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Lack of Association between Clinical Outcome of *Clostridium difficile* Infections, Strain Type, and Virulence-Associated Phenotypes[▽]

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***Clostridium difficile* strain NAP1/027 (North American pulsed-field gel electrophoresis [PFGE] type 1 and PCR ribotype 027 [R027]) has been associated with recent outbreaks in North America and Europe. It has been associated with more severe disease symptoms, higher mortality rates, and greater risk of relapse. This strain is thought to produce more toxins and sporulate to higher levels. However, recent studies suggest that this may not always be the case. The objective of our study was to assess, in a nonoutbreak situation, whether specific strains, such as NAP1/027, were associated with more severe disease symptoms, higher toxin production, and/or greater sporulation *in vitro*. We isolated and characterized *C. difficile* strains from 21 patients with mild to moderate, severe, or complicated symptoms of *C. difficile* infection (CDI). The isolates were characterized by different molecular typing methods, including PCR ribotyping, tandem repeat sequence typing (TRST), and sequencing of the *tcdC* gene. Fourteen isolates were of PCR ribotype 027 with deletions in *tcdC*, but no association with severity or clinical outcome was found. We show by immunodot blot detection of toxins with monoclonal antibodies that all R027 isolates produced more TcdA and TcdB than other strains. On the other hand, they consistently produced fewer spores than non-R027 isolates. Taken together, our data suggest that NAP1/027 isolates are not always associated with more severe disease, even though they may produce larger amounts of toxins. Our study also suggests that current assertions regarding the NAP1/027 may not apply to all isolates and that other factors may come into play.**

Clostridium difficile is the leading cause of nosocomial antibiotic-associated diarrhea in industrialized countries. The clinical presentation of *C. difficile* infection (CDI) varies in severity, with patients presenting with symptoms ranging from very mild diarrhea to fulminant colitis with complications such as toxic megacolon, bowel perforation, sepsis, and death (33).

The main virulence factors in *C. difficile* are TcdA and TcdB, two exotoxins encoded on a 19.6-kb pathogenicity locus, the PaLoc. The expression of the toxin genes is induced by the positive regulator TcdR (21) and repressed by TcdC (22), which is strongly expressed during early log phase (16). Deletions in the *tcdC* genes from various isolates have been reported, including a common 18-bp deletion and a 1-bp deletion at nucleotide 117 leading to the expression of a truncated TcdC. An emerging strain, referred to as NAP1/027, carries these deletions in *tcdC* and was associated with major outbreaks in North America and Europe and is now spreading worldwide (6, 18, 20). Truncation of TcdC is thought to be responsible for the higher toxin production by this strain (9, 11, 40). In addition, NAP1/027 as well as other strains encodes a binary toxin (CDT) that could possibly promote adhesion to colonic cells (31, 34). A number of studies suggest that epidemic NAP1/027 strains produce more spores, which could promote dissemination and persistence in hospital settings, thus exacerbating the problem of nosocomial transmission of CDI (1, 24, 41). However, sporulation in NAP1/027 is controversial, as shown by recent *in vitro* studies that did not show a correlation between strain type and sporulation rate (5, 27).

A current assumption is that patients infected with NAP1/027 strains develop more severe CDI symptoms and have greater risk of experiencing relapse, complications, and death (20, 23, 25, 30). However, the epidemiology of *C. difficile* is changing rapidly, and a number of recent studies suggest that strain type, including NAP1/027, is not associated with more severe disease in nonepidemic settings and that deletions in *tcdC* alone may not be good predictors of toxin production (7, 26, 42).

The objective of this study was to characterize 21 *C. difficile* isolates obtained from patients suffering from CDI. Patients were selected based on clinical outcome, with no prior assumption regarding the strain of *C. difficile* that caused disease. The isolates were typed with different methods, including PCR ribotyping and tandem repeat sequence typing (TRST), to study possible associations between strain type, disease severity, clinical outcome, and virulence-associated phenotypes, mainly toxin production and sporulation. The choice to use TRST was based on the fact that sequence data are generated, thus enabling interlaboratory comparisons.

MATERIALS AND METHODS

Patients, bacterial strains, and growth conditions. Patients were recruited during a nonoutbreak period between 16 September 2005 and 14 March 2006 at the Centre Hospitalier Universitaire de Sherbrooke (CHUS) in the province of Quebec, Canada, a 712-bed secondary and tertiary care hospital. Patients were initially selected based on CDI outcomes according to the 2010 Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) guidelines (8), and we included strains from patients with mild to moderate (n = 11), severe (n = 5), and complicated (n = 5) CDI. The

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TABLE 1. Primers used in this study

Primer	Target	Sequence (5′ → 3′)[a]	Reference
TpiA	*tpi*	GCW GGW AAY TGG AAR ATG MAY AA	10
TpiB		TTW CCW GTW CCD ATW GCC CAD AT	
TcdA-F	*tcdA*	AGA TTC CTA TAT TTA CAT GAC AAT A	19
TcdA-R		GTA TCA GGC ATA AAG TAA TAT ACT T	
TcdB-2F	*tcdB*	TGG TGA TAT GGA GGC ATC ACC ACT	This study
TcdB-2R		TGA GCT TTA GCT CTT GCA TCG TCA	
CdtA-F	*cdtA*	TGA ACC TGG AAA AGG TGA TG	37
CdtA-R		AGG ATT ATT TAC TGG ACC ATT TG	
CdtB-F	*cdtB*	CTT ATT GCA AGT AAA TAC TGA G	37
CdtB-R		ACC GGA TCT CTT GCT TCA GTC	
C1	*tcdC*	TTA ATT AAT TTT CTC TAC AGC TAT CC	36
C2		TCT AAT AAA AGG GA GAT TGT ATT ATG	
CD16S-1F	16S rRNA	GTG CGG CTG GAT CAC CTC CT	3
CD23S-2R	23S rRNA	CCC TGC ACC CTT AAT AAC TTG ACC	
TR6-F	TR6	TTT CAA CTT GTC CAG TTT TTA AGT C	43
TR6-R		ATG ACA TAG CGT TTG TGG AAT	
TR10-F	TR10	TGC ATC AAA TTG GTC AAG ACT	43
TR10-R		TGA AAT CAT TGA CTA TAA AGC AAA A	

[a] W, A or T; Y, C or T; R, A or G; M, A or C; D, A, G, or T.

institutional review board of CHUS had approved our study protocol, and informed consent was obtained from all patients. *C. difficile* was isolated from feces after alcohol shock and growth on CDMN selective agar (Oxoid, Canada) supplemented with 5% sheep blood, 0.1% taurocholate, and 1 mM glycine. The identity of presumptive *C. difficile* colonies was confirmed by PCR using primers TpiA and TpiB, which are specific to the triose phosphate isomerase gene (*tpi*) of *C. difficile*, as described before (10) (Table 1). *C. difficile* isolates were grown at 37°C under an anaerobic atmosphere (10% hydrogen, 5% CO_2, and 85% nitrogen) in an anaerobic chamber (Coy Laboratories). Bacteria were routinely grown in brain heart infusion (BHI) broth (Difco), BHI broth supplemented with 0.1% taurocholate and 1 mM glycine (BHI-tag) to favor spore germination, or tryptose yeast (TY) broth or agar (3% tryptose [Oxoid, Canada], 2% yeast extract [BioShop, Canada]). All media were prereduced overnight prior to use.

Molecular typing. Genomic DNA was extracted from 1.5-ml overnight cultures in BHI broth using the Bacteria GenomicPrep kit (GE Healthcare, Canada). All primers used in this study are listed in Table 1. The *tcdA* gene was PCR amplified as described previously (19). The *tcdB* gene was amplified with primers TcdB-2F and TcdB-2R, which we designed from a conserved region of the glucosyltransferase catalytic domain of TcdB and corresponding to nucleotides 788481 to 788505 of the *C. difficile* 630 genome (NCBI accession number NC009089). The reactions were performed in a 50-μl volume containing 1× amplification buffer (10 mM Tris-HCl [pH 9.0], 3.5 mM $MgCl_2$), 125 μM each deoxynucleoside triphosphate [dNTP], 1 μM each primer, 1.25 U *Taq* DNA polymerase, and 20 ng of purified genomic DNA. The PCR mixture was denatured (3 min at 94°C), and then 35 cycles (94°C for 30 s, 58°C for 30 s, and 72°C for 60 s) were performed, followed by a final extension step at 72°C for 5 min. The binary toxin genes *cdtA* and *cdtB* were amplified as described before (37). All PCR products were amplified in an Eppendorf Mastercycler Pro apparatus and were run on a 2% agarose gel, stained with ethidium bromide, and photographed.

Sequencing of *tcdC*. Primers C1 and C2 (Table 1) were used to amplify the entire *tcdC* gene as described elsewhere (36). The products were sequenced on an ABI 3730 xl DNA sequencer (Applied Biosystems, Canada) at the genomic platform of the CHUL Research Center (Québec, Canada). Sequences were analyzed and compared to the *tcdC* sequence from the reference strain VPI 10463 using BioNumerics v5.10 (Applied Maths, Belgium).

PCR-ribotyping. PCR-ribotyping was performed using primers CD16S-1F and CD23S-2R as described previously (3) (Table 1) with modifications described elsewhere (13). Patterns were compared using Gel Compar II (Applied Maths, Belgium), and profiles with ≤85% similarity using Pearson's correlation were considered different PCR ribotypes.

TRST. Tandem repeat sequence typing (TRST) was based on the method described by Zaiss et al. (43) except that loci TR6 and TR10 were amplified separately. PCR products were sequenced at the CHUL Research Center, and sequences were analyzed using BioNumerics v5.10 and the Repeat-Typing plug-in (Applied Maths, Belgium). The assignment of TRST types was based on associated repeat profiles and repeat sequences previously reported by Zaiss et al. (43).

MIC determination. Antibiotics tested were clindamycin (CLI), ceftriaxone (CRO), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), metronidazole (MTZ), vancomycin (VAN), piperacillin-tazobactam (TZP), and tigecycline (TIGE). MICs were determined using a broth microdilution method in 96-well plates. Overnight cultures in BHI broth were adjusted to a 0.5 McFarland standard and seeded into 96-well plates containing BHI broth and antibiotics to final concentrations ranging from 256 to 0.125 μg/ml. Plates were incubated for 18 h under an anaerobic atmosphere, and the optical density at 600 nm (OD_{600}) was recorded using a PowerWave XS microplate reader (BioTek Instruments, Canada). MIC results were interpreted according to the M11-S1 updated tables of the Clinical and Laboratory Standards Institute (CLSI) guidelines for anaerobes (28), using *C. difficile* breakpoints for the agar dilution method since no values were available for the broth method. Breakpoints for MXF were used to interpret CIP and LVX results. No breakpoints were available for ceftriaxone with *C. difficile*.

Evaluation of toxin production. Toxin production was assessed *in vitro*, as described previously (35), using an immunodot blot procedure. Briefly, an overnight culture in BHI broth was used to inoculate 50 ml of TY broth (2% inoculum). After 12, 24, 36, and 48 h of incubation, 500-μl culture samples were centrifuged at 13,000 × *g* for 1 min. Twenty microliters of the cleared supernatants was transferred onto a nitrocellulose membrane using a dot blotting apparatus. TcdA and TcdB toxins were detected using anti-TcdA and anti-TcdB monoclonal antibodies (Meridian Life Science), followed by detection with a monoclonal anti-IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling) and the ECL Plus chemiluminescence kit (GE Healthcare, Canada). Membranes were exposed to Hyperfilm ECL autoradiography films (GE Healthcare, Canada). Densitometry analysis of the spots was performed in three independent experiments with ImageJ v1.44o, and relative density units (arbitrary units [AU]) were given to each spot. The overall mean spot density at 48 h was

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TABLE 2. Molecular and phenotypic characteristics of *C. difficile* isolates

CDI clinical outcome	Strain	Ribotype	TRST	CDT[a]	*tcdC*[b]	Toxins at 48 h[c]				Spores at 48 h[d]
						TcdA		TcdB		
Complicated	383	27	27	+	+	466	H	616	H	L
	390	27	27	+	+	467	H	559	H	L
	391	27	27	+	+	579**	H	474	H	L
	398	18	19	+	wt	106	L	240	L	L
	403	27	27	+	+	571**	H	423	H	L
Severe	395	27	27	+	+	725***	H	500	H	L
	396	27	27	+	+	171	L	242	L	L
	384	53	3	−	wt	104	L	132	L	H
	392	23	14	−	wt	277	L	217	L	H***
	399	6	2	−	wt	95	L	156	L	H
Mild to moderate	386	27	27	+	+	670**	H	662*	H	L
	388	27	27	+	+	647**	H	551	H	L
	389	27	27	+	+	445	H	474	H	L
	393	27	27	+	+	605**	H	425	H	L
	394	27	27	+	+	503	H	416	H	L
	397	27	27	+	+	447	H	445	H	H
	387	27	27	+	+	425	H	379	H	L
	401	27	27	+	+	352	L	408	H	L
	385	18	19	+	wt	204	L	431	H	H
	400	24	65	−	wt	86	L	76	L	H
	402	6	2	−	wt	187	L	19	L	H***

[a] Presence (+) or absence (−) of the *cdtA* and *cdtB* genes, encoding the binary toxin CDT.
[b] wt, wild-type *tcdC* sequence identical to that of the reference strain VPI 10463; +, presence of the common 18-bp deletion in addition to the 1-bp deletion at nucleotide 117.
[c] Relative amounts of toxins detected by immunodot blotting; numbers indicate the mean density of the spots (arbitrary units [AU]) calculated from three independent blots. Letters indicate low (L) and high (H) toxin production compared to the mean densities for TcdA (387 AU [range, 292 to 483]) and TcdB (373 AU [292 to 455]). Asterisks indicate individual values that are significantly different from mean values for non-R027 isolates: *, = $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[d] L and H, isolates with spore counts at 48 h (spores/ml) below or above the mean for all isolates (1.4×10^7 spores/ml [range, 8.3×10^6 to 2.0×10^7]), respectively. Asterisks indicate isolates that produced significantly more spores than R027 isolates (***, $P < 0.001$).

then calculated by including all isolates, and low (L) and high (H) toxin production correspond to mean density values below or above the mean, respectively.

Evaluation of sporulation rates. The method for evaluation of sporulation rates consisted of inoculating 10 ml of TY broth with 2% of an overnight culture in BHI-tag broth. When the OD_{600} reached 0.5, 10 ml of fresh TY broth was inoculated at 2% and further incubated. After 24, 48, and 72 h of incubation, 100-μl samples were collected, fixed in 0.1% formaldehyde, and placed into a Petroff-Hausser chamber for counting. Under phase-contrast light, spores were

TABLE 3. MICs of antibiotics for *C. difficile* isolates

Strain[a]	MIC (μg/ml)[b]								
	CLI	CRO	CIP	LVX	MXF	MTZ	VAN	TZP	TIGE
383*	>256	64	>256	>256	32	1	0.5	8	<0.125
386*	>256	64	>256	256	32	1	1	16	<0.125
387*	>256	64	>256	256	32	1	1	8	<0.125
388*	>256	128	128	>256	32	1	1	16	<0.125
389*	>256	128	256	>256	32	2	2	16	<0.125
390*	>256	128	256	>256	32	4	2	16	<0.125
391*	>256	128	256	>256	32	0.5	2	16	<0.125
393*	256	128	256	256	32	4	2	16	<0.125
394*	256	128	256	256	16	1	1	16	<0.125
395*	256	128	256	256	32	2	1	16	<0.125
396*	256	128	>256	256	16	8	2	16	<0.125
397*	256	128	256	256	16	4	2	16	<0.125
401*	256	128	256	256	16	4	2	16	<0.125
403*	256	128	>256	256	16	2	2	16	<0.125
384	>256	64	16	8	2	1	1	8	<0.125
385	256	32	8	4	2	2	1	8	<0.125
392	>256	64	16	8	2	8	2	16	<0.125
398	>256	64	16	8	2	8	2	16	<0.125
399	>256	64	16	4	1	4	4	16	<0.125
400	256	64	16	8	2	4	2	16	<0.125
402	>256	64	16	8	2	4	4	16	<0.125

[a] NAP1/027 strains are marked with an asterisk.
[b] CLI, clindamycin; CRO, ceftriaxone; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; MTZ, metronidazole; VAN, vancomycin; TZP, piperacillin-tazobactam; TIGE, tigecycline.

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seen as bright objects. Free spores were counted in three independent experiments, and the sporulation rate was determined as spores/ml. The mean spore count at 48 h was calculated by including all isolates, and low (L) and high (H) sporulation rates correspond to spore counts below or above the mean, respectively.

Statistical analysis of the data. Statistical analysis of the data was done using GraphPad Prism v5.0d. To assess whether R027 isolates produced significantly more toxins than non-R027 isolates, TcdA and TcdB density values for each R027 isolate at 48 h were compared by one-way analysis of variance (ANOVA), followed by a Dunnett posttest using density values at 48 h for all non-R027 isolates as the comparator group. To assess whether non-R027 isolates produced more spores/ml than R027, spore counts for each non-R027 isolate at 24, 48, and 72 h were compared by one-way ANOVA, followed by a Dunnett posttest using the mean spore count for all R027 isolates as the comparator group. Statistical significance was obtained with a P value of <0.05.

RESULTS

Molecular typing versus clinical outcome. To have an idea of the genetic diversity of our *C. difficile* collection, strains were characterized first by PCR-ribotyping and subsequently by TRST (43). Most of the selected isolates (14; 67%) were of ribotype 027 (R027) and TRST type 027 (Table 2). The 7 other isolates represented 5 different ribotypes and 5 different TRST types (Table 2). Interestingly, no association between clinical outcome and strain type could be made, and the R027 isolates were found in all severity groups: mild to moderate ($n = 8$), severe ($n = 2$), and complicated ($n = 4$) (Table 2). TcdA and TcdB toxin genes were detected by PCR in all isolates (data not shown), and the binary toxin genes *cdtA* and *cdtB* were detected in all R027 isolates, as previously reported (40), and in two non-R027 isolates, CD385 and CD398 (both ribotype 18 and TRST 19). Finally, the *tcdC* gene was sequenced for all isolates and compared to that of the reference strain VPI 10463 (ATCC 43255). All and only R027 isolates had the characteristic deletion at nucleotide 117 in addition to the 18-bp deletion (Table 2).

Antibiotic susceptibility testing. Susceptibilities to 9 different antibiotics were determined for all isolates, and MIC values are summarized in Table 3. According to the CLSI guidelines, all 21 isolates were resistant to CLI (MIC $\geq$ 256 μg/ml) and showed high MIC values for ceftriaxone ($\geq$32 μg/ml) but were susceptible to MTZ, VAN, TZP, and TIGE. All R027 isolates were resistant to CIP, LVX, and MXF, while the non-R027 isolates were all susceptible (Table 3).

***In vitro* toxin production.** To determine whether NAP1/027 isolates in our set produced more toxins than other isolates, we compared the total *in vitro* extracellular toxin production of the 21 isolates using an immunodot blot method that enabled us to discriminate TcdA and TcdB. Densitometry analysis of the spots was performed on three independent blots (only one example is shown in Fig. 1), and the mean density was calculated after including all values at 48 h for all strains. As shown in Fig. 1 and Table 2, R027 isolates generally produced larger amounts of both TcdA and TcdB toxins, and most density values were above the mean (387 AU for TcdA and 373 AU for TcdB). Among the R027 isolates, 7 (50%) had values above the 95% confidence interval (CI) for TcdA (292 to 483 AU) and 7 (50%) had values above the 95% CI for TcdB (292 to 455 AU) (Table 2). In contrast, all non-R027 isolates produced much less toxin and all values were below the lower limit of the 95% CI for both toxins, except for CD385, which produced

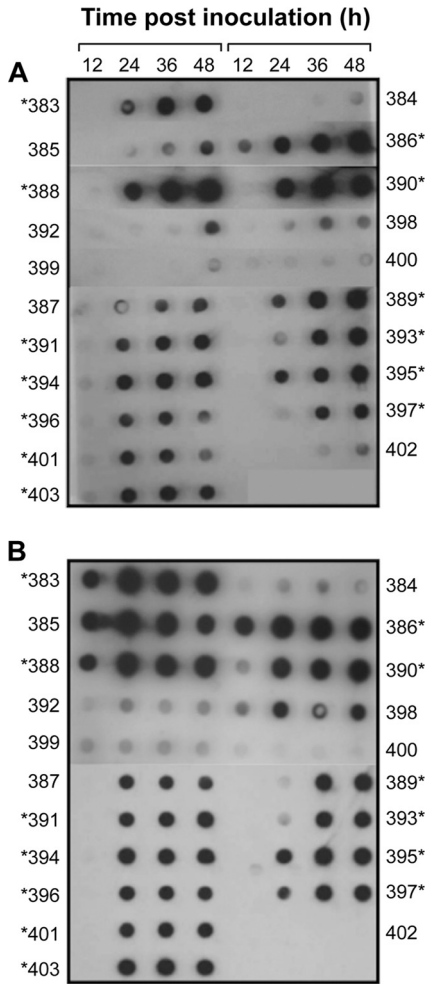

FIG. 1. Immunodot blot detection of TcdA (A) and TcdB (B) toxins released in culture supernatants by different *C. difficile* isolates. The experiment was done three times, and representative blots are presented. R027 isolates are marked with an asterisk.

TcdB amounts similar to those produced by R027 isolates (Table 2). Interestingly, the R027 isolate CD396 had density values for TcdA and TcdB below the lower limits of the 95% CI.

Sporulation rate. We performed a series of sporulation experiments *in vitro* to determine whether R027 isolates have greater sporulation capacities than non-R027 isolates. Interestingly, all non-R027 isolates except CD398 produced more spores after 48 h than the mean for all isolates (1.4×10^7 spores/ml) (Fig. 2 and Table 2). Moreover, 4 of these isolates (CD384, CD392, CD399, and CD402) had spore counts greater than the higher limit of the 95% CI (8.3×10^6 to 2.0×10^7 spores/ml). In contrast, 8 (57%) R027 isolates had a sporulation rate below the lower limit of the 95% CI, and only one R027 isolate (CD397) reached the mean sporulation rate, with 1.4×10^7 spores/ml (Fig. 2). Statistical analysis of the data showed that compared with R027 isolates, sporulation was significantly higher at 24 h for strain CD392 ($P < 0.001$). At 48 h, the difference was significant for strains CD392 ($P < 0.001$) and CD402 ($P < 0.001$), and at 72 h it was significant for

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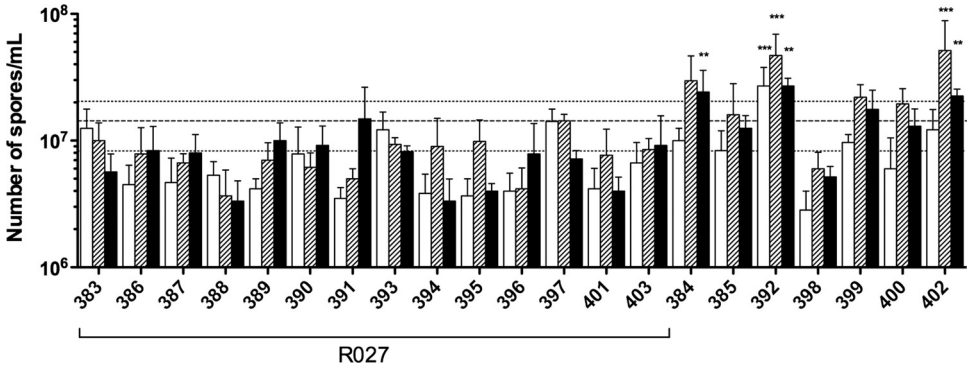

FIG. 2. Sporulation of *C. difficile* isolates as a function of time. Spores were counted at 24 h (white bars), 48 h (hatched bars), and 72 h (black bars) postinoculation. Values represent the mean spore count (spores/ml) ± standard error of the mean (SEM) from three independent experiments. The bracket indicates R027 isolates, and the dashed lines represent the mean and the lower and higher limits of the 95% CI. Asterisks indicate non-R027 isolates that sporulated significantly more than the mean for all R027 isolates combined at the corresponding sampling time: *, $P < 0.5$; **, $P < 0.01$; ***, $P < 0.001$.

strains CD384 ($P < 0.01$), CD392 ($P < 0.01$), and CD402 ($P < 0.05$) (Table 2).

DISCUSSION

A hypervirulent and epidemic strain called NAP1/027 has emerged over the last decade and has caused several outbreaks in North America and Europe (17, 20). These outbreaks have been associated with more severe disease symptoms, higher morbidity and mortality, and frequent relapses (23, 25, 30). This is generally thought to be due to increased toxin production (1, 40), increased sporulation capacity (1, 24), and resistance to fluoroquinolones (29) of the NAP1/027 strain. Hence, when a patient is infected with a NAP1/027 strain, it is generally assumed that the patient will experience more severe disease, with a higher risk of complication, relapse, and death. However, a growing number of studies suggest that this is not always true (7, 26, 42).

For example, Morgan et al. have compared severity of *C. difficile* disease for patients with R027 versus non-R027 ribotypes using a retrospective case-case study. The group included 123 patients admitted to a hospital in England during 2006. In a multivariable model, R027 was not associated with more severe disease than other PCR ribotypes (26). In a prospective study, Cloud and colleagues have typed by pulsed-field gel electrophoresis (PFGE) 236 isolates collected between 2004 and 2006 in a nonepidemic setting in a hospital in Boston, MA. NAP1 isolates composed 25% of their cohort, and in both univariate and multivariate analyses, patients infected with the NAP1 strain did not have worse outcomes than those infected with non-NAP1 strains (7). Wilson et al. have conducted a 1-year prospective study in England from 2007 to 2008. Of the 128 cases, 34 strains were R027, and again there was no association between this particular type and severe disease, relapse, early treatment failure, or attributable mortality (42). As suggested by Wilson et al., much of the evidence that NAP1/027 strains are more virulent and associated with more severe disease comes from studies involving outbreak settings (20, 23). Of the 21 isolates characterized in our setting, 14 corresponded to ribotype 027, and our data further support the conclusion that in nonepidemic settings, the NAP1/027 strains are not more virulent or associated with more severe disease and clinical outcome than other strains. Although our cohort was small compared to other epidemiological studies that addressed this question, our study was more exhaustive because it combined several molecular and phenotypic analyses that reinforce our conclusions.

Toxin production is an important virulence-associated phenotype, and previous studies suggested that R027 isolates produce more toxin *in vitro* (40), which led to the general assumption that NAP1/027 strains cause more severe disease (2, 24, 40). Warny et al. showed that toxin production by the epidemic NAP1/027 strain occurred earlier during growth and was stronger than that by nonepidemic toxinotype 0 strains (40). Curry et al. suggested that the Δ117 deletion in the *tcdC* gene led to severe truncation of TcdC, which probably explains the increased toxin production reported in NAP1/027 strains (9). However, Murray et al. also reported that two NAP1-related strains produced TcdA and TcdB earlier in exponential growth phase than five non-NAP1 strains, but the overall final toxin concentration in culture supernatants did not correlate with the presence or lack of the *tcdC* deletion (27). In two independent studies, Goldenberg and French (15) and Verdoorn et al. (38) came to the conclusion that *tcdC* deletions were not associated with disease severity or clinical outcome. In our study, all R027 isolates had the 18-bp and Δ117 deletions in *tcdC* and produced more toxins than non-R027 isolates *in vitro*, which is in agreement with the general observation mentioned above (24, 40). There were two exceptions, however: the R027 isolate CD396 produced smaller amounts of TcdA and TcdB despite the deletions, and strain CD385, a non-R027 isolate with a wild-type *tcdC*, had a TcdB production similar to that by R027 isolates, although the TcdA production was lower. It is noteworthy that strain ATCC 43255 (VPI 10463) has a wild-type *tcdC* gene and yet is a high toxin producer *in vitro* (reference 27 and our unpublished data). This further demonstrates that the sequence of *tcdC* alone should not be used to predict toxin production *in vitro*, although in our set there was a good correlation. The dynamics of toxin production have been shown to vary depending on the growth medium used and on the presence of rapidly metabolizable sugars such as glucose (12).

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Consequently, one must be careful when comparing results from different studies using different media. It should also be kept in mind that *in vitro* studies may not reflect what happens *in vivo* during an infection and that regulation of toxin synthesis could be different and influenced by multiple factors, as pointed out previously (2, 14).

Our study also revealed that non-R027 isolates sporulated faster and to greater extents than R027 isolates. This is in contradiction with other reports that suggested that R027 isolates have better sporulation capacities than non-R027 isolates (1, 24, 41). It is noteworthy that toxin A and B production and sporulation rates were generally inversely related in our assays. Hence, NAP1/027 isolates produced more toxins but sporulated less, whereas non-R027 isolates produced less toxin, likely because they sporulated to higher levels. This is in agreement with a previous study by Akerlund et al. (2, 24).

Recently, Burns et al. reported that R027 isolates did not sporulate more than non-R027 isolates and, furthermore, that some R027 isolates sporulated less than other types (5). Discrepancies in the sporulation capacities reported for NAP1/027 and other strains of *C. difficile* may be explained by the various methods and media used to isolate and recover spores. For example, Burns and colleagues observed significant interstrain differences in sporulation frequencies depending on whether heat shock or microscopy was used to count spores (5). Along the same line, Rodriguez-Palacios and LeJeune observed that heating spore suspensions at 85°C for 15 min significantly reduced the number of colonies recovered compared to heating at 63 or 71°C (32). In another study by Burns et al., spores from strain R20291 (a NAP1/027 strain) were shown to germinate less efficiently than those from strain 630Δ*erm* (4). Taken together, these reports suggest that the method used to isolate and recover spores in a sample has a significant impact on the sporulation rate calculated, and as a consequence, care should be taken when interpreting sporulation data. In the course of our work, we also determined sporulation rates using a method that involved heating spore suspensions for 10 min at 80°C followed by plating on BHI agar supplemented with taurocholate and glycine as germinants. Although our results (data not shown) were consistent with those reported here obtained by using the Petroff-Hausser method, we did see significant differences from one strain to another, and the interassay variability was higher than with the microscopic method. We think that methods relying on the microscopic observation of spores are more robust and representative of the real sporulation capacity of *C. difficile*. Thus, these methods should be favored to avoid any bias caused by interstrain differences in the germination capacity or heat resistance of spores.

The antibiotic associated with CDI was a quinolone in at least half of the cases in our study. Quinolones are often associated with CDI (29), and mutations in the *gyrA* and *gyrB* genes were shown to confer resistance, especially in NAP1/027 strains (39). We did not search for gyrase mutations, but resistance to fluoroquinolones was evident in all R027 isolates. Most other strains were susceptible to quinolones. Resistance to metronidazole or vancomycin was not observed.

In summary, there is a lack of association between severe CDI symptoms and the presence of the NAP1/027 strain in our study. Moreover, R027 strains sporulate less and produce more toxins than non-R027 strains, but no association was found between these phenotypes and CDI severity. Taken together, our data suggest that general assertions regarding the "hypervirulence" and "hypersporulation" of NAP1/027 strains should be avoided, especially in nonepidemic settings. One major limitation of our study is the small sample size compared to those in other epidemiological studies. On the other hand, our *in vitro* toxin production and sporulation experiments were done on a more diversified and larger set of strains than in previous studies, which strengthens our conclusions.

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