Genotypic investigation of *Clostridium difficile* in Prince Edward Island

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Clostridium difficile is an important cause of disease in Canada; however, little information is available about the disease in the Maritime provinces. The objective of the present study was to characterize C *difficile* isolates obtained from people hospitalized with C *difficile* infection in Prince Edward Island. One hundred twenty-six C *difficile* ELISA toxin-positive stool samples were obtained and cultured using an enrichment protocol. C *difficile* was isolated from 105 of 126 (83%) samples. Twenty-two different ribotypes were identified. The most common ribotype, ribotype W, was a North American pulsotype 2 (NAP2), toxinotype 0 strain, which represented 18% of isolates. The next most common ribotype was a NAP1, toxinotype III strain, which accounted for 11% of isolates. Ribotype 027/NAP1 only accounted for five (4.7%) isolates. Forty-five per cent of isolates possessed genes encoding production of binary toxin. Three different ribotypes, all NAP1, toxinotype III strains, had a frameshift mutation in the *tcd*C gene (Δ117), while one isolate (ribotype 078, NAP4, toxinotype V) had a truncating mutation (C184T) in the *tcd*C gene.

Key Words: Clostridium difficile; *Microbiology; Molecular epidemiology*

L'exploration génotypique du *Clostridium difficile* à l'Île-du-Prince-Édouard

Le *Clostridium difficile* est une cause importante de maladie au Canada. Cependant, on possède peu d'information sur la maladie dans les provinces maritimes. La présente étude visait à caractériser des isolats de C *difficile* prélevés sur des personnes hospitalisées en raison d'une infection à C *difficile* à l'Île-du-Prince-Édouard. Les auteurs ont obtenu 126 échantillons de selle positifs à la toxine ELISA mis en culture au moyen d'un protocole d'enrichissement. Ils ont isolé le C *difficile* dans 105 des 126 (83 %) des échantillons et repéré 22 ribotypes différents. Le ribotype le plus courant, le ribotype W, était un pulsotype 2 nord-américain (NAP2), de souche toxinotype 0, qui représentait 18 % des isolats. Le deuxième ribotype le plus courant, le NAP1, de souche toxinotype III, représentait 11 % des isolats. Le ribotype 027/NAP1 ne représentait que cinq (4,7 %) isolats. Quarante-cinq pour cent des isolats comportaient des gènes codant la production de toxine binaire. Trois ribotypes différents, tous NAP1 de toxinotype III, présentaient une mutation du cadre de lecture du gène *tcd*C (Δ117), tandis qu'un isolat (ribotype 078, NAP4, toxinotype V) présentait une mutation tronquée (C184T) du gène *tcd*C.

Clostridium difficile is an important cause of disease in Canada; it is responsible for both large outbreaks and endemic disease (1-3). Because of the significance of this pathogen and the apparent change in epidemiology noted in various countries (4,5), the need for microbiological surveillance is becoming clear. Recently, large studies of isolates from across Canada (6) and Ontario (7) were performed, and they described the types of C *difficile* that predominate in different regions. Studies, such as the present one, are important for the evaluation of the molecular epidemiology of C *difficile* infection (CDI) and to provide important microbiological data for comparison with epidemiological data. They also provide baseline information so that future changes in clinical aspects of CDI can be related to changes in predominant strains. The emergence and dissemination of a purportedly hypervirulent strain, designated ribotype 027 or North American pulsotype 1 (NAP1), has been associated with outbreaks and severe disease in various regions (2,8,9); however, little information about the disease is available in the Maritime provinces, including Prince Edward Island (PEI). The objective of the present study was to characterize C *difficile* isolates obtained from people hospitalized with CDI in PEI.

METHODS

Stool samples were submitted from 126 consecutive individuals diagnosed with CDI at Queen Elizabeth Hospital, Charlottetown, PEI, between October 31, 2005, and January 8, 2007, through a positive ELISA for C *difficile* toxins A and/or B (C *difficile* Tox A/B II, TechLab Inc, USA). Samples were stored aerobically at 4°C before processing. Enrichment culture was performed using an adaptation of the protocol described by Arroyo et al (10), with a seven-day enrichment in C *difficile* moxalactam norfloxacin with 0.1% sodium taurocholate broth before inoculation onto C *difficile* moxalactam norfloxacin agar. Plates were incubated for 48 h to 120 h at 37°C in an anaerobic chamber. Colonies with the characteristic morphology, odour and Gram stain appearance were subcultured onto Columbia blood agar and confirmed as C *difficile* by colony and Gram

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TABLE 1
Characteristics of the most common *Clostridium difficile* isolates (n=99) recovered from people with *C difficile* infection in Prince Edward Island (n=105)

Ribotype	Toxin A	Toxin B	CDT	*tcdC* deletion	*tcdC* mutation	PFGE	Toxinotype	Isolates (n)	Isolates (%)
W	+	+	–	–	–	NAP2	0	19	18.0
N	+	+	+	18 bp	Δ117	NAP1	III	12	11.4
C	+	+	+	–	–	0012	IX	12	11.4
AN	+	+	–	–	–	NAP2	0	12	11.4
Y	+	+	+	18 bp	Δ117	0098	III	11	10.5
027	+	+	+	18 bp	Δ117	NAP1	III	5	4.7
L	+	+	–	–	–	0042	0	5	4.7
017	–	+	–	–	–	0117	VIII	3	2.9
AK	+	+	–	–	–	NAP6	0	3	2.9
078	+	+	+	39 bp	C184T	NAP8	V	3	2.9
AA	+	+	–	–	–	NAP2	0	3	2.9
M	+	+	–	–	–	NAP2	0	3	2.9
A	+	+	+	–	–	0012	IX	2	1.9
AD	+	+	+	18 bp	Δ117	NAP1	III	2	1.9
AI	+	+	–	–	–	ND	0	2	1.9
0	+	+	–	–	–	ND	0	2	1.9

bp Base pair; NAP North American pulsotype; ND Not determined; PFGE Pulsed-field gel electrophoresis

stain morphology and production of l-proline-aminopeptidase (Pro Disc, Remel, USA).

Ribotyping was performed according to the methods described by Bidet et al (11). In situations in which the ribotype was known to be a recognized international ribotype from the Public Health Laboratory Service – Anaerobe Reference Unit (University Hospital of Wales, Cardiff, United Kingdom) by previous typing of reference strains, the appropriate numerical designation (ie, 027) was used. Otherwise, a letter that corresponded to an internal nomenclature for types not validated with the Public Health Laboratory Service reference strains was used. Detection of genes encoding toxin A (*tcdA*) and toxin B (*tcdB*) was performed by polymerase chain reaction (PCR) as previously described (12,13). CDT (*cdtB*) detection was performed using real-time PCR based on the conventional PCR method of Stubbs et al (14). The presence of a deletion in the *tcdC* gene was investigated on a representative isolate from each PCR ribotype by PCR amplification of the *tcdC* gene following the methods outlined by Spigaglia and Mastrantonio (15). The PCR product was purified and sequenced. A representative isolate from each PCR ribotype was also typed by toxinotyping (16). Ribotypes accounting for three or more isolates were also typed using pulsed-field gel electrophoresis (PFGE) (17) and classified as a NAP type or, in situations in which the PFGE pattern was not consistent with any of the NAP groups, the individual PFGE pattern number was reported.

RESULTS

C difficile was isolated from 105 of 126 (83%) samples. Twenty-two different ribotypes were identified. Details regarding strains represented by two or more isolates are presented in Table 1. Overall, toxinotype 0 strains were most common, accounting for 13 ribotypes and 54 (51%) isolates. Three distinct ribotypes were classified as NAP2. Toxinotype III strains accounted for four ribotypes and a total of 30 (30%) isolates. Ribotype 027/NAP1 accounted for five of these isolates, for an overall prevalence of 4.7%. The most common toxinotype III strain was a NAP1 strain classified as ribotype N. One toxinotype III strain was not associated with any of the NAP types; however, it had the same toxin gene profile as NAP1 and possessed the same *tcdC* gene alteration.

Overall, the toxin profile A+B+CDT– was the most common accounting for 52% of isolates, while toxin profiles A+B+CDT+ and A–B+CDT– accounted for 45% and 3%, respectively. Five different ribotypes, accounting for 33 (31%) isolates, possessed a deletion in the *tcdC*. Four types, all toxinotype III, had an 18 base pair deletion plus a single nucleotide deletion at position 117 that introduced a frameshift mutation (Δ117). A different *tcdC* alteration was present in one strain – a toxinotype V strain with a 39 base pair *tcdC* gene deletion known internationally as ribotype 078 and with a PFGE fingerprint that corresponded to NAP8. It contained a nonsense mutation at position 184 (C184T) of the *tcdC* gene. These mutations were not identified in isolates with an intact *tcdC* gene.

DISCUSSION

This is the first genotypic evaluation of *C difficile* in PEI. The predominance of ribotype W/NAP2 was not surprising because this ribotype is also the most common strain in Ontario (7); NAP2 was the most common clone in the Canadian Nosocomial Infection Surveillance Program (6). While the predominance of W/NAP2 was consistent with other Canadian studies, there were also significant differences in the strain distribution. Ribotype 027 only accounted for 4.7% of isolates, compared with 19.4% in Ontario (7). However, two other ribotypes were classified as NAP1, for a total prevalence of 18%, which is similar to the prevalence of NAP1 isolates in the Canadian Nosocomial Infection Surveillance Program study (27%) (6) and in Ontario (31%) (7). The most common NAP1 ribotype, ribotype N, only accounted for 6.6% of isolates in Ontario, but was the second most common strain in the present study. NAP1/ribotype 027 strains may be of particular concern because of reports of severe disease and outbreaks attributed to this strain (18,19); although some conflicting data are present (20). The mechanism of hypervirulence

associated with these strains was originally thought to be a deletion in the *tcdC* gene, a negative regulator of toxin A and B production, because strains possessing this deletion produced much higher levels of toxins in vitro (19). However, it has been subsequently demonstrated that strains with this deletion can produce a functional tcdC protein (21), and an upstream deletion (Δ117) in *tcdC* is more likely the cause of increased toxin production by NAP1/ribotype 027 because it encodes a stop codon (22,23). All NAP1 ribotypes possessed this *tcdC* mutation.

Ribotype 078/NAP8, a toxinotype V strain possessed a 39 base pair *tcdC* deletion and had a different *tcdC* mutation. This mutation, C184T, is a nonsense mutation that should result in severe truncation of the tcdC protein (23). The effect of this mutation on toxin production has not been clearly demonstrated, but toxinotype V strains have been reported to produce more toxins in vitro than toxinotype 0 strains, but less than toxinotype III strains (24). While uncommon, toxinotype V strains may be of concern because recent reports (24,25) have described an increase in CDI caused by the most common toxinotype strain – ribotype 078. Ongoing monitoring of the role of this strain in disease is indicated. The high prevalence of the toxinotype IX strain, ribotype C, was unexpected because this toxinotype is uncommon (26); toxinotype IX strains accounted for less than 1% of strains in Ontario (7). While it possessed a normal *tcdC* gene, this strain contained genes encoding production of binary toxin, which may be an important virulence factor (27). Overall, the prevalence of strains possessing binary toxin genes (45%) was higher than has historically been reported (28,29), but is consistent with recent reports describing dramatic increases in the prevalence of these strains internationally (6,7,30). The toxin A^-B^+ ribotype 017 was present, but uncommon, as is typically reported. Despite its inability to produce toxin A, this strain is clinically relevant and has been associated with outbreaks of CDI (31).

Consensus has not been achieved regarding optimal typing methodologies or combinations of methodologies. While PFGE is regarded as more discriminatory than ribotyping (32,33), this may not be the case when PFGE results are interpreted in the context of epidemic clones (NAP types). Multiple different ribotypes were identified within individual NAP clones, indicating that, as applied, ribotyping is more discriminatory. However, when individual PFGE patterns are evaluated in addition to NAP type designation, the discriminatory power of PFGE increases. PFGE has some clear advantages over ribotyping, including standardized methods and interpretation, and excellent interlaboratory reproducibility. However, because ribotyping is much easier and more amenable to rapid typing of large numbers of isolates, it is widely used. Toxinotyping is a less discriminatory technique that is useful as a secondary test and for broader comparison of isolates. It is not useful at the hospital level because of poor discriminatory power, but does provide useful information in larger epidemiological studies. Combinations of methods may be ideal for broad epidemiological studies and for evaluation of new strains.

The present study has provided the first evaluation of *C difficile* isolates in PEI, and has demonstrated both similarities and differences in the isolate types found in PEI versus other areas of Canada. Studies, such as the present one, provide important baseline information for future clinical and microbiological surveillance, which may be an important factor for elucidation of the pathogenesis and evaluation of infection prevention and control measures for this important pathogen.

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