Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe

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Summary
Background Toxins A and B are the primary virulence factors of Clostridium difficile. Since 2002, an epidemic of C difficile-associated disease with increased morbidity and mortality has been present in Quebec province, Canada. We characterised the dominant strain of this epidemic to determine whether it produces higher amounts of toxins A and B than those produced by non-epidemic strains.

Methods We obtained isolates from 124 patients from Centre Hospitalier Universitaire de Sherbrooke in Quebec. Additional isolates from the USA, Canada, and the UK were included to increase the genetic diversity of the toxinotypes tested. Isolate characterisation included toxinotyping, pulsed-field gel electrophoresis (PFGE), PCR ribotyping, detection of a binary toxin gene, and detection of deletions in a putative negative regulator for toxins A and B (tcdC). By use of an enzyme-linked immunoassay, we measured the in-vitro production of toxins A and B by epidemic strain and non-dominant strain isolates.

Findings The epidemic strain was characterised as toxinotype III, North American PFGE type 1, and PCR-ribotype 027 (NAP1/027). This strain carried the binary toxin gene cdhB and an 18-bp deletion in tcdC. We isolated this strain from 72 patients with C difficile-associated disease (58 [67%] of 86 with health-care-associated disease; 14 [37%] of 38 with community-acquired disease). Peak median (IQR) toxin A and toxin B concentrations produced in vitro by NAP1/027 were 16 and 23 times higher, respectively, than those measured in isolates representing 12 different PFGE types, known as toxinotype 0 (toxin A, median 848 μg/L [IQR 504–1022] vs 54 μg/L [23–203]; toxin B, 180 μg/L [137–210] vs 8 μg/L [5–25]; p<0·0001 for both toxins).

Interpretation The severity of C difficile-associated disease caused by NAP1/027 could result from hyperproduction of toxins A and B. Dissemination of this strain in North America and Europe could lead to important changes in the epidemiology of C difficile-associated disease.

Introduction Clostridium difficile infection results in a broad spectrum of disease ranging from mild diarrhoea to severe life-threatening conditions. Colonic injury and inflammation results from the production of two protein toxins: toxin A and toxin B. Isolates that produce neither toxin A nor B only and can cause pseudomembranous colitis.1 Moreover, some isolates produce toxin B and toxin A, and a holin-like protein (tcdD). The inverse is seen during the stationary phase, suggesting that tcdC negatively regulates toxin expression.2 Variations in the PaLoc sequence can be detected by toxinotyping:3 22 toxinotype variants (I–XXII), in addition to the reference toxinotype 0, have been characterised. Analysis of two large isolate collections showed that 78–88% isolates were toxinotype 0 and 2–3% were toxinotype III.4 Whether some toxinotypes are more virulent than others is not known.

In 2002, hospitals in Montreal and southern Quebec, Canada, began experiencing an epidemic of C difficile-associated disease. Between 2003 and 2004, about 14 000 nosocomial cases of the disease were reported.7 In January, 2005, 30 hospitals in Quebec reported rates of nosocomial disease higher than 15 per 10 000 patient-days, at least five times greater than the historical average.8 At the Centre Hospitalier Universitaire de Sherbrooke in Quebec, the proportion of patients with C difficile-associated disease who died within 30 days after diagnosis rose from 4·7% in 1991–92 to 13·8% in 2003, suggesting increased virulence of C difficile.9 The incidence of C difficile-associated disease per 100 000 individuals aged 65 years or more in Sherbrooke increased from 102 in 1991–92 to 210 in 2002 and 866 in 2003.9
Because toxins A and B are the primary virulence factors of *C difficile*, we postulated that increased virulence could be due to increased toxin production. Thus, we attempted to identify the epidemic strain at Sherbrooke and we compared toxin A and B production in this strain with that in other contemporary isolates.

**Methods**

We regarded *C difficile*-associated disease as health-care-associated in haemodialysis patients, in residents of a long-term care facility, and in hospital inpatients if symptoms developed more than 72 h after admission or within 2 months of discharge from a health-care facility. All other cases were regarded as community-acquired. We obtained isolates from 124 consecutive patients with *C difficile*-associated disease seen at Sherbrooke between June, 2004, and April, 2005. Faecal samples were diluted ten times in phosphate-buffered saline, centrifuged, and filtered. The supernatant was tested for cytotoxicity by use of MRC-5 cells. *C difficile* was isolated with cycloserine cefoxitin fructose agar (CCFA) plates (Quelabs, Montreal, Canada) after ethanol treatment.10

Additional isolates from recent outbreaks were obtained from the US Centers for Disease Control and Prevention (n=17), Hôpital Maisonneuve-Rosemont, Montreal (n=7), and the UK Anaerobe Reference Laboratory (n=6). This selection was meant to include isolates geographically distant from Sherbrooke to increase the genetic diversity of the sample.

*C difficile* isolates were characterised by toxinotyping, pulsed-field gel electrophoresis (PFGE), and PCR ribotyping with methods previously described.8,11 PFGE and PCR-ribotyping have greater discriminating power than toxinotyping. PFGE dendrograms were created with Bionumerics 4.0 (Applied Maths Austin, TX). Additionally, we detected deletions in tcdC by PCR using in-house primers.12 Finally, the B fragment (cdtB) of binary toxin gene was detected by PCR.11

*In-vitro* production of toxin A and toxin B was measured by enzyme-linked immunosorbent assays (ELISA) in culture supernatants from a 1 mL sample of the culture. All isolates were initially frozen at -80°C; aliquots of cell suspensions were then prepared by the anaerobic culture of isolates, undertaken three times for 20–24 h at 37°C in 50 mL Acambis proprietary broth medium (Cambridge, MA, USA). A sample of the third culture was mixed with an equal volume of glycerol and stored at -80°C until testing. Frozen aliquots were thawed and subcultured three times as done previously to ensure equilibration of every isolate with the culture medium and to dilute the storage medium. Finally, a sample of the third culture (5% inoculum) was cultured for 3 days, during which growth and toxin production were measured. Variations in inoculum cell densities had no effect on cell density at the stationary phase. The kinetics of the logarithmic phase and stationary phase were assessed in separate experiments. We measured growth and toxin production at 24 h, 48 h, and 72 h. Growth was measured by absorbance at 600 nm (Genesys 20, Thermo Electron Corporation, Rochester, NY, USA). One optical density unit corresponded to $1\times10^8$ colony-forming units (CFU) per mL. Spores were detected by light microscopy.

We measured toxin A and B concentrations by capture ELISA with specific polyclonal antibodies prepared at Acambis as previously described.13 Briefly, microplates were coated overnight with antitoxin A or antitoxin B goat IgG (2 µg/mL) in carbonate-bicarbonate buffer (pH 9.8). Plates were blocked with 2.5% skim-milk buffer in Dulbecco’s phosphate-buffered saline and 0.05% Tween 20 (blocking buffer) at 37°C for 90 min. We then prepared culture supernatants, standards and controls, using blocking buffer, and these were incubated for 1 h at 37°C (100 µL/well). After washing, cultures were incubated with antitoxin A or antitoxin B mouse-specific IgG coupled to alkaline phosphatase (Southern Biotech, Birmingham, AL, USA), with the use of diolamine as substrate. Purified toxins A and B were used as standards (with 95% purity, as measured by high-pressure liquid chromatography). The coefficients of variation (CV) of toxin production by the control strain *C difficile* ATCC 43255 were 8-2% for toxin A production and 26% for toxin B production, based on five independent cultures and ELISA readings. Interassay CV of toxin B ELISA was 13-6%. Isolate testing for toxin production was masked. Culture medium, primary antibodies, and toxin standards were developed at Acambis for industrial production of a toxoid vaccine against *C difficile*.

Differences in cell growth were assessed with the Student’s t test. We compared toxin production using the Mann-Whitney test, since the distribution of toxin production values was skewed.

**Role of the funding source**

The study had no funding source. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

**Results**

To identify the epidemic strain at Sherbrooke, we characterised an initial group of *C difficile* isolates obtained from 13 consecutive patients with *C difficile*-associated disease who presented with the infection during June and July, 2004. 12 (92%) isolates were toxinoftype III, all showing identical or highly related PFGE patterns. This strain carried an 18-bp *tcdC* deletion and the *cdtB* gene. We sequenced the *tcdC* deletion and found that it was identical to one previously described.15 The PFGE and PCR-ribotype patterns of these isolates belong to the North American PFGE type 1 (NAP1) and PCR-ribotype 027.
respectively; this genotype (NAP1/027) also encompassed all eight isolates that were toxinotype III, had tcdC deletions, and had the binary toxin gene from other locations (France, UK, and USA [Maine, Georgia, Pennsylvania, New Jersey]). Figure 1 shows the representative PFGE patterns and the resulting dendrogram. Isolates that were toxinotype III, had the tcdC deletion, contained the binary toxin gene, and had PCR-ribotype 027 exhibit two PFGE patterns with 94% similarity (NAP1a and NAP1b; figure 1). By contrast, the six toxinotype 0 isolates from Canada and other countries each present a different PFGE pattern with less than 80% similarity (figure 1).

After the initial 13 isolates were characterised, we toxinotyped and tested 111 additional isolates from consecutive patients for deletions in tcdC. Of 86 isolates that were probably health-care-associated, 58 (67%) were toxinotype III. Of 38 isolates that were probably community-acquired, 14 (37%) were toxinotype III. All but one of the toxinotype III strains showed the 18-bp tcdC deletion.

To compare the production of toxins A and B, the test group of 15 NAP1/027 strains included seven from Sherbrooke, one from Montreal, Canada, two from the UK, four from the USA, and one from Paris (CD196). Historically, toxinotype 0 represents 78–88% of hospital toxigenic isolates; therefore, we investigated whether NAP1/027 produces more toxins A and B than a control group of 25 contemporary isolates of toxinotype 0, randomly selected from the same geographical areas where the toxinotype III isolates were recovered (13 from the USA, nine from Canada, and three from the UK). With 80% similarity by PFGE as a cut-off, the six toxinotype 0 isolates from Canada and other countries each present a different PFGE pattern with less than 80% similarity (figure 1).

Growth kinetics were very similar and showed a peak in cell density at 24 h in both groups (figure 2). Cell density was 17% higher in NAP1/027 than in control strains. At 24 h, mean (SD) absorbance at 600 nm was 1.77 (0.41) in controls and 2.07 (0.51) in NAP1/027 (p = 0.045, Student’s t test). At 48 h, the difference in density was not significant (p = 0.07). Spores were detected at 48 h and 72 h in 18 (72%) toxinotype 0 and 14 (93%) NAP1/027 isolates. In this batch culture method, isolates reached the stationary phase by 24 h (data not shown). Toxin A and toxin B production was much faster and greater in NAP1/027 than in controls. In toxinotype 0, only 14 (56%) isolates produced more than 0.5 μg/L of toxin A at 24 h.

At 24 h, median toxin A concentration was 4 μg/L in toxinotype 0 (IQR 0–64) and 543 μg/L in NAP1/027 (430–873; figure 3). In 11 (44%) toxinotype 0 isolates, toxin A production was not detectable after 24 h (<0.5 μg/L), whereas toxin B ranged from 2 to 3 μg/L. At 24 h, median toxin B concentration was 3 μg/L in toxinotype 0 (2–6 μg/L) and 149 μg/L in NAP1/027 (137–210; figure 3). ELISA values correlated with toxin band intensity by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; data not shown). Toxin A and toxin B production did not differ significantly between isolates from Canada and other areas (data not shown). At 48 h, median toxin A was 54 μg/L (IQR 23–203) in toxinotype 0 and 848 μg/L (504–1022) in NAP1/027. Median toxin B at 48 h was 8 μg/L (5–25) in toxinotype 0 and 180 μg/L (137–210) in NAP1/027. Thus, the peak median (IQR) concentration of toxin A was 16 times higher in NAP1/027 than in toxinotype 0 control strains; toxin B concentrations were 23 times higher in the epidemic strain than in the control strain (toxin A, median 848 μg/L [504–1022] vs 54 μg/L [23–203]; toxin B, 180 μg/L [137–210] vs 8 μg/L [5–25]; p < 0.0001 for both toxins, Mann-Whitney test).

**Figure 1:** PFGE analysis of C difficile study isolates from various geographical locations

**Figure 2:** Growth curves of toxinotype 0 and toxinotype III (NAP1/027)

Mean cell density and SDs are shown.
deletion in this unusual toxin production profile needs further investigation.

In-vitro toxin production in different \textit{C. difficile} isolates was investigated elsewhere\cite{footnote} by use of an early phenotyping method.\cite{footnote} A correlation between toxin production and type was recorded, as well as a correlation between toxin A and toxin B production. However, toxin A production was reported as either low ($\leq$40 $\mu$g/L) or high ($>40$ $\mu$g/L) and toxin B was semiquantified with a cytotoxicity assay rather than ELISA, which made comparison with our data difficult.

Historically, about 6\% of \textit{C. difficile} isolates have carried the binary toxin genes and these are toxinotype variants (ie, non-toxinotype 0).\cite{footnote} By contrast, we found that NAP1/027 uniformly carries the genes for binary toxin. Previous studies have not established conclusively that patients infected with binary-toxin-producing isolates present a more severe disease than those infected with other strains.\cite{footnote} In the USA and Europe, binary-toxin-producing isolates belonged to at least nine toxinotype variants, but the presence of a deletion in \textit{tcdC} was not investigated.\cite{footnote} The role of the binary toxin in the virulence of NAP1/027 remains unclear and we did not measure binary toxin production in vitro. Although strains that are positive for binary toxin but negative for toxins A and B are not pathogenic in hamsters, their culture supernatant causes fluid secretion in rabbit ileum, but not mucosal damage.\cite{footnote} The severity of colonic inflammation in patients with \textit{C. difficile}-associated disease in Quebec\cite{footnote} does not support the hypothesis that the binary toxin plays a key role in NAP1/027-induced disease since this toxin seems to act mostly on the ileum. Further studies are needed to determine the pathogenic role of the binary toxin in human beings.

The epidemic strain at Sherbrooke and presumably in many hospitals in Quebec belongs to PFGE type NAP1, PCR-ribotype 027, and is toxinotype III. Until recently, toxinotype III strains represented 2–3\% of hospital isolates of \textit{C. difficile}.\cite{footnote} The ability of this strain to produce binary toxin in vitro was reported in 1988.\cite{footnote} With restriction endonuclease analysis, the same genotype as NAP1/027 (also known as type BI) was found in only 14 of more than 6000 US historic isolates obtained before 2001.\cite{footnote} To our knowledge, NAP1/027 was not reported to cause either severe disease or outbreaks until recently, when it was identified as the cause of several outbreaks in the USA, some predating the Quebec epidemic.\cite{footnote} Moreover, retrospectively the strain has been identified in isolates from sporadic US cases obtained in the early 1980s.\cite{footnote} The finding of an association between NAP1/027 (or BI) and high toxin production in the context of an epidemic associated with a high case-fatality ratio confirms the suspicion that the epidemic in Quebec is caused by a more virulent strain. In Sherbrooke, between 2003 and 2004, as many as a sixth of inpatients with health-care-
associated *C. difficile* as a direct or indirect consequence of this infection.21

A dominant strain that was PFGE type NAP1, toxinotype III, and contained a *tcdC* deletion and *ctdB* was also discovered in samples from Montreal and from outbreaks associated with increased morbidity, frequent need for colectomy, and mortality in the USA.22,23,24 In the UK, where the number of reported cases of *C. difficile*-associated disease doubled over 3 years,25 NAP1/027 is the cause of ongoing outbreaks in at least three hospitals where a high case-fatality ratio has been noted. Although the detailed distribution of NAP1/027 in the UK remains to be determined, data recently released by the UK Department of Health are worrying; in 2004, nationwide rates of *C. difficile* reports per 1000-bed-days for patients aged 65 years or more were 1·69 for general acute-care trusts and 1·96 for specialist trusts.26 In the Netherlands, NAP1/027 was identified in two severe outbreaks also associated with fatalities.27 The outbreaks in Quebec and the UK have been widely reported in national media and have raised public concern leading to increased emphasis on surveillance and control measures.

The mechanisms behind the emergence and high transmissibility of NAP1/027 are not fully understood, but it seems plausible that the severe diarrhoea induced by this strain could help the dissemination of spores in the hospital environment by incontinent patients. In this study, none of the patients with community-acquired disease had been admitted during the preceding year, suggesting that NAP1/027 could have spread from hospitals into the community. Importantly, antimicrobial susceptibility testing of contemporary and historic isolates of NAP1/027 indicates a substantial increase in resistance to all fluoroquinolones.28 A cohort study of Sherbrooke inpatients recorded that fluoroquinolone use in patients recorded that fluoroquinolone use in the preceding year, suggesting that NAP1/027 could be a major shift in the epidemiology of *C. difficile*-associated disease. Clinicians need to be vigilant in the prevention, diagnosis, and treatment of the disorder.

Conflict of interest statement

M Warny and A Fang are Acambis employees and are involved in the development of a vaccine against *C. difficile*. M Warny owns Acambis stock.

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