MAJOR ARTICLE

Health Care–Associated *Clostridium difficile* Infection in Canada: Patient Age and Infecting Strain Type Are Highly Predictive of Severe Outcome and Mortality

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Background. *C. difficile* infection (CDI) has become an important and frequent nosocomial infection, often resulting in severe morbidity or death. Severe CDI is more frequently seen among individuals infected with the emerging NAP1/027/BI (NAP1) strain and in the elderly population, but the relative importance of these 2 factors remains unclear. We used a large Canadian database of patients with CDI to explore the interaction between these 2 variables.

Methods. The Canada-wide CDI study, performed in 2005 by the Canadian Nosocomial Infection Surveillance Program (CNISP), was used to analyze the role of infecting strain type and patient age on the severity of CDI. A severe outcome was defined as CDI requiring intensive care unit care, colectomy, or causing death (directly or indirectly) within 30 days after diagnosis.

Results. A total of 1008 patients in the CNISP database had both complete clinical data and infecting strain analysis documented. A total of 311 patients (31%) were infected with the NAP1 strain, 83 (28%) were infected with the NAP2/J strain, and the rest were infected with various other types. The proportion of NAP1 infections correlated with the incidence and the severity of CDI when analyzed by province. Thirty-nine (12.5%) of the infections due to the NAP1 strain resulted in a severe outcome, compared with only 41 (5.9%) of infections due to the other types ($P < .001$). The patient's age was strongly associated with a severe outcome, and patients 60–90 years of age were approximately twice as likely to experience a severe outcome if the infection was due to NAP1, compared with infections due to other types.

Conclusions. Our study confirms the strong age association with infection due to the NAP1 strain and severe CDI. In addition, patients 60–90 years of age infected with NAP1 are approximately twice as likely to die or to experience a severe CDI-related outcome, compared with those with non-NAP1 infections. Patients >90 years of age experience high rates of severe CDI, regardless of strain type.

A new era for *Clostridium difficile* infection (CDI) began early this millennium in the United States and Canada, causing problems for patients and challenges for health care workers [1–3]. Epidemics of CDI were documented in Quebec, Canada [2, 4, 5]; Western Europe [6]; and the United States [7] that resulted in total colectomies for some patients and CDI-related deaths of other patients and dramatically illustrated the importance of infection-control interventions and antibiotic stewardship [4]. Recent outbreaks have been largely, although not exclusively, driven by the newly recognized North American pulsed-field type 1 (NAP1) strain, alternately known as ribotype 027, restriction endonuclease type BI, toxinotype III, or the "hypervirulent strain" (depending on the typing methodology and nomenclature used). These strains demonstrate: (1) genetic mutations in the tcdC toxin regulator gene;

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(2) binary toxin genes; and (3) fluoroquinolone resistance mutations [2, 3, 8]. The NAP1 strain has also been recognized as the cause of outbreaks across 6 European countries (United Kingdom, the Netherlands, Belgium, France, Austria, and Ireland) and has been detected sporadically in at least 6 more [9].

The baseline for CDI frequency and severity in Canada was established in the Canadian Nosocomial Infection Surveillance Program (CNISP) study of 2062 patients from 19 institutions in 8 provinces during 1997 [10, 11]. Increases in CDI frequency and mortality in Quebec were first widely reported in mid-2004 [5], although recent studies have retrospectively identified increases from as early as December 2002 [4]. A study of 12 Quebec hospitals during the first half of 2004 showed that CDI mortality varied directly with patient age [2], whereas another prospective study from Quebec found that severe disease was twice as common among patients infected with NAP1 strains [4, 12], although other studies did not find a correlation with strain type and patient outcome [13–15].

Two recent analyses described the epidemiology of CDI in Canada in a follow-up CNISP study of 34 hospitals across 9 Canadian provinces during the period November 2004–April 2005 [16, 17]. We used this combined clinical and laboratory database to describe the molecular epidemiology of the infecting strains and to undertake an analysis of the bacterial phenotypes and genotypes that predict severe outcomes and attributable mortality associated with CDI in adults.

PATIENTS AND METHODS

The CNISP is a collaborative effort of the Canadian Hospital Epidemiology Committee (CHEC), a subcommittee of the Association of Medical Microbiologists and Infectious Disease, the National Microbiology Laboratory, and the Centre for Infectious Diseases Prevention and Control of the Public Health Agency of Canada. A total of 34 hospitals participated in a prospective surveillance for health care–associated CDI (HA-CDI) from 1 November 2004 through 30 April 2005. The epidemiologic analyses of this surveillance is described elsewhere [16, 17]. In brief, the following case definition was used for CDI: (1) diarrhea over a 2-day period or fever, abdominal pain, and/or ileus plus laboratory confirmation of a positive *C. difficile* toxin A or B assay result, or (2) diagnosis of pseudomembranous colitis on colonoscopic examination, or (3) histological or pathological diagnosis of CDI. The infection was considered to be health care–associated if the patient's symptoms began at least 72 h after hospital admission; or symptoms resulted in readmission of a patient who had been hospitalized within 60 days before the symptom onset date and who was not a resident in a long-term care facility or nursing home. Data regarding severe CDI were collected 30 days after the diagnosis of a positive case and included death (all-cause deaths and deaths attributable to CDI as judged by local case review), intensive care unit admission, and colectomy. All deaths that occurred within 30 days after diagnosis of a CDI episode were assessed by the CHEC member or a designated physician at

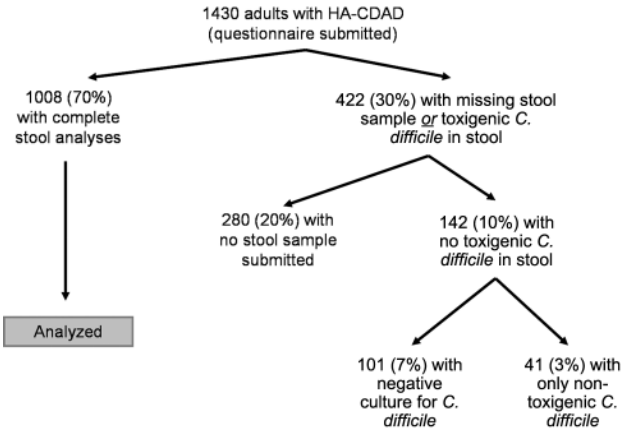

Figure 1. Origin of *Clostridium difficile* isolates analyzed in the surveillance project. HA-CDAD, health care–associated *C. difficile*–associated disease.

Table 1. Primers Used in this Study for Characterization of *Clostridium difficile* Isolates

Primer	Sequence (5′ to 3′)	Gene	Size, base pairs	Study
cdtBpos	CTTAATGCAAGTAAATACTGAG	*cdtB*	510	Terhes et al [31]
cdtBrev	AACGGATCTCTTGCTTCAGTC			
tcdA-F	AGATTCCTATATTTACATGACAATAT	*tcdA*	369/110	Lemee et al [32]
tcdA-R	GTATCAGGCATAAAGTAATATACTTT			
tcdB-3	AATGCATTTTTGATAAACACATTG	*tcdB*	329	Present study
tcdB-4	AAGTTTCTAACATCATTTCCAC			
Tpi-F	AAAGAAGCTACTAAGGGTACAAA	*tpi*	230	Lemee et al [32]
Tpi-R	5CATAATATTGGGTCTATTCCTAC			
PaL15	TCTCTACAGCTATCCCTGGT	*tcdC*	673	Spigaglia and Mastratonio [33]
PaL16	AAAAATGAGGGTAACGAATTT			

NOTE. cdtB, binary toxin subunit B; tcdA, toxin A gene; tcdB, toxin B gene; tcdC, negative regulator gene C; tpi, triose phosphate isomerase gene.

Table 2. Results of Pulse-Field Gel Electrophoresis (PFGE) Typing of *Clostridium difficile* Isolates, Including the Most Common and Epidemiologically Important Types

PFGE type	No. (%) of isolates (n = 1005)
NAP1[a]	311 (31)
NAP2	293 (29)
NAP3	11 (1.1)
NAP4	61 (6.1)
NAP5	3 (0.3)
NAP6	31 (3.1)
NAP7[b]	4 (0.4)
NAP8[c]	1 (0.1)
Other	290 (29)

[a] This NAP1 group includes 12 strains with fingerprint type 0012, which additional evidence shows may not belong to the NAP1 group of strains (see Discussion).
[b] Two of the 4 NAP7 isolates are also ribotype 078.
[c] This NAP8 isolate is also ribotype 078.

that site to determine whether the death was (1) directly related to CDI (ie, the patient had no other underlying condition that would have caused death during this hospitalization); (2) indirectly related to CDI (ie, the CDI contributed to the patient's death but was not the primary cause); or (3) not related to CDI.

For every case of CDI, a stool sample at the time of diagnosis was submitted to the National Microbiology Laboratory. The *C. difficile* culture was performed by exposing the stool to an alcohol shock procedure [18] and then subculturing onto prereduced *C. difficile* Moxalactam Norfloxacin agar (Oxoid); the specimen was then incubated anaerobically at 35°C until there was visible growth (for up to 72 h). If no growth was visible at 72 h, the culture was repeated from the stool sample. Suspected colonies of *C. difficile* were confirmed using standard techniques. Specifically, an isolate was considered to be *C. difficile* if it was an anaerobic, gram-positive, spore-forming bacillus with typical macroscopic and microscopic morphology, produced a horse stable–like odor, fluoresced a bright yellow-green under long-wave UV light, and had positive latex agglutination assay results using the Microscreen *C. difficile* Latex Agglutination Kit (Microgen Bioproducts). Isolates were stored on Microbank beads (Pro-Lab Diagnostics) at −70°C.

Pulsed-field gel electrophoresis was performed as previously described elsewhere [19], with minor modifications. In brief, 6–8-h-old cells in brain heart infusion broth were suspended in cell lysis buffer (6 mM Tris-HCl, pH 8.0; 100 mM edetic acid [EDTA], pH 9.0; 1 M NaCl; 0.2% deoxycholate; 0.5% sarkosyl; 0.5% Brij 58) and plugs were made by mixing equal volumes of cells and molten 1% Seakem Gold agarose containing 1% sodium dodecyl sulfate in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Cells were lysed by incubating plugs at 37°C overnight in cell lysis buffer containing 2 mg/mL lysozyme, 20 μg/mL RNase, and 12.5 units mutanolysin. Plugs were then incubated at 55°C for 2–3 h in 500 mM EDTA, pH 9.0, and 1% sarkosyl, containing 50 μg/mL proteinase K. Washed plugs were digested with *Sma*I for 2 h to overnight at 25°C. DNA was separated on 1% agarose gels (BioRad pulsed-field grade) in 0.5 × tris-borate-EDTA buffer containing 50 μM thiourea at 6 V/cm with 1- to 40-s switch time for 22 h. Gels were stained with ethidium bromide, destained with water, and digitally photographed. Images of gels were analyzed with BioNumerics software, version 4 (Applied Maths).

DNA was isolated by mixing 200 μL Instagene matrix (BioRad) with 400 μL of cells and boiling for 20 min. The supernatant was used directly in polymerase chain reaction (PCR). Primers used in PCR analysis are listed in Table 1. Analysis was conducted on each isolate with 2 multiplexes using Qiagen Multiplex Master Mix (Qiagen). Multiplex 1 contained 0.3 μM of the *cdtB* primers (binary toxin subunit B), 0.3 μM of the *tcdA* primers (toxin A), and 0.2 μM of the *tcdB* primers (toxin B). Multiplex 2 contained 0.2 μM of both the *tpi* primers (triose phosphate isomerase housekeeping gene) and *tcdC* primers (negative regulator of PaLoc). Cycling conditions were 94°C for 15 min followed by 30 cycles of 94°C for 30 sec, 58°C for 90 sec, and 72°C for 90 sec, then 72°C for 7 min.

Antimicrobial susceptibilities to 12 antimicrobials were determined using agar dilution following the Clinical Laboratory Standards Institute guidelines [20]. Resistant breakpoints used were as follows: metronidazole, >16 mg/L; vancomycin, >16 mg/L; teicoplanin, >16 mg/L; clindamycin, >4 mg/L; ciprofloxacin, >4 mg/L; levofloxacin, >4 mg/L; gatifloxacin, >4 mg/L; moxifloxacin, >4 mg/L; cefazolin, >16 mg/L; cefuroxime, >32 mg/L; ceftriaxone, >32 mg/L; and cefotaxime, >32 mg/L.

Although this surveillance project was observational and was considered to be within the usual scope of institutional Infection Prevention and Control programs, not involving any alteration in patient care, ethics approval was obtained at some of the participating hospitals.

Descriptive and univariate analyses were performed. To assess differences between patient populations, continuous variables were expressed by mean values and compared using the Student t test and/or the Mann-Whitney U test. Categorical variables were expressed as proportions and were compared using the χ^2 test and Fisher exact test when necessary. All tests were 2-tailed, and a P value of <.05 was considered to be statistically significant. Relative risks with corresponding 95% confidence intervals were calculated according to standard methods. Severe outcome was defined as an admission to the intensive care unit for complications of CDI, colectomy due to CDI, and/or death (directly or indirectly) related to CDI. Statistical analysis was conducted using SAS software, version 9.1 (SAS Institute).

RESULTS

A total of 1493 patients with HA-CDI were identified in the 34 hospitals during the 6-month surveillance period. Of these, 1430 (96%) were adults 18 years of age or older, and these patients are the focus of this analysis. Not all case patients had a stool sample submitted for analysis, and not all submitted stool samples yielded a toxigenic strain of *C. difficile* when cultured. Figure 1 shows the source of the 1008 *C. difficile* isolates used in this analysis. Of the 1150 stool samples that were received for culture, 101 (8.8%) showed no growth of *C. difficile,* whereas another 41 (3.6%) showed only growth of a nontoxigenic *C. difficile* strain. Thus, the stool culture yield for a toxigenic *C. difficile* isolate was 1008 of 1150 submitted stool specimens, or 87.7%. The geographic origin of the toxigenic *C. difficile* isolates, by province, is as follows: British Columbia, 81 isolates; Alberta, 111 isolates; Saskatchewan and Manitoba (combined), 37 isolates; Ontario, 451 isolates; Quebec, 225 isolates; and Atlantic Canada (combined), 103 isolates. The pulsefield typing results of 1005 isolates are shown in Table 2. Thirty-one percent of isolates were typed as NAP1, with another 29% being NAP2 (also known as the J type, by restriction endonuclease typing). The remaining 40% of isolates belonged to NAP groups 3 to 6 or did not cluster with known NAP types. Among the 311 isolates designated as NAP1, 12 have fingerprint type 0012, which is quite similar to NAP1-type fingerprints and is clustered with them by the BioNumerics software. However, additional data, such as ribotyping, toxinotyping, and *tcdC* sequence analysis, suggest that type 0012 strains do not belong to the NAP1 group. Excluding these presumptive type 0012 strains, 298 (99.7%) of the 299 NAP1 strains demonstrated the presence of the binary toxin gene and a previously described 18 base-pair deletion in the *tcdC* gene [2, 3].

Table 3. Distribution of NAP1 Strain Type, by Clinical Outcome

Outcome	No. (%) of patients with infection due to NAP1	No (%) of patients with infection due to non-NAP1 strains	Total
Severe	39 (12.5)	41 (5.9)	80
Nonsevere	272 (87.5)	656 (94.1)	928
Total	311 (100)	697 (100)	1008

NOTE. Severe outcome was defined as intensive care unit admission for *C. difficile* infection (CDI), colectomy due to CDI, or CDI-attributable death. $P < .001$ for the difference in outcome between patients with NAP1 infections and those with non-NAP1 infections.

The geographic distribution of the NAP1 strain, as a proportion of each provincial total, is shown in Figure 2: Quebec had the highest proportion of NAP1 (76% of isolates), whereas Saskatchewan and Manitoba had the lowest proportion (7%). The CDI incidence, attributable mortality, and NAP1 proportion, superimposed by province, are also shown in Figure 2.

Univariate analysis of the association of strain type with CDI outcome can be seen in Table 3. Thirty-nine (12.5%) of 311 patients infected with a NAP1 strain experienced a severe outcome, compared with only 41 (5.9%) of 697 patients infected with other strains ($P < .001$). The association of strain type with all severe outcomes, or with attributable mortality alone, as analyzed per decade of age of the infected case patients, is shown in Figure 3. Under the age of 60 and over the age of 90 years, there was no statistically significant difference in the incidence of severe outcomes between patients infected with NAP1 and

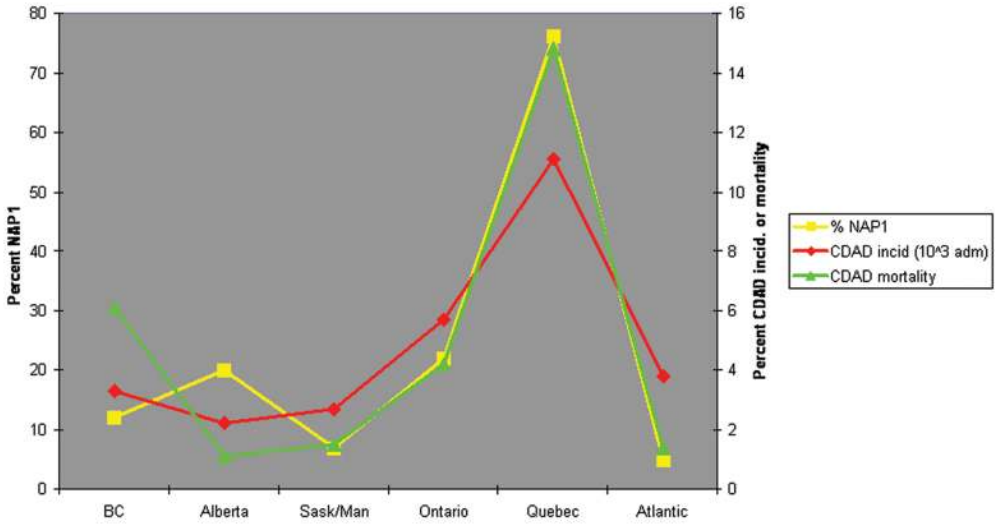

Figure 2. *Clostridium difficile* infection (CDI) incidence, CDI-attributable mortality, and fraction of NAP1 isolates (as a proportion of provincial total), by province. BC, British Columbia; CDAD, *C. difficile*–associated disease; Sask/Man, Saskatchewan and Manitoba.

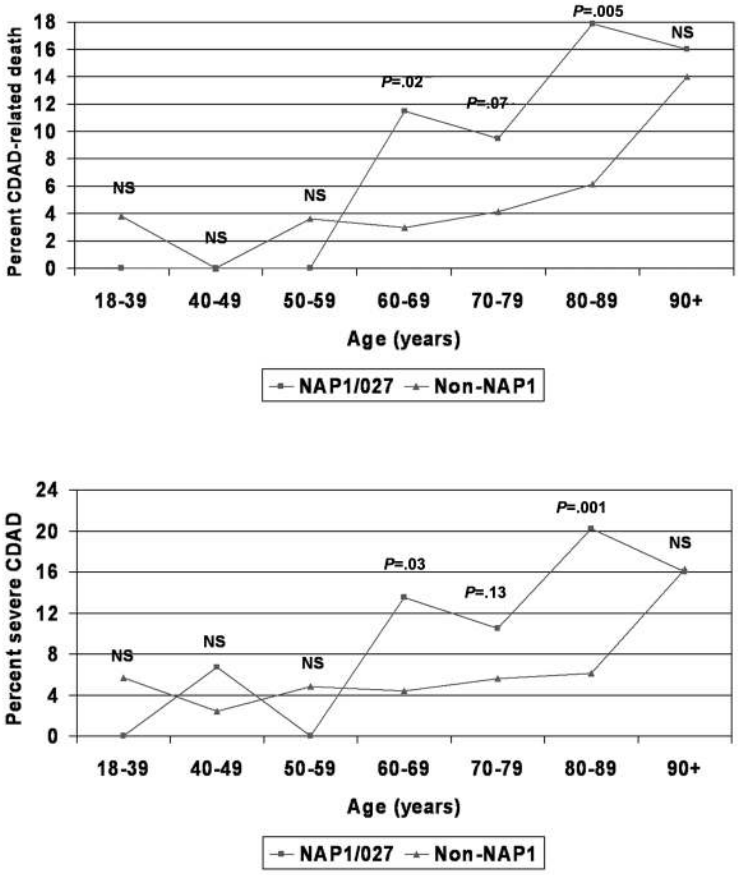

Figure 3. Incidence of death attributable to *Clostridium difficile*–associated disease (CDAD), by age and infecting *C. difficile* strain type *(top)* and incidence of severe outcomes (*C. difficile*–associated colectomy, admission to intensive care unit, or death), by age and infecting *C. difficile* strain type *(bottom)*. NS, not significant ($P > .05$).

those infected with non-NAP1 strains. However, at every age range >60 and <90 years of age, patients infected with a NAP1 strain were more likely to experience a severe outcome or CDI-attributed death, this difference being highly statistically significant except in the 70–79-year-old age group.

The antimicrobial susceptibility results for the 1008 isolates can be seen in Table 4. None of the isolates demonstrated resistance to metronidazole, vancomycin, or teicoplanin. All isolates were resistant to ciprofloxacin, cefuroxime, and cefotaxime. A statistically significant difference in the susceptibility profile was seen between NAP1 and non-NAP1 isolates for clindamycin, the "respiratory fluoroquinolones" (ie, levofloxacin, gatifloxacin, and moxifloxacin), cefazolin , and ceftriaxone. In addition, a 1-dilution "drift" upwards was seen in the 50% and 90% minimum inhibitory concentration results for metronidazole between the NAP1 and non-NAP1 isolates, as has been previously documented [12].

DISCUSSION

This analysis used a large national database of patients with CDI with clinical variables and outcome measures linked to the genotypic and phenotypic analysis of the infecting strain. With this database, there is a strong correlation between the presence of the NAP1 strain and the incidence of and mortality associated with CDI when the data are analyzed by province. In addition, we have confirmed the strong relationship between patient age and severe CDI outcome, which is strikingly similar to what has been previously found in studies with fewer individuals in a single Canadian province (Table 5) [2]. Although strain type has been suggested as an additional cause of excess morbidity and severe outcomes in other analyses [12, 21, 22], the lack of large data sets has precluded a firm conclusion on this hypothesis. A recent analysis from the United Kingdom failed to show increased virulence of the NAP1 strain in a case-case comparison [23], but their patient numbers were small, they used their own novel definition of "severe disease" (which did not include attributable death), and they lacked mortality data on 5% of their study subjects. However, in our study, which included >1000 evaluable patients with CDI, the NAP1 strain is clearly correlated with more-severe disease and outcomes among patients at almost all ages, except among younger adults (<60 years of age) and extremely elderly individuals (>90

Table 4. In vitro Antimicrobial Susceptibility Results for *Clostridium difficile* Isolates, by Strain

	NAP1			Non-NAP1			
Antibiotic	Resistant, % of isolates	MIC_{50}	MIC_{90}	Resistant, % of isolates	MIC_{50}	MIC_{90}	*P*
Metronidazole	0	1	2	0	0.5	1	NS
Vancomycin	0	1	2	0	1	2	NS
Teicoplanin	0	0.5	0.5	0	0.5	0.5	NS
Clindamycin	82	8	256	88	>256	>256	.02
Ciprofloxacin	100	>64	>64	100	64	>64	NS
Levofloxacin	92	>64	>64	66	>64	>64	<.001
Gatifloxacin	83	32	64	59	32	64	<.001
Moxifloxacin	83	32	32	60	16	64	<.001
Cefazolin	99	64	128	93	64	128	<.001
Cefuroxime	100	>256	>256	100	>256	>256	NS
Cefotaxime	100	256	>256	100	>256	>256	NS
Ceftriaxone	79	64	64	59	64	128	<.001

NOTE. MIC_{50}, 50% minimum inhibitory concentration; MIC_{90}, 90% minimum inhibitory concentration; NS, not statistically significant.

years of age). It is of note that patients ⩾90 years of age do not fare well with this infection, regardless of the infecting strain type, and demonstrate a CDI-attributable mortality rate of 16% when infection is due to NAP1 and 14% when infection is due to a non-NAP1 strain. A multivariate analysis was not performed, because other patient-specific clinical variables and strain-specific data were not collected as part of this study.

One hundred and forty-two (12.3%) of the submitted stool specimens did not yield growth of toxigenic *C. difficile*. It is possible that some of the negative culture results represent the absence of *C. difficile* in stool specimens that were erroneously identified as toxin positive by local enzyme immunoassays, because these tests are not 100% specific. However, our yield of toxigenic *C. difficile* isolates was 87.7%, which is similar to that of other studies investigating the yield of stool cultures for this pathogen in symptomatic adults [24].

We noted that 12 of the 311 strains designated as NAP1 by the BioNumerics software were different from the other NAP1 strains by 3 other molecular markers. The patients with these strains were analyzed in the NAP1 group, because most epidemiologic analyses would have designated them as having infection due to NAP1. However, as typing techniques become more discriminatory (such as multilocus variable-number tandem-repeat analysis or multilocus sequence typing, among others) [25, 26], it may be easier to allocate patients into purer and more-distinct groups by their infecting strains, thus allowing more robust analyses of these infections by strain type and subtype.

Four isolates were typed as NAP7 and 1 isolate was typed as NAP8 in our study. Because these NAP types are found among the group of emerging ribotype 078 strains, we performed ribotyping on these 5 isolates. Two of the 4 NAP7 isolates and the single NAP8 isolate were confirmed as ribotype 078.

The susceptibility pattern of the NAP1 and non-NAP1 isolates are consistent with previously published data, except that our collection of NAP1 strains were more susceptible to clindamycin than previously shown [27]. This may be a reflection of a wider geographic sampling of NAP1 strains in our survey than in the previous Quebec survey. The 1-fold dilution decrease in metronidazole susceptibility that we found among NAP1 strains has also been shown in a smaller set of Canadian isolates [12] and suggests an upward "minimum inhibitory concentration creep" among the hypervirulent NAP1 group.

Canadian hospitals that participated in the CNISP CDI project may not be representative of Canadian hospitals as a whole. CNISP hospitals are more likely to be university-affiliated, larger, and possess more specialty medical and surgical pro-

Table 5. Age-Specific Mortality due to *Clostridium difficile* Infection

	Mortality due to *C. difficile* infection, %	
Age, years	Present study	Loo et al [2]
<40	3.0	2.6
41–50	0	1.2
51–60	2.8	3.2
61–70	5.4	5.1
71–80	5.9	6.2
81–90	10.4	10.2
>90	14.7	14.0

grams. As well, not all geographic areas of Canada were well-represented in the surveillance. For instance, Atlantic Canada was relatively underrepresented, as were Quebec and Saskatchewan. Thus, the patient population and the infecting strain types included in this surveillance project may not reflect the actual Canadian experience with CDI at the time of the surveillance. However, we feel that enough patients were recruited from diverse hospitals and populations to make the analyses robust, and the strong association between NAP1 infection and severe disease is unlikely to be affected by the choice of CNISP hospitals.

The definition of CDI used in this surveillance project [12] is slightly different from that used by the Centers for Disease Control and Prevention [28] and the European Centre for Disease Prevention and Control [6], but only in a minor way (ie, number of diarrheal stools). This definition was retained for the current surveillance, because it had been used in 1997, and direct comparison with historical Canadian rates was desired.

Since this 2005 surveillance project was completed, similar CDI surveillance in Canada in 2007 showed a decrease in the proportion of NAP1 infections and CDI deaths in Quebec [29], presumably because of the increased funding for and implementation of enhanced CDI control across the entire province. However, there was an increase in the incidence of NAP1 infection in British Columbia, Ontario, and the Atlantic provinces, accompanied by an increase in CDI-related mortality in those provinces [29]. The results of the 2008 and 2009 Canadian CDI surveys are currently being analyzed.

Despite the fact that we and others have found correlations between severe CDI and patient age, it is not yet clear which host factors may explain this phenomenon. Similarly, the correlation of NAP1 infections with severe outcomes has not yet been satisfactorily explained. Despite the increased production of toxin demonstrated in this strain [30], it remains unknown if this finding is the explanation for its increased virulence. Current efforts at strain sequencing may explain the variable clinical syndromes associated with the different strain types.

In conclusion, a large Canadian CDI database has been instrumental in confirming the strong age association with severe CDI. In addition, the proportion of NAP1 infections in a given province is associated with the incidence of CDI and of severe CDI. We have also shown that patients 60–90 years of age are approximately twice as likely to die or experience severe CDI if infected with the NAP1 strain, whereas those >90 years of age experience high rates of severe CDI, regardless of strain type. Efforts to control the spread of the NAP1 strain may lead to decreased rates of CDI and of severe CDI. The bulk of the morbidity and mortality associated with this disease continues to occur in adults >60 years of age, for whom CDI prevention is crucial.

MEMBERS OF THE CANADIAN NOSOCOMIAL INFECTION SURVEILLANCE PROGRAM WHO PARTICIPATED IN THE SURVEILLANCE FOR *CLOSTRIDIUM DIFFICILE* INFECTION

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