Genetic and antigenic analysis of invasive serogroup C *Neisseria meningitidis* in Canada: A decrease in the electrophoretic type (ET)-15 clonal type and an increase in the proportion of isolates belonging to the ET-37 (but not ET-15) clonal type during the period from 2002 to 2009

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BACKGROUND: Serogroup C meningococcal disease has been endemic in Canada since the early 1990s, with periods of hyperendemic disease documented in the past two decades. The present study characterized invasive serogroup C meningococci in Canada during the period from 2002 to 2009.

METHODS: Serogroup C meningococci were serotyped using monoclonal antibodies. Their clonal types were identified by either multilocus enzyme electrophoresis or multilocus sequence typing.

RESULTS: The number of invasive serogroup C *Neisseria meningitidis* isolates received at the National Microbiology Laboratory (Winnipeg, Manitoba) for characterization has dropped from a high of 173 isolates in 2001 to just 17 in 2009, possibly related to the introduction of the serogroup C meningococcal conjugate vaccine. Before 2006, 80% to 95% of all invasive serogroup C meningococci belonged to the electrophoretic type (ET)-15 clonal type, and the ET-37 (but not ET-15) type only accounted for up to 5% of all isolates. However, beginning in 2006, the percentage of the ET-15 clonal type decreased while the ET-37 (but not ET-15) type increased from 27% in 2006 to 52% in 2009. The percentage of invasive serogroup C isolates not belonging to either ET-15 or ET-37 also increased. Most ET-15 isolates expressed the antigenic formula of C:2a:P1.7,1 or C:2a:P1.5. In contrast, the ET-37 (but not ET-15) isolates mostly expressed the antigens of C:2a:P1.5,2 or C:2a:P1.2.

CONCLUSION: A shift in the antigenic and clonal type of invasive serogroup C meningococci was noted. This finding suggests vigilance in the surveillance of meningococcal disease is warranted.

Key Words: ET-15; ET-37; Meningococci; Serogroup C

The epidemiology of invasive serogroup C meningococcal disease has an interesting history in Canada. Before the mid-1980s, serogroup C *Neisseria meningitidis* was responsible for only a small fraction (approximately 12%) of all invasive meningococcal disease (IMD) cases (1). Beginning in 1986, there was a surge in the number of serogroup C IMD cases (2), which coincided with the identification of a new clone of serogroup C *N meningitidis*, termed electrophoretic

L'analyse génétique et antigénique du *Neisseria meningitidis* invasif du sérotype C au Canada : une diminution du groupe clonal de type électrophorétique (ET)-15 et une augmentation de la proportion d'isolats appartenant au groupe clonal ET-37 (mais pas ET-15) entre 2002 et 2009

HISTORIQUE : La maladie à méningocoque du sérotype C est endémique au Canada depuis le début des années 1990, des périodes de maladie hyperendémique étant attestées depuis vingt ans. La présente étude caractérise des groupes de méningocoques invasifs du sérotype C au Canada entre 2002 et 2009.

MÉTHODOLOGIE : Les chercheurs ont sérotypé les méningocoques du sérotype C au moyen d'anticorps monoclonaux. Ils ont déterminé leur groupe clonal par électrophorèse d'enzyme multilocus ou par typage séquentiel multilocus.

RÉSULTATS : Le nombre d'isolats de *Neisseria meningitidis* invasifs du sérotype C envoyés au *National Microbiology Laboratory* de Winnipeg, au Manitoba, en vue de leur caractérisation a fléchi d'un pic de 173 isolats en 2001 à seulement 17 en 2009, peut-être en raison de l'adoption du vaccin conjugué contre le méningocoque du sérotype C. Avant 2006, de 80 % à 95 % de tous les méningocoques invasifs du sérotype C appartenaient au groupe clonal de type électrophorétique ET-15, et l'ET-37 (mais pas l'ET-15) ne représentait que jusqu'à 5 % de tous les isolats. Cependant, à compter de 2006, le pourcentage de groupe clonal ET-15 a diminué, tandis que l'ET-37 (mais pas l'ET-15) est passé de 27 % en 2006 à 52 % en 2009. Le pourcentage d'isolats invasifs du sérotype C n'appartenant ni à l'ET-15 ni à l'ET-37 a également augmenté. La plupart des isolats d'ET-15 exprimaient la formule antigénique C:2a:P1.7,1 ou C:2a:P1.5. Par contre, les isolats d'ET-37 (mais pas d'ET-15) exprimaient surtout les antigènes C:2a:P1.5,2 ou C:2a:P1.2.

CONCLUSION : Les chercheurs ont remarqué une transition du groupe antigénique et clonal des méningocoques invasifs du sérotype C. En raison de cette observation, il serait judicieux d'être vigilant en matière de surveillance de la maladie à méningocoque.

type (ET)-15, as determined by the technique of multilocus enzyme electrophoresis (MLEE) (3). Since the mid-1980s, there had been two brief periods (1989 to 1993 and 2000 to 2001) with increased incidence of IMD and, in both instances, it was associated with an increase in serogroup C disease due to the ET-15 clone (4). The first localized outbreak due to this clone was documented in the winter of 1988/1989 in Victoria County, Ontario (5). Since that time, it has

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quickly spread to other parts of the country leading to implementation of intensive targeted vaccination campaigns in several provinces (Ontario, Quebec, Prince Edward Island and British Columbia) between 1991 and 1993 (6-9). Aside from causing an increase in the incidence of IMD in Canada (10) and the United States (11), ET-15 was also responsible for outbreaks in various parts of the world in the 1990s, including Israel, the Czech Republic, Iceland, Finland, Norway, England and Australia (12).

The ET-15 meningococci were identified by MLEE to be a member or subtype of the hypervirulent clone of the ET-37 complex, which has long been known to be associated with epidemic disease (13-15). The distinguishing feature of the ET-15 clone from the ET-37 clonal complex is the presence of a unique genetic allele for the housekeeping enzyme fumarase (*fumC* gene allele 2), which differs from that found in the ET-37 meningococci, which has the *fumC* gene allele 1. This difference in the *fumC* gene alleles, first observed by MLEE, has now been confirmed at the genetic level by a single base pair change (from G for allele 1 to A for allele 2) at position 640 of the *fumC* gene (16). Other than this change in the housekeeping enzyme fumarase, the original ET-15 variant that first appeared in Canada expressed the same antigenic formula (C:2a:P1.5,2) as members of the serogroup C ET-37 clonal complex (15).

In Canada, the vaccination campaigns launched after the initial appearance of the ET-15 clone were successful in reducing the extent of meningococcal disease between 1994 and 1999 (17,18). The meningococcal vaccines administered during the outbreaks were the bivalent (A,C) and the quadrivalent (A,C,W-135,Y) polysaccharide vaccines. While effective in reducing disease in the short term, these plain polysaccharide vaccines do not offer long-lasting immunity (19,20). In 2000/2001, a second wave of serogroup C disease appeared, with the responsible ET-15 clone expressing genetic and antigenic variations (21) that might be responsible for the significant increases in meningococcal disease in some provinces, including British Columbia, Alberta, Manitoba, Ontario and Quebec (22). These variations in the serogroup C ET-15 meningococci included mutations in the serotype 2a antigen and recombination events in the PorA antigens (21). Since this second wave of serogroup C IMD, all provinces and territories have introduced a meningococcal serogroup C (Men-C) conjugate vaccine into their routine immunization schedules for infants and school children (Public Health Agency of Canada, Provincial and territorial immunization programs <www.phac-aspc.gc.ca/ptimprog-progimpt/index.html>).

Surveillance studies performed after implementation of the Men-C conjugate vaccine policy have found a substantial decrease in serogroup C disease (23) as well as an indirect effect on herd immunity (24). However, during our routine laboratory characterization of serogroup C meningococci from IMD cases, we noticed early in 2006 a shift in invasive serogroup C meningococci from the ET-15 variant to the ET-37 (but not ET-15) type, such that by 2007, of all the serogroup C disease cases identified, more cases were caused by the ET-37 clone than by the ET-15 variant. Furthermore, we also noticed an overall slight increase in the proportion of serogroup C cases due to the non-ET-15 and non-ET-37 clones. Therefore, the objective of the present study was to describe this temporal and geographic change and to discuss the potential implications of this shift in the genetic clone of invasive serogroup C meningococci in Canada. The present article also describes the characteristics of invasive serogroup C isolates in Canada during the period from 2002 to 2009.

METHODS

Isolates

Isolates of *N meningitidis* recovered from normally sterile body sites (eg, blood, cerebrospinal fluids, joint fluids, etc) of patients with IMD were provided by provincial public health laboratories across the country as part of the national strategy for surveillance of IMD in Canada (25). Serogrouping was performed at the local public health laboratories and confirmed at the National Microbiology Laboratory (NML).

In the current surveillance system for IMD, all provinces submit their IMD isolates to the NML via their provincial public health laboratories. Also, it has been estimated that isolates from 80% to 90% of IMD cases were submitted to the NML for analysis, and in recent years, the NML has also received isolates from IMD cases not reported to the National Notifiable Disease Surveillance System (26,27). In the present study, all invasive *N meningitidis* isolates received at the NML during the period from 2002 to 2009 were included.

Serotyping and serosubtyping

Serotyping and serosubtyping of meningococci was performed by an indirect whole cell ELISA (28) with monoclonal antibodies to the following serotype and serosubtype antigens: 1, 2a, 2b, 4, 14, 15, 17, 19, P1.1, P1.2, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P1.14, P1.15, P1.16 and P1.19 (Rijksinstituut voor Volksgezondheid en Milieu, National Institute of Public Health, Bilthoven, The Netherlands; and kind gifts from Dr WD Zollinger).

Genetic characterization of strains

Determination of the genotype of isolates before 2004 was performed by MLEE (13,29) and, since 2004, by multilocus sequence typing (MLST) (30). During 2004, MLST was conducted on all serogroup C *N meningitidis* in parallel with MLEE, with the intention of replacing MLEE with MLST. For discrimination of the ET-15 variant from the ET-37 clone, extended DNA sequencing of the *fumC* gene was performed as described (16). In 2009, 20 serogroup C *N meningitidis* isolates that were formerly identified as ET-15 were retrieved from the NML strain collection and their identities as ET-15 were confirmed by MLST using the protocol described by Vogel et al (16). Throughout the present study, no disagreement was found between the results obtained by MLEE and MLST.

RESULTS

From 2002 to 2009, 294 serogroup C individual case isolates were recovered from IMD patients in Canada. The distribution of isolates by year according to the genetic clones of ET-15, ET-37 (but not ET-15) and non-ET-15/non-ET-37 is presented in Table 1. The clonal type of ET-15 was predominant among the invasive serogroup C isolates from 2002 to 2005, accounting for 81% to 96% of all invasive serogroup C isolates collected. By 2006, the percentage of the ET-15 clonal type had decreased to 61%, with further decreases to 48% observed in 2007, and 26% in 2008. In 2009, the percentage of the ET-15 clonal type among all invasive serogroup C isolates was 35%. Relative to the decrease in the ET-15 clonal type, the number of isolates and the percentage of the ET-37 (but not ET-15) clonal type increased from nine (27%) in 2006 to 12 (48%) in 2007, 18 (67%) in 2008 and back to nine (53%) in 2009. Aside from the overall temporal change in the numbers and percentages of ET-37 clonal type, geographical differences in the appearance of the ET-37 clonal type were also noticed. For example, in British Columbia, the increase of the ET-37 clonal type went from one case isolate in 2002 and 2005 to three, four and nine cases in 2006, 2007 and 2008, respectively. In Ontario, the increase was also from one case isolate per year in 2003 and 2005 to four, eight, two and eight cases in 2006, 2007, 2008 and 2009, respectively. Similar increases have not been observed in other provinces such as Alberta and Quebec (data not shown).

To examine if the shift from the ET-15 to non-ET-15 clonal type was related to certain antigenic types, the expression of serotype and serosubtype antigens in the ET-15 and the non-ET-15 isolates were compared (Table 2). While more (58%) ET-15 isolates expressed the PorA antigens of P1.5 and P1.7,1; only 6% of the ET-37 (but not ET-15) isolates expressed these serosubtype antigens. On the contrary, 69% of the ET-37 (but not ET-15) isolates expressed PorA antigens of P1.2 and P1.5,2; while only 24.3% of the ET-15 isolates expressed these serosubtype antigens.

During this study period, 22 (8%) of the 294 invasive serogroup C isolates were typed as neither ET-15 nor ET-37, and their characteristics

TABLE 1
Invasive meningococcal disease (IMD) and clonal genetics of invasive serogroup C *Neisseria meningitidis* in Canada, from 2002 to 2009

Year	IMD cases*, n	Cases due to serogroup C	Serogroup C isolates belonging to		
			ET-15	ET-37 (but not ET-15)	Non-ET-15/ non-ET-37
2002	186	72 (39)	69 (96)	2 (3)	1 (1)
2003	145	36 (25)	29 (81)	2 (6)	5 (14)
2004	156	45 (29)	41 (91)	0 (0)	4 (9)
2005	164	39 (24)	34 (87)	2 (5)	3 (7)
2006	154	33 (21)	20 (61)	9 (27)	4 (12)
2007	186	25 (13)	12 (48)	12 (48)	1 (4)
2008	158	27 (17)	7 (26)	18 (67)	2 (7)
2009	173	17 (10)	6 (35)	9 (53)	2 (12)
All years	1322	294 (22)	218 (74)	54 (18)	22 (8)

Data presented as n (%) unless otherwise indicated. *The number of IMD cases was based on the number of invasive *N meningitidis* isolates received at the National Microbiology Laboratory (Winnipeg, Manitoba). ET Electrophoretic type

are described in Table 3. The overall genetic and antigenic diversity among these 22 non-ET-15/non-ET-37 serogroup C isolates was in contrast to the ET-15 and ET-37 serogroup C meningococci. There were 14 different sequence types represented among these 22 isolates belonging to nine different clonal complexes.

DISCUSSION

N meningitidis is known to have the capability to change and evolve via a number of genetic mechanisms, such as slipped-strand mispairing for phase variations in the expression of virulence factors such as capsule and lipo-oligosaccharide (31), inactivation of genes via insertion (or excision) of genetic mobile elements such as IS-1301 (32), and genetic mutations and recombinations (33,34). Since the ET-15 clone was first identified in Canada in 1986, variants of this clone have been described in subsequent years. These variants include a mutation hot-spot identified in the serotype 2a antigen, which led to the phenotype of nonserotypeable (35,36), PorA serosubtype antigenic variants expressing the antigenic formula of C:2a:P1.1,7 (37) and C:2a:P1.5 (21,38), and capsule switching of the C:2a:P1.5,2 ET-15 to B:2a:P1.5,2 ET-15 (39,40). Some of these antigenic changes have been implicated as potential causes of increased serogroup C disease activities in North America (21,41). Aside from the variability in the organism itself, incidences of IMD are known to fluctuate in an unpredictable cyclical pattern, which varies in different geographical regions, affected by the season, humidity and viral infections such as influenza (14). The frequency and distribution of the different serogroups responsible for causing IMD may also change over time (42).

As part of our routine laboratory surveillance activities, which include serogrouping, serotyping, serosubtyping and antibiotic susceptibility testing, all serogroup C isolates are also tested by either MLEE and/or MLST with extended DNA sequencing of the *fumC* gene to detect the ET-15 variant (16) to track the presence and prevalence of the ET-15 clone. The typing method of MLEE was introduced into our laboratory in the early 1990s when the first wave of serogroup C ET-15 disease appeared (3); before that, information about the clonal type of invasive serogroup C strains did not exist. However, since the ET-15 clonal type was identified, it has been the dominant type found in invasive serogroup C isolates in Canada. Even between the first and second waves of increased serogroup C disease activity, such as during periods of endemic disease (eg, in 1995 and 1996), 92% of the invasive serogroup C isolates in Canada belonged to the ET-15 clonal type (17).

In the present study, we documented a change in the invasive serogroup C meningococci, with the ET-15 type being replaced by the ET-37 type. The decrease in the ET-15 clonal type appeared to be related to the sharp drop in serogroup C isolates expressing all

TABLE 2
Comparison of the serotype and serosubtype antigens among the electrophoretic type (ET)-15 and ET-37 (but not ET-15) serogroup C invasive meningococci in Canada from 2002 to 2009

Antigens	Isolates expressing the different antigenic combinations among clonal groups		
	ET-15	ET-37 (but not ET-15)	ET-15 + ET-37
C:2a:P1.2	25 (11)	14 (26)	39 (14)
C:2a:P1.5	52 (24)	2 (4)	54 (20)
C:2a:P1.5,2	28 (13)	23 (43)	51 (19)
C:2a:P1.7,1	74 (34)	1 (2)	75 (28)
C:2a:P1.-	19 (9)	9 (17)	28 (10)
C:NT:P1.-	6 (3)	2 (4)	8 (3)
Others	14 (6)*	3 (6)†	17 (6)
All antigenic combinations, n	218	54	272

Data presented as n (%) unless otherwise indicated. *Includes two isolates each of C:2a:P1.15, C:NT:P1.2, C:NT:P1.5, and C:NT:P1.5,2; and one each of C:2a:P1.14, C:2a:P1.5,16; C:NT:P1.4; C:NT:P1.7,1; C:NT:P1.1, and C:NT:P1.-;

†Includes two isolates of C:NT:P1.2, and one isolate of C:NT:P1.5,2

TABLE 3
Characteristics of non-electrophoretic type-37 invasive serogroup C meningococci in Canada from 2002 to 2009

Case number	Year of isolation	Source	Antigenic formula	Sequence type	Clonal complex
1	2003	Blood	C:14:P1.-	ST-437	ST-41/44
2	2004	Blood/CSF	C:15:P1.9	ST-571	ST-41/44
3	2005	Blood	C:14:P1.-	ST-136	ST-41/44
4	2006	Blood	C:NT:P1.10	ST-34	ST-32/ET-5
5	2006	Brain swab	C:4:P1.-	ST-33	ST-32/ET-5
6	2002	Blood	C:4:P1.-	ST-4025	ST-8/cluster A4
7	2007	CSF	C:2b:P1.2	ST-8	ST-8/cluster A4
8	2003	CSF	C:17:P1.19	ST-1095	ST-269
9	2003	CSF	C:17:P1.19	ST-269	ST-269
10	2004	CSF	C:17:P1.19	ST-269	ST-269
11	2005	Blood	C:NT:P1.-	ST-60	ST-60
12	2006	CSF	C:NT:P1.2,5	ST-60	ST-60
13	2004	Blood	C:4:P1.13	ST-278	ST-35
14	2004	Blood	C:4:P1.-	ST-278	ST-35
15	2006	Eye	C:4:P1.-	ST-278	ST-35
16	2008	Blood	C:4:P1.-	ST-278	ST-35
17	2009	Blood	C:15,19:P1.13	ST-278	ST-35
18	2003	Blood	C:NT:P1.6	ST-4109	ST-334
19	2003	CSF	C:NT:P1.6	ST-4109	ST-334
20	2008	Blood	C:NT:P1.9	ST-4109	ST-334
21	2009	Blood	C:NT:P1.5	ST-2006	ST-103
22	2005	Blood	C:1:P1.9	ST-6209	ST-212

CSF Cerebrospinal fluid

antigenic types, and by 2009 there were only six invasive serogroup C ET-15 case isolates and four (67%) of these were expressing the C:2a:P1.5 antigenic combination. In contrast, there were only two (3.6%) C:2a:P1.5 isolates among the 55 invasive serogroup C ET-37 (but not ET-15) isolates, and the majority of the ET-37 (but not ET-15) isolates expressed the antigenic type of C:2a:P1.2,5, as originally described by Wang et al (15). Therefore, this shift from the ET-15 clonal type to the ET-37 (but not ET-15) clonal type appeared to also be associated with antigenic changes taking place in the invasive serogroup C isolates. Because we did not examine other virulence factors in our serogroup C ET-15 and ET-37 isolates, such as FetA and Opa proteins, it is uncertain whether there might be other changes associated with this genetic shift in the clonal type observed in the invasive

serogroup C meningococci. A similar shift from the ET-15 variant to the ET-37 clonal type has not been reported in other parts of the world. This might be because the routine MLST protocol does not call for extended DNA sequencing of the *fumC* gene to cover the single point mutation site at position 640 that differentiates between ET-15 and ET-37 (16) and, therefore, such changes could have gone undetected elsewhere.

The overall percentage of the non-ET-15/non-ET-37 type also increased since our last study (43) (Table 3). In that study, with isolates recovered from 1999 to 2003, 15 (3.4%) of 441 invasive serogroup C isolates belonged to the non-ET-15/non-ET-37 clonal type while in the present study, 22 (9%) belonged to this category. It is also interesting to note that during the period from 1999 to 2003, one-third (five of 15) of the non-ET-15 and non-ET-37 serogroup C isolates belonged to the ST-8/cluster A4 clonal group, and since then, there has been only one additional ST-8/cluster A4 isolate recovered in 2007 from a serogroup C IMD case. Replacing the ST-8/cluster A4 was the ST-35 clonal complex as being the most common non-ET-15 and non-ET-37 clonal type among invasive serogroup C isolates. Five such isolates have been recovered since 2004, all of which belonged to the sequence type of ST-278. Isolates of ST-278 were not found in our previous study (1999 to 2003), although there were two isolates belonging to the ST-35 clonal complex and these two isolates, ST-35 and ST-4126, were recovered in 1999 and 2000, respectively. Finally, the three ST-269 isolates with the antigenic formula of C:17:P1.19 were related to an outbreak in Quebec in the winter of 2004/2005, which was due to an emerging clone of serogroup B meningococci ST-269 expressing the antigens B:17:P1.19 (44). It was interesting to note that this clone of ST-269 expressing the antigens of B:17:P1.19 first emerged in Quebec in the spring of 2003, and has expanded in subsequent years. These three serogroup C ST-269 isolates expressing antigens 17:P1.19 have been shown to be potential capsule switched strains (44), although it was not possible to determine whether the switch was from B:17:P1.19 to C:17:P1.19 or vice versa.

Although the present study was based on laboratory isolates collected passively from submitting provincial public health laboratories across the country, the sample of isolates collected in this system can be regarded as representative because we have been able to describe emerging clones (36,37,44) as well as unusual isolates (45) under this system of case isolates collection. Therefore, we believe that this shift in the invasive serogroup C strains in Canada is not due to a reporting or strain collection artifact.

Men-C conjugate vaccine was approved for use in Canada in 2001 (46), and routine Men-C conjugate vaccine programs have now been implemented in all jurisdictions in Canada, with most jurisdictions having implemented programs by 2005 and the remaining by 2007 (Public Health Agency of Canada, Provincial and Territorial Immunization Programs <www.phac-aspc.gc.ca/ptimprog-progimpt/index.html>). The success of the Men-C conjugate vaccine programs resulted in a significant reduction in the number of invasive serogroup C isolates received and analysed at the NML from a high of 173 isolates in 2001 (47) to just 17 in 2009 (the present study). Although surveillance studies in the post-Men-C conjugate vaccine period did not show any capsule replacement (23,24), most IMD cases in Canada are now caused by serogroup B strains (NML, unpublished data).

The significant antigenic and genetic diversity that exist in *N meningitidis* and the ability of this organism to change may both contribute to its success as a human pathogen, although in most situations it exists in the upper respiratory tract as a harmless commensal to the human host (48). This remarkable adaptability of *N meningitidis* should caution against any complacency in the monitoring of this human pathogen. The regular findings of unusual IMD isolates, such as the report of null mutants as causes of IMD (49), capsule switching and subcapsular antigenic shift (21,41), unusual capsular antigens (45,50) as well as the shift from a predominant ET-15 clonal type to the non-ET-15 form, as described in the present study, all speak to the need for continued vigilance in our surveillance of IMD.

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