strains isolated in Alberta in 1989 and 1990.

The genetic diversities at the 13 enzyme loci examined in serogroup C isolates are expressed separately for ETs 1 to 24 and ETs 25 to 34 (Table 2). *H* values per locus for ETs 1 to 24 and ETs 25 to 34 were 0.171 and 0.372, respectively. Among ETs 1 to 24, 10 were represented by multiple isolates (2 to 201); consequently, there was less genetic diversity, on average, among isolates ($H = 0.089$) than among ETs. Since ETs 25 to 34 were represented by single isolates, genetic diversity ($H = 0.372$) was the same for both ETs and isolates. FUM was the most useful enzyme for the distinction of serotype 2a isolates among ETs 1 to 24. The frequencies of FUM-1, FUM-2, and FUM-3 were 0.340, 0.557, and 0.103, respectively, for isolates and 0.709, 0.208, and 0.083 for ETs 1 to 24, respectively. ETs 1 to 12, 14, 19, 20, 23, and 24 were characterized by the presence of FUM-1. ETs 13 and 15 to 18, which included isolates from the focal outbreak in 1989 and involved in the spread across Ontario, exhibited FUM-2. ETs 21 and 22 were characterized by the presence of FUM-3.

The distribution of ETs among the 258 serotype 2a isolates and 5 nontypeable (carrying P1.2) isolates associated with group C disease in Ontario between 1986 and 1990 is shown in Table 3. In 1986, isolates belonging to ETs 5 and 21 were

TABLE 2. Genetic diversity at 13 enzyme loci in ETs and isolates of *N. meningitidis* serogroup C

Enzyme locus	Genetic diversity in:		
	ETs 1 to 24	Isolates belonging to ETs 1 to 24	ETs 25 to 34 or isolates belonging to ETs 25 to 34
MAE	0.192	0.041	0.623
G6P	0.040	0.004	0.279
PEP	0.366	0.188	0.623
IDH	0.265	0.020	0.623
ACO	0.184	0.041	0.534
GD1	0.040	0.012	0.000
GD2	0.322	0.222	0.589
FUM	0.423	0.563	0.000
ALP	0.040	0.004	0.534
IPO	0.000	0.000	0.000
ADK	0.040	0.004	0.400
PGM	0.192	0.030	0.534
GOT	0.115	0.030	0.723
Mean	0.171	0.089	0.372

the most predominant and accounted for 63.3% of the serotype 2a isolates. In 1987, group C disease was caused more uniformly by isolates belonging to ETs 1, 5, 15, and 21. In 1988, isolates belonging to ET 15 began to replace those belonging to ETs 1, 5, and 21 as the major cause of group C, serotype 2a disease. ET 15 was responsible for the focal outbreak of group C disease which occurred in Victoria County, Ontario, early in 1989 and accounted for 72.3% of the serotype 2a isolates for that year. ET 15 accounted for 85.1% of serotype 2a disease strains isolated in Ontario and 74.0% of all the group C disease strains isolated in that province in 1990.

Although the collection of meningococcal disease isolates from other provinces was probably less comprehensive than that of such isolates from Ontario, the same trend in incidence of ET 15 occurred. For example, ET 15 was not found in the isolates from other provinces in 1986 and 1987. ET 15 was present in Québec as early as 1988 and in all provinces by 1990. ET 15 accounted for 50% of the group C disease isolates by 1990 and, including Ontario, for 65% of all the isolates. ET 5, the modal genotype described by Caugant et al. (7), was more uniformly distributed across Canada, while ET 21 was mainly restricted to Ontario and Québec and was responsible for small focal outbreaks in each province in 1986. ET 21 was present in Québec as early as 1983.

DISCUSSION

The incidence of group C meningococcal disease has been increasing in Canada since 1986 (20). Analysis of disease-associated strains showed that group C disease is caused mainly by serotype 2a strains belonging to a single clonal complex.

One particular member (ET 15) of the clonal complex was responsible for the focal outbreak of group C disease in Victoria County, Ontario, in January 1989 and for the subsequent spread of group C disease throughout Ontario by 1990. While ET 15 was not found in the collection of isolates from other provinces in 1986 and 1987, it accounted for half of the group C disease isolates in 1990. Thus, with MEE, it was possible to identify an individual virulent clone which is currently responsible for much of the group C disease in Canada.

TABLE 3. Distribution of ETs among *N. meningitidis* serogroup C, serotype 2a strains isolated in Ontario between 1986 and 1990

Yr	No. of isolates	No. (%) of isolates of ET:					
		1	5	9	15	21	Others
1986	22	2 (9.1)	7 (31.8)	1 (4.6)	2 (9.1)	7 (31.8)	3 (13.6)
1987	25	8 (32.0)	4 (16.0)	0 (0.0)	4 (16.0)	5 (20.0)	4 (16.0)
1988	39	7 (18.0)	1 (2.6)	2 (5.1)	21 (53.9)	2 (5.1)	6 (15.4)
1989	90	3 (3.3)	2 (2.2)	7 (7.8)	65 (72.3)[a]	3 (3.3)	10 (11.1)
1990	87	1 (1.2)	2 (2.3)	6 (6.9)	74 (85.0)[b]	2 (2.3)	2 (2.3)
Total	263	21 (8.0)	16 (6.1)	16 (6.1)	166 (63.1)	19 (7.2)	25 (9.5)

[a] Includes one serogroup C, nontypeable (carrying P1.2) isolate.
[b] Includes four serogroup C, nontypeable (carrying P1.2) isolates.

The most distinguishing feature of the ET 15 isolates is the presence, for FUM, of an allele variant which is rarely found in serogroup C isolates causing disease globally (7). This unique property of these isolates will permit the monitoring of any spread to other countries.

A serogroup C, serotype 2a strain of ET 15 was also isolated from a Canadian student with meningococcemia at a hospital in Mannheim, Germany, 2 days after entry into Germany (17). The student lived in Ontario in an area of known incidence of ET 15 and was part of a school group which had been in Italy for 3 days before arriving in Germany. Since ET 15 isolates have not been found in Italy (13) and since the occurrence of FUM-2 is rare, it is likely that the patient came in contact with the organism while still in Canada or while in Europe through exposure to schoolmates who might have been carriers of the organism. The latter explanation may be more feasible, since Edwards et al. (9) have shown that invasive group C meningococcal disease occurs primarily in persons who are newly infected with the organism. The short carriage period prior to infection is supported by the fact that ET 15 isolates could not be found in group C meningococcal carriers during the outbreak in Victoria County in 1989 (12).

Our studies showed that the introduction of the ET 15 clone into the population in Ontario occurred at least as early as 1986 and within 3 years was responsible for the widespread increase in the incidence of group C disease in that province. Introduction of the clone into the population may have occurred through contact with sources outside Canada. However, the rare occurrence of isolates of ET 15 in other countries (7) would tend to disfavor this hypothesis. Because of its close genetic relationship to the modal genotype (7), which is also common in Canada, ET 15 may have arisen through a single mutational change from FUM-1 to FUM-2, as described by Caugant et al. (4) for single enzyme changes for clones of serotype 2b meningococci causing group B disease in The Netherlands.

A more complex process, based on stepwise single mutations for FUM and GD2, may also be hypothesized if one considers the fact that isolates exhibiting FUM-3 and GD2-2 (e.g., ET 21) were mainly restricted to the provinces of Ontario and Québec and were responsible for small focal outbreaks prior to the major onset of disease due to ET 15 isolates. The latter hypothesis involves sequential changes from clones ET 21 to ET 17 and then to ET 15 (see Table 2 for individual electrophoretic enzyme profiles) and necessitates a mutational change from FUM-3 (ET 21) to FUM-2 (ET 17) followed by a mutational or recombinational event change from GD2-2 (ET 17) to GD2-4 (ET 15). However, this possibility is less tenable, since only one strain belonging to ET 17 was found, and it was isolated in Ontario in 1988, 2 years after the first known isolation of ET 15 in that province. Unfortunately, the extent of frequency of ET 17 isolates prior to 1986 is unknown, since only a few group C disease isolates prior to 1986 were available for analysis.

The discriminatory power of MEE has permitted the identification of a major virulent clone of *N. meningitidis* serogroup C associated with group C disease in Canada and the province of Ontario in particular. Additional studies are needed to determine whether the emergence of this virulent clone can be attributed to enhanced or novel changes in cell surface characteristics (16) or other pathogenic mechanisms.

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