Characterization of *Clostridium difficile* Strains Isolated from Patients in Ontario, Canada, from 2004 to 2006\(^\text{\dag}\)

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*Clostridium difficile* is the bacterium most commonly surmised to cause antimicrobial- and hospital-associated diarrhea in developed countries worldwide, and such infections are thought to be increasing in frequency and severity. A laboratory-based study was carried out to characterize *C. difficile* strains isolated from persons in Ontario, Canada, during 2004 to 2006 according to toxin type (enterotoxin A, cytotoxin B, and binary toxin [CDT]), *tcdC* gene characterization, ribotyping, pulsed-field gel electrophoresis, and toxotyping. *Clostridium difficile* was isolated from 1,080/1,152 (94%) samples from 21 diagnostic laboratories. Isolates with toxin profiles A\(^+\) B\(^-\) CDT\(^+\), A\(^+\) B\(^-\) CDT\(^-\), A\(^+\) B\(^+\) CDT\(^+\), and A\(^+\) B\(^+\) CDT\(^-\) accounted for 63%, 34%, 2.4%, and 0.6% of isolates, respectively. Alterations in *tcdC* were detected in six different ribotypes, including ribotype 027. A total of 39 different ribotypes were identified, with ribotype 027/North American pulsotype 1 (NAP1), an internationally recognized outbreak strain associated with severe disease, being the second most common ribotype (19% of isolates). Transient resistance to metronidazole was identified in 19 (1.8%) isolates. While a large number of ribotypes were found, a few predominated across the province. The high prevalence and wide distribution of ribotype 027/NAP1 are disconcerting in view of the severity of disease associated with it.

*Clostridium difficile*, a gram-positive anaerobic bacterium, is the species most commonly surmised to cause antimicrobial- and hospital-associated diarrhea in developed countries worldwide (20). Toxigenic strains of *C. difficile* typically produce two toxins: toxin A (an enterotoxin) and toxin B (a cytotoxin) (46). A small percentage of strains produce only toxin B, but these are still clinically relevant (1, 21). Some strains may also produce another toxin, termed binary toxin (or CDT) (5, 13). Recently, there have been reports of increases in morbidity and mortality associated with *C. difficile* (17, 33). The increased in vitro toxin production was swabs proportionate to their share of reported results reported to Quality Management Program—Laboratory Services in 2004.

While CDAD is a common disease in hospitals in Ontario, Canada, and outbreaks have been reported, there is little information regarding the epidemiology of CDAD in Ontario or the strains that cause disease in this population.

The objective of this study was to characterize clinical isolates of *C. difficile* across Ontario that are associated with diarrhea with regard to their toxin profile, molecular typing, and antimicrobial susceptibility.

**MATERIALS AND METHODS**

**Samples.** Diagnostic laboratories in Ontario were asked to participate by an invitation included in a routine mailing from the Ontario Medical Association Quality Management Program—Laboratory Services in 2004. Participating laboratories were requested to submit a number of serial stool swabs proportionate to their share of reported *C. difficile* toxin-positive stool results reported to Quality Management Program—Laboratory Services in 2004. Participating laboratories were allowed to choose their own starting date but were required to submit serial samples once collection had started. Inclusion criteria were that stool samples were to be submitted for *C. difficile* toxin testing by the attending physician and that *C. difficile* toxin testing results were positive. Only one sample per patient was submitted. Cotton-tipped culture swabs were dipped into stool samples, placed in liquid Stuart’s medium, and stored aerobically at 4°C until shipping. Samples were shipped approximately every 2 weeks.

**Bacterial culture.** Enrichment culture was performed using an adaptation of the protocol described by Arroyo et al. (2). Swabs were immersed in *C. difficile* moxalactam-norfloxacin broth with taurocholate and incubated aerobically at 35°C for 7 days. A total of 2 ml of culture broth was then removed and subjected to alcohol shock by the addition of an equal volume of 95% (vol/vol) ethanol. Following a 1-h incubation at room temperature, tubes were centrifuged at 4,000 × g for 10 min, and the pellet was inoculated onto *C. difficile* moxalactam-norfloxacin-
merics software version 4.0 (Applied Maths, Austin, TX). Each gel was stan-

purity and use of the Etest was repeated.

was present (37), the area of growth around the Etest was subcultured to test for

values were used:

Colonies with the morphology, odor, and Gram stain appearance characteristic

cin agar. Plates were incubated for 48 to 120 h at 37°C in an anaerobic chamber.

metronidazole were

Biodisk, Solna, Sweden) (26) on Mueller-Hinton agar with 5% sheep blood

to metronidazole, vancomycin, levofloxacin, and clindamycin by using Etest (AB

and sequenced. A representative isolate from each PCR ribotype was also sub-

methods outlined by Spigaglia and Mastrantonio (43). PCR products were purified

was investigated by PCR amplification of the

negative controls were used for all PCRs. The presence of a deletion and

tation of the conventional PCR technique of Stubbs et al. (44). Positive and

using Sybr green was used to detect CDT (encoded by

DNA extraction was performed using a commercial kit

C. difficile colonies were subcultured onto Columbia blood agar and confirmed to be C.

cin agar. Plates were incubated for 48 to 120 h at 37°C in an anaerobic chamber.

Colonies with the morphology, odor, and Gram stain appearance characteristic

RESULTS

A total of 1,152 stool samples were received from 21 particip-

ating diagnostic laboratories. The number of samples re-

ceived from each laboratory ranged from 1 to 374 (mean ±

standard deviation, 51 ± 230). C. difficile strains were isolated from

1,080 of 1,152 (94%) samples, of which 962 (89%) were

from inpatients. The mean age of patients was 70.2 ± 18.1

years (median, 75 years; range, 2 to 101 years). Ten patients

(0.93%) were under the age of 18 years, while 713 (66%) were

over the age of 65 years. Females accounted for 54.4% of

patients.

There were 39 distinct PCR ribotype patterns. Ribotypes

accounting for 10 or more isolates are presented in Table 1. The numbers of different ribotypes per laboratory ranged from

1 to 37 (mean ± standard deviation, 12 ± 52). For most

laboratories, one or two ribotypes accounted for greater than

50% of isolates. The most common ribotype, W, accounted for

<table>
<thead>
<tr>
<th>Ribotypea</th>
<th>Presence ofc:</th>
<th>tcdC deletion size (bp)</th>
<th>tcdC alteration type</th>
<th>PFGE type</th>
<th>Toxinotype</th>
<th>No. of regions with ribotypec</th>
<th>No. (%) of isolates</th>
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<tbody>
<tr>
<td>W</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NAP2</td>
<td>III</td>
<td>7</td>
<td>275 (25.5)</td>
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<tr>
<td>027</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NAP1</td>
<td>III</td>
<td>7</td>
<td>209 (19.4)</td>
</tr>
<tr>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>III</td>
<td>5</td>
<td>71 (6.6)</td>
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<tr>
<td>L</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>0042</td>
<td>0</td>
<td>6</td>
<td>63 (5.8)</td>
</tr>
<tr>
<td>017</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>0011</td>
<td>VIII</td>
<td>5</td>
<td>58 (5.4)</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0012</td>
<td>IX</td>
<td>7</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>III</td>
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<td>+</td>
<td>−</td>
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<td>IX</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>−</td>
<td>0077</td>
<td>XII</td>
<td>1</td>
<td>10 (0.9)</td>
</tr>
<tr>
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<td>+</td>
<td>−</td>
<td>0046</td>
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<td>10 (0.9)</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>0047</td>
<td>IX</td>
<td>2</td>
<td>10 (0.9)</td>
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<td>U</td>
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<td>+</td>
<td>−</td>
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<tr>
<td>AB</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

- *: ribotype had two different toxin gene patterns.
- +: present; −: absent.
- NAP designation or individual pattern number if not part of a recognized NAP type.
- There were seven regions in total.
26% of isolates and was found in samples from 17 (81%) laboratories. This strain possessed genes encoding toxins A and B but not CDT (A$^+$ B$^+$ CDT$^-$), had a normal tcdC gene, was of toxinotype 0, and was classified as NAP2 by PFGE (Fig. 1). The second most common ribotype, 027, was the internationally recognized outbreak strain, which accounted for 19% of isolates. It was found in samples from 20 (95%) of the 21 laboratories. It had a profile of A$^+$ B$^+$ CDT$^+$, had an 18-bp deletion in the tcdC gene plus a single-nucleotide deletion at position 117 ($\Delta$117), which introduced a frameshift mutation, and was classified as NAP1 by PFGE (Fig. 1). The third most common ribotype, N, accounted for 6.6% of all isolates. Interestingly, it also had the toxin profile A$^+$ B$^+$ CDT$^+$, possessed an 18-bp tcdC gene deletion and $\Delta$117, was of toxinotype III, and was classified as NAP1 by PFGE (Fig. 1). In addition to the two strains described above, four other ribotypes also possessed an altered tcdC gene. Two had a profile of A$^+$ B$^+$ CDT$^+$ as well as an 18-bp tcdC gene deletion and $\Delta$117 and was toxinotype III but was not NAP1 by PFGE. Another was an A$^+$ B$^+$ CDT$^+$ toxinotype XIV strain with an 18-bp tcdC deletion and $\Delta$117. A different tcdC alteration was present in one strain, a toxinotype V strain with a 39-bp tcdC gene deletion; the ribotype is known internationally as ribotype 078. It contained a nonsense mutation at position 184 (C184T) of the tcdC gene.

The toxin profile A$^+$ B$^+$ CDT$^-$ accounted for 62.9% of all isolates, while toxin profiles A$^+$ B$^+$ CDT$^+$, A$^+$ B$^+$ CDT$^-$, and A$^-$ B$^+$ CDT$^+$ accounted for 34.2%, 2.3%, and 0.6%, respectively. A$^-$ B$^+$ strains were found only in samples from six (29%) laboratories in two of the seven provincial health regions (Table 2).

The prevalences of ribotype 027 in the various provincial health regions differed, with significantly high prevalences in the central west and southwest regions and a significantly low prevalence in the Toronto region ($P < 0.001$). Ribotype W/NAP2, the most common ribotype, was significantly more common in the central east and east regions than in the southwest region ($P < 0.001$). There was no association between 027 and age, gender, and inpatient/outpatient status.

Overall, CDT was present in 34.8% of isolates. The prevalence of CDT in the Toronto region was low, and the prevalence in the southwest region was high compared to that seen in other regions ($P < 0.001$). There was no effect of age on CDT ($P = 0.14$); however, isolates containing CDT genes were more common in females ($P = 0.036$).

Seventeen (1.6%) isolates were found to be resistant to metronidazole in vitro on initial testing but were subsequently found to be susceptible when testing was repeated. For one isolate, resistance (MIC > 256 $\mu$g/ml) was demonstrated twice, but the isolate was seen to be susceptible thereafter. The remaining isolate was resistant (MIC, 32 to >256 $\mu$g/ml) for three passages; however, resistance was eventually lost during serial passage. Contamination was not identified on subculture from around the Etest strip on any isolate with suspected resistance. Of these apparently metronidazole-resistant isolates, five were ribotype 027 (A$^+$ B$^+$ CDT$^+$, toxinotype III), five were ribotype W (A$^+$ B$^+$ CDT$^-$, toxinotype 0), four were ribotype A (A$^+$ B$^+$ CDT$^-$, toxinotype IX), two were ribotype AD (A$^+$ B$^+$ CDT$^+$, toxinotype III), and there was one each of ribotype 078 (A$^+$ B$^+$ CDT$^+$, toxinotype V), ribotype C (A$^+$ B$^+$ CDT$^+$, toxinotype IX), and ribotype Y (A$^+$ B$^+$ CDT$^+$, toxinotype III). The MIC$_{50}$ and MIC$_{90}$ for metronidazole were 0.5 and 1.0 $\mu$g/ml, respectively, with an MIC range of 0.064 to >256 $\mu$g/ml. Four (0.4%) isolates had reduced susceptibility to vancomycin. The MIC$_{50}$ and MIC$_{90}$ for vancomycin were 0.5 and 1.0 $\mu$g/ml, respectively, with an MIC range of 0.19 to 4 $\mu$g/ml. No isolates were susceptible to levofloxacin. Seventeen (1.6%) were of intermediate susceptibility (MIC, 4 $\mu$g/ml), while the remaining 98.4% were resistant. The MIC$_{50}$ and MIC$_{90}$ for levofloxacin were 4.0 and <32 $\mu$g/ml, respectively, with an MIC range of 3.0 to >32 $\mu$g/ml. Only 4 (0.4%) isolates were susceptible to clindamycin, while 94 (8.7%) were intermediate. The MIC$_{50}$ and MIC$_{90}$ for clindamycin were 12 and >256 $\mu$g/ml, respectively, with an MIC range of 1.0 to >256 $\mu$g/ml.

### TABLE 2. The seven Ontario health regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Main cities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central east</td>
<td>Barrie, Newmarket, Peterborough, Port Hope, Whitby</td>
</tr>
<tr>
<td>Central south</td>
<td>Brantford, Hamilton, Simcoe, St. Catharines</td>
</tr>
<tr>
<td>Central west</td>
<td>Brampton, Belwood, Oakville, Waterloo</td>
</tr>
<tr>
<td>East</td>
<td>Belleville, Brockville, Cornwall, Kingston, Ottawa, Pembroke</td>
</tr>
<tr>
<td>North</td>
<td>Kenora, New Liskeard, North Bay</td>
</tr>
<tr>
<td>Southwest</td>
<td>Chatham, Clinton, London, Owen Sound, Point Edward, St. Thomas, Stratford, Windsor, Woodstock</td>
</tr>
<tr>
<td>Toronto</td>
<td>Toronto</td>
</tr>
</tbody>
</table>
DISCUSSION

Ribotype 027, a strain that has been associated with outbreaks of CDAD internationally (27, 32, 45), was commonly found in Ontario, accounting for 19% of isolates. When all NAP1 strains are considered, this prevalence increases to 26%. The high prevalence and widespread distribution of this strain in Ontario hospitals were surprising and are concerning in light of international outbreaks and possible increased morbidity and mortality associated with this strain (15, 27, 32, 45). It is unclear whether this is associated with anecdotal reports of increased severity of CDAD in the province, since historical strain data are not available. With serial sampling, it is possible for an outbreak to bias results and elevate the prevalence of an outbreak strain. While this could have had some effect, we did not receive any reports of outbreaks of CDAD in participating hospitals during the study period, and a large number of hospitals from different regions participated, so there should have been limited potentially confounding effects.

Interestingly, four different ribotypes had an 18-bp deletion in the tcdC gene and the Δ117 mutation and were toxino type IIIi, yet only one was classified as ribotype 027 and only two were identified as NAP1 by PFGE. As shown in Fig. 1, the ribotype patterns of these presumably related isolates were quite distinct. This indicates that strains with different lineages or greater evolutionary divergence may possess this potentially important alteration in tcdC. The role of alterations in tcdC in the pathogenesis of disease has not been clearly established; however, it is likely that they are the cause of the increased toxin production that has been studied in vitro for NAP1 (45). Δ117 introduces a frameshift mutation that results in truncation of the TcdC protein (28). Considering the fact that TcdC is a negative regulator of toxin production, it is reasonable to assume that this mutation could have a significant effect on toxin production. Ribotype 078 possessed a different alteration in the tcdC gene. It contained a nonsense mutation at position 184 that is predicted to result in a severe truncation of the TcdC protein (12). The effect of this mutation has not been reported in vivo or in vitro; however, it is reasonable to suspect that there would be an effect on toxin production. Further study of this mutation is warranted, particularly as there is evidence that the ribotype 078 strain may be increasing in prevalence (16, 19).

One aspect of studying C. difficile that is controversial is typing. A consensus has not been achieved regarding optimal typing methodologies, or combinations of methodologies, and all available options have limitations. While PFGE is regarded as more discriminatory than ribotyping (7, 23, 36), this study shows that this is not necessarily so 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 can be more discriminatory. However, when individual PFGE patterns are evaluated in addition to NAP type designations, the discriminatory power of PFGE increases. Conversely, there were instances when isolates within a distinct ribotype had different toxin gene profiles (i.e., A−B− and A−B+). While it is possible that a recent change in toxin profile occurred within a ribotype, it is probably more likely that this represents the inadequate discriminatory power of ribotyping. This complicates the interpretation of ribotyping and identifies potential problems with selecting a representative isolate from distinct ribotypes for additional testing, as was performed here. These limitations do not mean that ribotyping is not useful. Being a rapid and easy technique, it is amenable to widespread use. While techniques such as REA and multilocus variable-number tandem-repeat analysis are more discriminatory than either ribotyping or PFGE (23), neither is a currently widely used primary typing technique. REA is a cumbersome technique and is not widely used (23), while multilocus variable-number tandem-repeat analysis may be too discriminatory (23; M. Mulvey, unpublished data) and more useful as a secondary typing tool. However, results of this study indicate that the use of multiple different typing methodologies can be useful and should be considered, particularly when evaluating new strains.

The ribotype 027 designation originates from the PHLS Anaerobic Reference Unit in Cardiff, Wales. Because of the nature of ribotyping, each individual laboratory must test reference strains to be able to communicate with the same nomenclature. As opposed to the case for most of the ribotypes in this study, reference strains of ribotype 027 have been tested in the laboratory of J. S. Weese and H. Martin, so the widely used 027 designation was used.

The high prevalence (35%) of CDT was not surprising, considering the prevalence of ribotype 027/NAP1. Earlier studies have reported a prevalence of CDT of up to 12% (14, 40, 42), but more recent studies have reported higher numbers consistent with the dissemination of ribotype 027/NAP1 (15, 34). Whether this is associated with changes in severity of disease is unclear, although CDT-containing strains have been reported to be associated with more serious disease (3).

The low prevalence of A−B+ strains (2.3%) was consistent with previous studies (22); however, it was interesting that these strains appeared to have been associated mainly with small local outbreaks (data not presented). Based on the nature of the study, it cannot be definitively stated that these were true outbreaks; however, temporal clustering in samples from individual laboratories is suggestive.

This study raises questions about the possible presence of metronidazole resistance. Concerns are highlighted by recent reports of poor responses to this drug. Reduced susceptibility to metronidazole has been previously reported (4, 8, 9, 37, 47) but has been controversial, particularly because of a lack of repeatability. Recently, inducible resistance, heteroresistance and loss of resistance with serial passage have been described (30, 37, 38). Those studies suggest that transient in vitro resistance may be real and could be clinically relevant. In this study, isolates would have undergone multiple serial passages and typically at least one freeze-thaw cycle before susceptibility testing was performed. If resistance was real but transient and lost during handling, metronidazole resistance could represent a significant unidentified clinical issue. However, while contamination was not identified, it cannot be ruled out, nor can a clinically irrelevant in vitro effect. The presence of apparent metronidazole resistance in different strains is concerning but has been reported in isolates from horses (18, 29). Further study is required to determine whether this is an in vitro or potentially relevant in vivo effect.

Care must be taken when interpreting these microbiological results in the context of apparent changes in epidemiology and

Bacteria. Approved standard M11–A7. 7th ed. Clinical and Laboratory Standards Institute, Wayne, PA.


