Multilocus Variable-Number Tandem-Repeat Analysis of *Clostridioides difficile* Clusters in Ribotype 027 Isolates and Lack of Association with Clinical Outcomes

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ABSTRACT The epidemiology of *Clostridioides difficile* infection (CDI) has drastically changed since the emergence of the epidemic strain BI/NAP1/027, also known as ribotype 027 (R027). However, the relationship between the infecting *C. difficile* strain and clinical outcomes is still debated. We hypothesized that certain subpopulations of R027 isolates could be associated with unfavorable outcomes. We applied high-resolution multilocus variable-number tandem-repeat analysis (MLVA) to characterize *C. difficile* R027 isolates collected from confirmed CDI patients recruited across 10 Canadian hospitals from 2005 to 2008. PCR ribotyping was performed first to select R027 isolates that were then analyzed by MLVA ($n = 450$). Complicated CDI (cCDI) was defined by the occurrence of any of admission to an intensive care unit, colonic perforation, toxic megacolon, colectomy, and if CDI was the cause or contributed to death within 30 days after enrollment. Three major MLVA clusters were identified, MC-1, MC-3, and MC-10. MC-1 and MC-3 were exclusive to Quebec centers, while MC-10 was found only in Ontario. Fewer cases infected with MC-1 developed cCDI (4%) than those infected with MC-3 and MC-10 (15% and 16%, respectively), but a statistically significant difference was not reached. Our data did not identify a clear association between subpopulations of R027 and different clinical outcomes; however, the data confirmed the utility of MLVA's higher discrimination potential to better characterize CDI populations in an epidemiological analysis. For a patient with CDI, the progression toward an unfavorable outcome is a complex process that probably includes several inter-related strain and host characteristics.

KEYWORDS *Clostridioides difficile*, *Clostridium difficile*, MLVA typing, ribotype 027, complication, hypervirulent

Clostridioides difficile remains the primary cause of health care-acquired antibiotic-associated diarrhea in industrialized countries (1, 2). The increase in mortality and morbidity has been attributed to the emergence of a strain that caused multiple outbreaks in North America and Europe since the 2000s (3, 4). This strain, BI/NAP1/027 (R027), was initially thought to be more virulent than other isolates due to its multidrug resistance profile and its greater potential for toxin production (4, 5). However, some studies have reported variable virulence-associated phenotypes of

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R027 isolates (6–10). Also, the association between R027 isolates and unfavorable clinical outcomes is still controversial. While some studies have reported an association between BI/NAP1/027 isolates and higher 30-day mortality, recurrence, complicated CDI (cCDI), and CDI severity (3, 11–15), other large studies did not show any statistically significant association (16–20).

In a large prospective multicenter cohort of 1,380 adults, we failed to identify a significant association between cCDI and R027 strains (16). A potential hypothesis to explain these inconsistent findings is the presence of genetic variability within a given ribotype cluster. Other more discriminant typing techniques, such as multilocus variable-number tandem-repeat analysis (MLVA), have been used to identify the transmission dynamics of *C. difficile* in health care settings (21, 22). This method relies on the PCR amplification of 7 different genetic loci comprising repetitive sequences. The number of repetitions in each locus can be determined using high-resolution chromatographic methods such as capillary electrophoresis, thus allowing discrimination of closely related isolates. Previous studies have identified several subtypes with R027 isolates using MLVA (23–25). In the present study, we used MLVA to discriminate subtypes within a group of R027 isolates collected from 10 hospital centers in Québec and Ontario between 2005 and 2008 and sought to investigate potential associations between cCDI and specific subtypes.

(Part of the results was presented at the 5th International *Clostridium difficile* Symposium [ICDS], 19 to 21 May 2015, in Bled, Slovenia, by J. R. Garneau, C. N. Abou Chakra, L.-C. Fortier, A.-C. Labbé, A. McGeer, J. Pépin, and L. Valiquette, entitled “Analysis of the relationship between MLVA genetic types and clinical outcomes following infection by *Clostridium difficile* ribotype 027.”)

MATERIALS AND METHODS

Patients with confirmed CDI, hospitalized in one of 10 acute care hospitals in the provinces of Quebec and Ontario, Canada, were enrolled prospectively between June 2005 and October 2008 (16). The study was approved by the research ethics boards of all participating institutions. CDI was defined as having at least six unformed stools over 36 h or having a diagnosis of paralytic ileus, and either positive *C. difficile* toxin detection in a stool sample or pseudomembranous colitis demonstrated by endoscopy. Patients were eligible if they were 18 years of age or older, hospitalized at the time of diagnosis, and if the subject or proxy provided written consent to participate. Subjects already experiencing one of the predefined outcomes at the time of enrollment or receiving palliative care were excluded. At the time of enrollment, we recorded demographics, data on hospital admission, chronic comorbidities, immunosuppression, and surgical procedures or gastrointestinal instrumentation. Information was collected from medical charts and patient interviews about antimicrobial therapy, gastric acid suppression, and antiperistaltic agents used within 2 months prior to the diagnosis of CDI. The most abnormal value of vital signs and laboratory tests within 12 h before or 24 h postenrollment was abstracted. Detailed methodology on laboratory and diagnostic tests and variable definitions were previously described elsewhere (16).

Bacterial strain typing. *C. difficile* was isolated from frozen fecal samples after alcohol shock and growth on selective *C. difficile* agar base supplemented with moxalactam and norfloxacin (CDMN; Oxoid, Canada), 0.1% taurocholate, and 1 mM glycine and then grown at 37°C in an anaerobic chamber (Coy Laboratories, USA). All media were prerduced overnight prior to use. Capillary-based PCR ribotyping was performed in a final volume of 10 μ l using primers CD16S-1F and CD23S-2R (6, 20) and 1 $\times$ PCR buffer, 200 μ M deoxynucleoside triphosphates (dNTPs), 1.5 mM MgCl₂, 0.6 μ M each primer, 0.2 units of Platinum Taq DNA polymerase (Invitrogen), and 20 ng of purified genomic DNA. The cycling conditions were and initial incubation of 2 min at 95°C followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The amplification was completed by a 2-min incubation at 72°C. The amplified products were analyzed by automated chip-based microcapillary electrophoresis on a Caliper LC-90 instrument (Caliper Life Sciences). Chromatograms were converted into band profiles and integrated into the GelCompar II database version 5.1 (Applied Maths NV) for cluster analysis. New ribotype groups were assigned for strains not matching any of the reference strain profiles in our library (electrophoresis profile with Pearson correlation of <85% with reference profiles). MLVA typing was conducted on strains identified as R027 using the method described by van den Berg et al. (26). Five variable-number tandem-repeat (VNTR) loci, designated A6, B7, C6, E7, and G8, were targeted and amplified by PCR from all *C. difficile* isolates. Loci F3 and H9 were excluded from the analysis, as they were found to be invariant in R027 strains (25–27). Analyses of MLVA profiles were performed using BioNumerics V.5.01 (Applied Maths NV). The unweighted pair group method with arithmetic mean (UPGMA) dendrogram for the clustering and assignment of the MLVA types was constructed using multistate categorical coefficients with a tolerance value of zero. Following the type assignment for each strain, the clustering of MLVA types into clonal clusters and the creation of a minimum-spanning tree (MST) was done using the Manhattan coefficient (MC) (28–30), which takes into account the sum of tandem repeat difference (STRD) for the five loci analyzed. Neighbor strains showing an STRD of ≤ 2 were considered clonal and were grouped to form an MLVA clonal complex, further used in the association analyses (26, 31–33). For strains with equal

STRDs, a priority rule was used to give a closer relationship to strains varying only at a single locus (23, 26). An MLVA complex was established and identified if it contained at least four strain types.

CDI clinical outcomes. At the time of enrollment, severe CDI was defined as having a white blood cell count (WBC) of $>15,000$ cells/ μ l or a serum creatinine increase of $\geq 50\%$ from baseline (34) (adjusted to age and sex in white race patients [35]). Within 30 days of follow-up, complicated CDI (cCDI) was defined by the occurrence of any of (i) admission to an intensive care unit (ICU), (ii) colonic perforation, toxic megacolon, colectomy, or hemicolectomy, or (iii) CDI was the cause or contributed to death within 30 days after enrollment. Recurrent CDI (rCDI) was defined by the reappearance of diarrhea and the presence of toxin, compatible endoscopy, or a prescription of an empirical CDI treatment, in alive patients at least 48 h after the completion of the treatment for the enrollment episode.

Statistical analyses. Statistical analyses were performed using SPSS Statistics 22.0 (SPSS Inc., Chicago, IL) and SAS/STAT 9.4 software (SAS Institute Inc., Cary, NC, USA). Proportions were compared with two-tailed χ^2 and Fisher exact tests when appropriate. Logistic regression was used to identify predictors of severe and cCDI with 95% confidence intervals (CI), and survival analyses (Kaplan-Meier method, log rank test, and Cox hazards modeling) were used for recurrences. The time to event variable was defined as the time until the first recurrence, and censoring was used at death or date of last known contact. Multivariate forward models were built up sequentially with variables significant at a P value of ≤ 0.1 in univariate analyses and variables with P values of < 0.05 were kept in the final models.

RESULTS

A total of 1,053 stool samples were obtained, and the PCR ribotype was assigned to 922 isolates (614 from Ontario centers and 308 from Quebec). Among those, 52.4% ($n = 483$) were classified as R027 (262 from Ontario centers and 221 from Quebec). MLVA typing was completed only for 450 (93%) patients with R027. The main clinical characteristics of these patients are shown in Table 1. Most patients were 65 years or older (73%; $n = 329$) with the presence of at least one chronic disease in 88% and two or more in 66%. Almost two-thirds of patients (62%; $n = 277$) were exposed to two or more antimicrobials within 2 months prior to the CDI diagnosis. At enrollment, CDI was the first episode in 80% of patients ($n = 361$), was health care acquired in 92% ($n = 412$), and a majority of patients were treated with metronidazole either alone (85%; $n = 383$) or in combination with oral vancomycin.

MLVA clusters. A total of 371 different MLVA types were identified using our stringency criteria. MLVA type 31 was the most frequent and included 10 strains exhibiting the same locus profiles. Three types included five strains (types 4, 32, and 212), two types had 4 strains (types 13 and 33), five types had 3 strains (types 14, 26, 52, 152, and 202), and 42 types were composed of only two strains. All remaining 315 types consisted of single isolates. We evaluated the diversity index (DI) for each locus. Locus C6 was the most heterogeneous, with 47 different states resulting in a high DI of 0.955. Locus A6 had 35 different states for a DI of 0.933. Locus B7 showed 21 states and a DI of 0.853. Locus G8 demonstrated 19 states (DI = 0.699). Locus E7 was the least variable locus, with only 10 different observed states (DI = 0.631). Strains with highly similar MLVA locus profiles (STRD ≤ 2) were grouped into clusters using the Manhattan coefficient. The resulting MST is shown in Fig. 1.

A total of 14 MLVA clusters were identified. Among these, three major clusters were observed: MC-1 ($n = 69$), MC-3 ($n = 53$), and MC-10 ($n = 50$) (see Table S1 in the supplemental material). The other 11 clusters ($n = 183$) comprised between 5 and 15 strains, with profiles closely related to those of ≤ 4 other types, and were not grouped into any complex (labeled MC-other).

The distribution of major groups according to the province and year of diagnosis of CDI is shown in Table 2. Important differences were observed between the provinces and across the years of CDI diagnosis ($P < 0.001$ for all comparisons). Clusters MC-1, MC-4, MC-6, and MC-7 comprised strains originating exclusively from a single center located in Montreal (Fig. 1; Table S1). Infection with MC-1 started in 2006 and represented 38% of strains in this center and 15% of all strains. MC-3 was only present in both Quebec centers. The MC-1 and MC-3 complexes represented 33% and 25%, respectively, of the strains collected in the Province of Quebec, whereas MC-10 strains were only present in Ontario centers and represented 21% of all the strains collected in that province. Patients who acquired CDI within hospital settings (hospital onset-

TABLE 1 Characteristics of patients with R027 *Clostridioides difficile* infection

Variable	No./total no. (%) or median value (IQR) ^a for group in which:	
	MLVA was obtained (n = 450)	MLVA was not obtained (n = 33)
Age (years)	74.4 (63.4–83.1)	67.1 (50.5–77.0)
Sex		
Female	209 (46.4)	11 (33.3)
Male	241 (53.6)	22 (66.7)
Charlson comorbidity index		
0–3	247 (54.9)	18 (54.6)
4–6	140 (31.1)	9 (27.3)
≥7	63 (14.0)	6 (18.2)
Antimicrobial exposure ^b	401 (89.1)	28 (84.9)
Cephalosporins	244 (54.2)	12 (36.4)
Fluoroquinolones	181 (40.2)	11 (33.3)
Carboxy-ureidopenicillins	85 (18.9)	9 (27.3)
Macrolides-clindamycin	83 (18.4)	8 (24.2)
Antistaphylococcal-aminopenicillins	71 (15.8)	2 (6.1)
CDI diagnosis method ^c		
Conventional toxins A+B EIA	243 (54.0)	25 (75.8)
GDH+toxin A detection	97 (21.6)	1 (3.0)
Rapid toxins A+B EIA	89 (19.8)	7 (21.2)
Cytotoxicity assay	21 (4.7)	
Origin of CDI		
Hospital onset-HCFA ^d	375 (83.3)	26 (78.8)
Community onset-HCFA	37 (8.2)	2 (6.1)
Community acquired	38 (8.4)	5 (15.2)
CDI treatment		
Metronidazole (p.o. or i.v.) ^e	347 (77.1)	23 (71.9)
Vancomycin	45 (10.0)	3 (9.4)
Metronidazole and vancomycin	36 (8.0)	3 (9.4)
None	19 (4.2)	3 (9.4)
CDI outcomes		
All-cause 30-day mortality	61/445 (13.7)	3/32 (9.4)
CDI-associated 30-day mortality	31/441 (7.0)	
cCDI ^f	51/442 (11.5)	2/32 (9.4)
Recurrence of CDI ^g	146/440 (33.2)	12/32 (37.5)

^aIQR, interquartile range.^bEach patient could have received more than one class of antimicrobials within 2 months of enrollment.^cTechLab *C. difficile* Tox A/B IITM kit was used in all Ontario centers. Samples from patients in Quebec centers were mostly tested by Biosite Diagnostics Triage Micro *C. difficile* panel (64%), Premier Toxins A&B Meridian Bioscience (22%), ImmunoCard Toxins A&B, Meridian Bioscience (12%), cytotoxin assay (10%), or a combination of tests (1%). EIA, enzyme immunoassay; GDH, glutamate dehydrogenase.^dHCFA, health care facility associated.^ep.o., per os; i.v., intravenous.^fcCDI defined as one or more of the following: admission to an intensive care unit for complications associated with CDI, colonic perforation, toxic megacolon, colectomy or hemicolectomy, or CDI was the cause or contributed to death within 30 days after enrollment.^gRecurrence was defined by the presence of diarrhea and *C. difficile* toxin or compatible endoscopy or prescription of an empirical CDI treatment at least 48 h after the completion of the last CDI treatment.

FIG 1 Minimum-spanning tree of MLVA data from 450 R027 *C. difficile* isolates. Clustering of MLVA profiles was performed using a Manhattan coefficient (MC), and each circle represents a unique MLVA complex. MLVA clusters were assigned if 2 neighboring types had an STRD of ≤ 2 and if at least 4 types fulfilled this criterion. The size of each circle indicates the number of isolates with this particular complex. Circles are color coded by location of the strain sampling (participating center) and labeled MC. The R027 reference strains CD196 and R20291 were included in the clustering (black circles). Bold solid lines, STRD = 1; solid lines, STRD = 2; dashed lines, STRD ≥ 3 to ≤ 10 ; light dashed lines, STRD > 10 .

health care facility associated [HCFA]) were mostly infected by strains that were part of the MC-1 and MC-10 clusters (87% and 90%, respectively).

Association with clinical outcomes. Overall, severe CDI at the time of enrollment was observed in 43% of patients ($n = 188$) and cCDI in 11.5% ($n = 51$) within 30 days of the admission (Table 1). The frequencies of severe cases were similar across all MLVA clusters (range, 39.6% to 44.9%). MC-1 was associated with a lower frequency of cCDI (4%) than cases infected with MC-3 or MC-10 (15% and 16%, respectively) or other clusters (12%). This trend, however, was not statistically significant in univariate logistic regression with a crude odds ratio (OR) for MC-1 versus other clusters of 0.34 (95% confidence interval [CI], 0.1 to 1.1; $P = 0.08$) (Table 3). The same trend with nonsignificant associations was also obtained between MLVA clusters and 30-day attributable mortality when considered alone as an outcome (3% in MC-1 versus 12% in both MC-3 and MC-10; $P = 0.13$).

The frequencies of recurrence were 42% in MC-1, 40% in MC-3, 49% in MC-10, and 32% in the other clusters. Neither overall nor pairwise comparisons of clusters were associated with recurrence in survival analyses (log rank, $P = 0.1$) (see Fig. S1).

Clinical characteristics of patients in each MC group were investigated (see Table S2). Patients infected with strains of MC-1 were younger than those infected with strains of MC-10 (median age 71 versus 83 years; $P = 0.013$), and they less frequently presented with signs of confusion (12% mild or severe confusion versus 43% in MC-10; $P = 0.006$). These patients were also less likely to have abnormal levels of WBC ($\geq 20 \times 10^9$

TABLE 2 Geographical and temporal distribution of major MLVA groups

Province or category	No. (%) for group:				
	MC-1	MC-3	MC-10	Other	Total
Total	69 (15.3)	53 (11.8)	50 (11.1)	278 (61.8)	450
Quebec	69 (100)	53 (100)		90 (32.4)	212 (47.1)
Sherbrooke ^a		21 (39.6)		10 (3.6)	31 (7.0)
Montreal ^b	69 (100)	32 (60.4)		80 (28.8)	181 (40.2)
Year of enrollment					
2005	2 (2.9)	18 (34)		7 (2.5)	27 (6.0)
2006	40 (58.0)	24 (45.3)		33 (11.9)	97 (21.6)
2007	13 (18.8)	10 (18.9)		31 (11.2)	54 (12.0)
2008	14 (20.3)	1 (1.9)		19 (6.8)	34 (7.6)
Ontario			50 (100)	188 (67.6)	238 (52.9)
Toronto Central ^c			32 (64.0)	113 (40.6)	145 (32.2)
Toronto North-East ^d			18 (36.0)	75 (27.0)	93 (20.7)
Year of enrollment					
2005				3 (1.1)	3 (0.7)
2006			1 (2.0)	16 (5.8)	17 (3.8)
2007			25 (50.0)	80 (28.8)	105 (23.3)
2008			24 (48.0)	89 (32.0)	113 (25.1)
Origin of CDI					
Hospital onset-HCFA	60 (87.0)	36 (67.9)	45 (90.0)	234 (84.2)	375 (83.3)
Community onset-HCFA	2 (2.9)	11 (20.8)	3 (6.0)	21 (7.6)	37 (8.2)
Community acquired	7 (10.1)	6 (11.3)	2 (4.0)	23 (8.3)	38 (8.4)
Patient location at the time of CDI					
symptoms onset					
Medical ward	45 (65.2)	38 (71.7)	41 (82.0)	159 (57.2)	283 (62.9)
Surgical ward	10 (14.5)	4 (7.5)	4 (8.0)	35 (12.6)	53 (11.8)
Emergency room	10 (14.5)	5 (9.4)	1 (2.0)	13 (4.7)	29 (6.4)
Intensive care	3 (4.3)	5 (9.4)	1 (2.0)	37 (13.3)	46 (10.2)
Community	1 (1.4)	1 (1.9)	3 (6.0)	34 (12.2)	39 (8.7)

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cells/liter, 4% versus 15% in MC-3 and 12% in MC-10; $P = 0.003$) and of serum albumin (<25 g/liter, 6% versus 21% and 20%, respectively; $P < 0.001$). However, although the overall difference was not significant, the proportion of immunosuppressed individuals was higher in this group than in MC-10 (36% versus 18%). Patients infected with MC-10 were less likely to have C-reactive protein (CRP) levels ≥ 150 mg/liter (6% versus 12% in MC-1 and 19% in MC-3; $P < 0.001$) and increased serum creatinine (18% versus 27% in MC-1 and 32% in MC-3; $P = 0.03$). No major differences were observed in comorbidities, previous exposure to antimicrobials, surgical procedures, other symptoms, or blood tests.

In a univariate logistic regression, cCDI was mainly associated with tachycardia, a respiratory rate of ≥ 20 breaths/min, and several abnormal laboratory results (Table 3; Table S3). Only four factors remained significantly associated with cCDI in a multivariate model: increased heart and respiratory rates, abnormal WBC ($<4 \times 10^9$ /liter and $\geq 20 \times 10^9$ /liter), and increased blood urea nitrogen (BUN; >11 mmol/liter). Even if there was a trend toward a protective effect of MC-1, none of MLVA clusters was associated with cCDI when adjusted for other patients' characteristics (Table 3).

DISCUSSION

This study aimed to better understand the association between *C. difficile* strains and unfavorable CDI outcomes. Among strains identified as R027 from a large multicenter cohort, we identified three major MLVA clusters. These clusters showed

TABLE 3 Factors associated with cCDI in univariate and multivariate logistic regression

Factor	No. cCDI/total no. (%)	Crude OR ^a (95% CI)	P value	AOR (95% CI) ^b
MLVA clusters ^c				
MC-1	3/69 (4.4)	0.34 (0.10–1.14)	0.081	0.40 (0.11–1.49)
MC-3	8/53 (15.4)	1.36 (0.59–3.14)	0.475	1.22 (0.43–3.48)
MC-10	8/50 (16.0)	1.42 (0.61–3.30)	0.412	1.30 (0.47–3.62)
MC-other	32/276 (11.8)	Reference		Reference
Comorbidity with chronic heart disease				
No	21/239 (8.8)	Reference		
Yes	30/203 (14.8)	1.80 (0.99–3.26)	0.052	
Charlson comorbidity index				
0–3	22/242 (9.1)	Reference		
4–6	22/138 (15.9)	1.90 (1.01–3.57)	0.047	
≥7	7/62 (11.3)	1.27 (0.52–3.13)	0.600	
Previous exposure to fluoroquinolones				
No	16/189 (8.5)	Reference		
Yes	35/252 (13.9)	1.74 (0.93–3.26)	0.081	
Heart rate (beats/min)				
≤90	20/292 (6.9)	Reference		Reference
>90	31/146 (21.2)	3.67 (2.01–6.70)	<0.001	2.80 (1.39–5.62)
Respiratory rate (breaths/min)				
≤20	31/365 (8.5)	Reference		Reference
>20	19/72 (26.4)	3.86 (2.04–7.33)	<0.0001	2.25 (1.03–4.90)
WBC ^d (10 ⁹ /liter)				
<4	5/24 (20.8)	5.07 (1.60–16.12)	0.006	5.77 (1.56–21.31)
4–11.9	11/223 (4.9)	Reference		Reference
12–19.9	15/117 (12.8)	2.83 (1.26–6.39)	0.012	1.88 (0.74–4.79)
≥20	20/66 (30.3)	8.38 (3.76–18.68)	<0.001	4.05 (1.67–9.82)
Serum albumin (g/liter)				
≤25	22/110 (20.0)	7.75 (1.76–34.17)	0.007	
26–35	20/198 (10.1)	3.48 (0.79–15.33)	0.099	
>35	2/64 (3.1)	Reference		
Missing	7/70 (10.0)	3.44 (0.69–17.23)	0.132	
BUN ^e (mmol/liter)				
<7	9/214 (4.2)	Reference		Reference
7–10.9	6/71 (8.5)	2.10 (0.72–6.13)	0.173	1.86 (0.56–6.14)
≥11	31/109 (28.4)	9.05 (4.12–19.88)	<0.001	6.64 (2.79–15.78)
Dialysis	0/12			
Missing	5/36 (13.9)	3.67 (1.16–11.68)	0.027	3.03 (0.80–11.38)
CRP ^f (mg/liter)				
<50	10/141 (7.1)	Reference		
50–149.9	13/149 (8.7)	1.25 (0.53–2.96)	0.608	
≥150	16/44 (36.4)	7.49 (3.08–18.22)	<0.001	
Missing	12/108 (11.1)	1.64 (0.68–3.95)	0.272	
Procalcitonin (ng/ml)				
<0.5	15/245 (6.1)	Reference		
≥0.5	24/89 (27.0)	5.66 (2.81–11.42)	<0.001	
Missing	12/108 (11.1)	1.92 (0.87–4.25)	0.109	
Severe CDI at enrollment				
No	13/247 (5.3)	Reference		
Yes	38/183 (20.7)	4.72 (2.43–9.15)	<0.001	

^aOR, odds ratio.^bAOR, adjusted odds ratio; CI, confidence interval.^cMC, Manhattan coefficient.^dWBC, white blood cell count.^eBUN, blood urea nitrogen.^fCRP, C-reactive protein.

an interesting time-space distribution. Some clusters were exclusive to a center or a province (MC-1 in Montreal, Quebec, and MC-10 in Ontario centers). CDI cases within cluster MC-1 were enrolled between 2006 and 2008, cases within MC-3 were between 2005 and 2007, and strains within MC-10 cluster were between 2007 and 2008.

The strong geographical clustering of strains is an interesting aspect of the MST built using the Manhattan coefficient. Every Manhattan cluster except for one (MC-13) was able to perfectly distinguish strains sampled in each province (14 strains from Ontario). The Manhattan approach was also able, to a certain extent, to cluster strains according to the health care center from which they were sampled. These observations suggest that MLVA typing is able to discriminate different genetic profiles among R027 strains, previously considered highly homogenous or virtually identical. The existence of R027 genetic variants has also been suspected in the past using pulsed-field gel electrophoresis (PFGE) typing (5, 27). The capacity of MLVA to differentiate genetic clusters inside a unique ribotype might reinforce our understanding of dissemination routes and the identification of initial reservoirs.

Eyre et al. have suggested that MLVA and whole-genome sequencing (WGS) have a good level of concordance and were both useful to investigate transmission and genetic relatedness of *C. difficile* strains in hospital settings (22). Constant progress in sequencing technologies could soon allow for whole-genome comparisons and/or *in silico* determination of MLVA profiles in a cost-efficient manner. In a large retrospective study using WGS, it was shown that sources of infections are much more diverse than previously thought (36). In contrast, our analysis identified large clusters of indistinguishable strains ($\text{STRD} \leq 2$), suggesting a greater extent of dissemination after the introduction of strains in health care centers or possibly a common exterior reservoir for some specific strains. On the other hand, the detection of different MLVA clusters within the same hospital over a short time frame could also be explained by the occurrence of a significant genetic change (acquisition or loss of many tandem repeats at once) followed by rapid dissemination of the new profile in the hospital. A smaller study in France also showed a spatiotemporal distribution of MLVA subtypes from R027 strains collected between 2005 and 2014 (37). However, this study did not aim to associate strains with clinical outcomes. It is also noteworthy to mention that a distinction between health care centers was not possible with strains from Ontario. A possible explanation for this is that many of the health care centers from this region are physically interconnected and characterized by an important flow of patients and medical staff, possibly facilitating the transmission of nearly clonal strains. Clustering of the strains according to the time of inclusion was not perfect, although some interesting observations could be made. For instance, Manhattan clusters appeared to be somewhat discriminatory, since they clustered strains sampled less than 2 years apart. However, the relationship between MLVA type and time of sampling is conceivably obscured by the fact that *C. difficile* sporulates and that loci targeted by MLVA (as the rest of the genome) can remain unchanged for a prolonged period while in the sporulated form.

Identified MLVA clusters were not associated with CDI severity at enrollment or with the occurrence of cCDI. A trend for more frequent cCDI cases in MC-3 and MC-10 than in MC-1 was observed in our study without reaching statistical significance. Also, in these groups, some differences were found in certain variables suggesting a stronger inflammatory response (WBC, serum albumin levels, and heart and respiratory rates). However, these differences were not consistent across other inflammation markers measured (CRP and procalcitonin). MC-1 cluster seemed to be associated with a lower risk of cCDI than other clusters, and patients in this group had fewer biological abnormalities. However, higher proportions of these patients were younger and immunosuppressed, which could explain the differences in clinical characteristics and outcomes. Our analyses were hampered by the small sample size included in each major cluster despite the large sample of R027 isolates ($n = 450$), a consequence of the high discriminatory potential of MLVA

(several small clusters of <3 strains). Among the cases for which MLVA typing could not be performed, the main patients' characteristics were similar, and only 2 cases met the cCDI definition. Cultivation bias likely had very little effect on the study findings. As in our previous report, which includes the whole cohort, cCDI was associated with increased WBC and BUN as well as elevated heart and respiratory rates (16).

Common typing methods currently used to characterize *C. difficile* isolates, such as PCR ribotyping, PFGE, multilocus sequence typing (MLST), or MLVA, are useful for epidemiological studies and outbreak monitoring (38). However, these methods do not provide direct information about virulence genes or virulence-associated characteristics, and this might explain why there is so much controversy regarding the link between strain types and CDI severity or clinical outcomes. More powerful approaches, such as WGS, could, on the other hand, provide detailed information about genetic variations between strains that could eventually be linked with clinical outcomes or severity (4, 39). However, several of the recent WGS projects applied to *C. difficile* focused on the core genome, on single nucleotide polymorphism, and relied on referenced-based sequence assembly (22, 40, 41). Hence, mobile genetic elements such as plasmids, transposons, and prophages, which account for much of the genetic variations between strains (42), were overlooked.

In our study, several subtypes were identified within R027, as demonstrated by the identification of multiple MLVA clusters. WGS performed on these R027 variants and detailed sequence analysis using *de novo* assembly could, therefore, allow for direct identification of genetic characteristics that could discriminate strain subtypes and that could be associated with clinical outcomes (42, 43). It is also important to keep in mind that in addition to the virulence of the strain *per se*, multiple factors can influence the evolution of the clinical issue following CDI. The immunity and inflammatory response of each infected individual, as well as their age and other comorbidities, can have a substantial impact on the presentations or outcomes of the infection (16, 44, 45). However, ascertaining the relative contributions of immunity and the immune response in clinical outcomes following CDI is still a truly challenging task, since no standardized methods have been developed and applied to measure related biomarkers. Hence, we reiterate that it is of the utmost importance to measure as many patient and strain characteristics as possible in order to maximize our understanding of the different variables possibly impacting the outcome of CDI.

Conclusions. Despite the lack of statistical association with clinical outcomes, MLVA typing performed in this study allowed the discrimination of 3 major genetic clusters within R027 isolates that exhibited a strong association with the geographical location of sampling. The MC-1 group was associated with a trend toward a lower level of cCDI. These findings might lead to further research to elucidate the association between R027 and severe and complicated CDI by supporting the hypothesis that not all R027 isolates are equally virulent.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.01724-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We have no competing interest to declare.

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