**Clostridium difficile** Ribotype Does Not Predict Severe Infection

Seth T. Walk,¹ ² Dejan Micic,³ Ruchika Jain,¹ ² Eugene S. Lo,¹ Itishree Trivedi,¹ Eugene W. Liu,⁴ Luay M. Almassalha,¹ Sarah A. Ewing,¹ Cathrin Ring,¹ ² Andrzej T. Galecki,¹ ³ ⁴ Mary A. M. Rogers,¹ Laraine Washer,¹ ⁵ Duane W. Newton,⁶ ⁷ Preeti N. Malani,¹ ² ⁹ Vincent B. Young,¹ ² ⁸ and David M. Aronoff¹ ² ⁸

¹Department of Internal Medicine, ²Division of Infectious Diseases, ³Division of Geriatric and Palliative Medicine, ⁴Department of Biostatistics, ⁵Department of Infection Control and Epidemiology, ⁶Department of Pathology, and ⁷Department of Microbiology, University of Michigan Health System, and ⁹Veteran’s Affairs Ann Arbor Healthcare System, Geriatric Research Education and Clinical Center (GRECC), Ann Arbor, Michigan

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**Background.** Studies of *Clostridium difficile* outbreaks suggested that certain ribotypes (eg, 027 and 078) cause more severe disease than other ribotypes. A growing number of studies challenge the validity of this hypothesis.

**Methods.** We conducted a cross-sectional study of *C. difficile* infection (CDI) to test whether ribotype predicted clinical severity when adjusted for the influence of other predictors. Toxigenic *C. difficile* isolates were cultured from stool samples, screened for genes encoding virulence factors by polymerase chain reaction (PCR) and ribotyped using high-throughput, fluorescent PCR ribotyping. We collected data for 15 covariates (microbiologic, epidemiologic, and laboratory variables) and determined their individual and cumulative influence on the association between *C. difficile* ribotype and severe disease. We then validated this influence using an independent data set.

**Results.** A total of 34 severe CDI cases were identified among 310 independent cases of disease (11.0%). Eleven covariates, including *C. difficile* ribotype, were significant predictors of severe CDI in unadjusted analysis. However, the association between ribotypes 027 and 078 and severe CDI was not significant after adjustment for any of the other covariates. After full adjustment, severe cases were significantly predicted only by patients’ white blood cell count and albumin level. This result was supported by analysis of a validation data set containing 433 independent CDI cases (45 severe cases; 10.4%).

**Conclusions.** Ribotype is not a significant predictor of severe CDI when adjusted for the influence of any other variables separately or in combination. White blood cell count and albumin level are the most clinically relevant predictors of severe CDI cases.

An increase in the incidence and severity of *Clostridium difficile* infection (CDI) throughout the United States, Canada, and Europe coincided with the emergence of a previously rare genotype [1]. This genotype, known as polymerase chain reaction (PCR) ribotype 027, North American Pulsed-field type 1 (NAP1), or restriction endonuclease analysis (REA) type BI, was reported to harbor an intrinsic ability to cause more severe disease compared to other pathogenic isolates [1, 2]. At least one other *C. difficile* lineage, ribotype 078, has been referred to as “hypervirulent” [3]. Laboratory studies identified numerous microbiologic properties to explain the increased virulence of 027 and/or 078 isolates, including antibiotic resistance [4], increased toxin production [5], enhanced ability for toxin B isoforms to bind target cells [6], and increased sporulation ability [7].

More recent data do not support the hypervirulent hypothesis [8–12], although the clinical definition of severe CDI or the methods used for data analysis are not consistent across all studies. Because institutions are often limited to retrospective review of patient...
records, it is important to define severe CDI using commonly recorded information. Such a definition has been recommend-

We sought to quantify the prevalence of *C. difficile* ribotypes at a single institution and to determine whether specific ribotypes were associated with severe disease. In particular, we tested the hypothesis that *C. difficile* ribotype predicts severe CDI cases even after adjustment for other clinical and laboratory variables. To do so, we developed models using an initial (derivation) data set and then validated our results with the same model fitted to a validation data set.

**MATERIALS AND METHOD**

**Setting**
The University of Michigan Health System (UMHS) includes a 930-bed, tertiary care inpatient facility and 5 off-site ambulatory care facilities. This study was approved by the University of Michigan Institutional Review Board.

**Clinical Epidemiology**
Severe CDI was defined as recommended by McDonald et al (intensive care unit admission, interventional surgery, or death within 30 days of diagnosis) [13]. We tested whether microbiologic, epidemiologic, and laboratory factors were predictive of severe CDI. Microbiologic factors included CDI cases caused by isolates of ribotypes 027 and 078–126 (hereafter referred to as 078) and/or those carrying previously recognized virulence factors (binary toxin and insertions/deletions, premature stop codons, or deletions of the tcdC gene). Epidemiologic factors included patient age, sex, and the setting of CDI onset or CDI surveillance definition [13] (healthcare facility-onset, healthcare facility-associated [HO-HCFA]; community-onset, healthcare facility associated [CO-HCFA]; community-associated [CA]; or indeterminate [IND]). Other epidemiologic factors included comorbid conditions as defined by the Charlson comorbidity index (CCI) [14]. Renal disease and a history of cancer were defined using the CCI and combined with age to create a modified age, renal function, and history of cancer (ARC) comorbidity score [15]. Laboratory values were recorded for white blood cell count, albumin level, creatinine, hematocrit, platelet count, total bilirubin, and blood urea nitrogen. Leukocytosis and leukopenia were defined as >12,000 and <4000 cells/mL, respectively. All laboratory values were collected within 72 hours of CDI diagnosis.

Two independent data sets were generated for this study. An initial data set of 310 cases of CDI (derivation set) was used for preliminary unadjusted and adjusted analyses and to derive a final model (see data analysis section below). A second data set (validation set) of 433 cases of CDI was used to validate the model fitted to the derivation data set. Data for each set were collected in the same manner.

**Stool Samples and *C. difficile* Isolates**
Suspected CDI-positive stool samples, defined as clinician-ordered specimens that were submitted for testing for the presence of toxigenic *C. difficile*, from hospitalized inpatients and ambulatory outpatients presenting to the main hospital or UMHS off-site facilities, were obtained from the Clinical Microbiology Laboratory between 14 January 2010 and 2 March 2011 (derivation set) and between 2 March 2011 and 5 March 2012 (validation set). No asymptomatic colonization cases were included.

Samples were cultured anaerobically on taurocholate-cycloserine-cefoxitin-fructose agar at 37°C [16]. A single colony was then subcultured in brain-heart infusion broth [16]. Aliquots were diluted in sterile water (UltraPure Distilled Water, Invitrogen) and used for PCR and ribotyping. The presence of other microbial causes of colitis or gastroenteritis was not evaluated.

**Taxonomic and Toxigenic Verification**
*C. difficile*-specific 16S ribosomal RNA (rRNA)-encoding gene PCR was used to verify isolate taxonomy [17]. A 5-plex PCR assay was also used to screen for the presence of an independent *C. difficile*-specific 16S locus along with *C. difficile* toxin (tcdA and tcdB) and binary toxin (cdtA and cdtB) genes [18]. Only tcdA- and/or tcdB-positive *C. difficile* isolates were analyzed. Toxin gene negative isolates were confirmed to be nontoxigenic using a Vero-cell cytotoxicity assay [19]. Published primers were used to amplify and sequence the tcdC gene [20]. Sequences were obtained in both directions and aligned/edited using SeqMan Pro (DNASTAR Lasergene 8.1.5, DNASTAR, Inc, Madison, Wisconsin). The MEGA5 program [21] was then used to identify tcdC mutations according to published alleles [22].

**Fluorescent PCR Ribotyping**
PCR ribotyping primers [23] were synthesized with a fluorescent label (Integrated DNA Technologies, Inc) and adjusted to 10 pmol/µL. A 25 µL PCR was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems) and the following conditions: 95°C (10 minutes); 35 cycles of 95°C (30 seconds), 55°C (30 seconds), and 72°C (1 minute 30 seconds); final extension of 72°C (10 minutes). Amplicons were analyzed using an ABI3730xl DNA Analyzer and MapMaker 1000 ROX DNA sizing standard (BioVentures, Inc).

Reference strains included REA types BI-1, J-4, K-19, G-6, Y-6, CF-3, and BK-2 (PCR ribotypes 027, 001,
Isolates of hyphenated ribotypes cluster into the same REA groups [24], so it is unclear whether they represent distinct genotypes. A similar ribotyping procedure was developed elsewhere [25]. Important differences are highlighted as Supplemental Data.

**Data Analysis**

Unconditional logistic regression was used to test our main hypothesis and to assess the predictive strength of factors discussed in the Clinical Epidemiology section above on severe CDI. Unadjusted and adjusted analyses were conducted with the glm() function using R software [26]. To generate models, only significant covariates on unadjusted analysis were considered. In addition, we performed likelihood ratio tests to evaluate significance for the effects of covariates and to develop a final model. Two-tailed level of significance was set at .05. Per our a priori hypothesis, ribotype was included in all models, and odds ratios were calculated for both the derivation and validation data sets. The rationale for deriving and validating a model was to assess the reproducibility of the results. In secondary analyses, the data were combined to minimize type 2 error, and a final model was fitted in the adjusted analysis. All covariates, regardless of their significance in the unadjusted analysis, were included in the model of the combined data set.

**RESULTS**

**Inclusion of C. difficile Isolates and CDI Cases**

We identified 331 C. difficile isolates obtained from symptomatic patients during the initial study period. Nontoxigenic isolates were excluded (n = 12 [4%]) along with samples representing repeat testing of the same patient (n = 9 [3%]). In total, 310 isolates from 310 different patients were considered for the derivation set. For the validation set we identified 460 isolates of hyphenated ribotypes cluster into the same REA groups [24], so it is unclear whether they represent distinct genotypes. A similar ribotyping procedure was developed elsewhere [25]. Important differences are highlighted as Supplemental Data.
additional *C. difficile* isolates in the second study period, of which 27 were excluded because they were nontoxigenic (n = 12 [3%]) or represented repeat sampling of the same patient (n = 15 [3%]). In total, 433 *C. difficile* isolates from 433 different patients were considered in the validation set. Patient demographics were similar in each data set (Table 1).

**Ribotype Abundance**

The 310 *C. difficile* isolates of the derivation set belonged to 75 distinct ribotypes (Figure 1A). The most common ribotypes observed were 014–020 (17%) and 027 (14%). Other ribotypes accounted for <20 cases (7%) each. Similarly, 91 distinct ribotypes were observed in the validation set (Figure 1B) and 014–020 and 027 isolates were again the most common (34%). These results illustrate that the *C. difficile* population at our institution was relatively stable over the sampling period, diverse, and not dominated by a single ribotype.

**Predictors of Severe CDI**

Unadjusted logistic regression implied that 2 microbiologic, 3 epidemiologic, and 6 laboratory covariates were significant predictors of severe CDI (Table 2). We then adjusted for covariates separately to determine their influence on the effect of *C. difficile* ribotype (Table 3). Each of the models resulted in a decreased adjusted odds ratio for *C. difficile* ribotype and a nonsignificant association with severe CDI.

In the fully adjusted analysis (Table 4), we adhered to the following strategy. First, we developed a “full” model consisting of the 11 significant covariates in unadjusted analysis. Because of missing data, primarily for laboratory tests, this model was fit to complete data for 148 cases (48%) in the derivation data set. Covariates were evaluated for their significance relative to a model where they were excluded in step-wise fashion (see Methods). *C. difficile* ribotype was included as a covariate, regardless of significance, to assess whether its effect was influenced by adjusting for other covariates. This analysis implied that the effect of *C. difficile* ribotype is not significant after adjusting for the effects of white blood cell count (leukocytosis/leukopenia) and albumin level. Results for this reduced model are shown in Table 4. In a second step of validation, we fit the reduced model to complete data for 244 cases (56%) in the validation data set (Table 4). This analysis again supported the hypothesis that adjustment for other factors attenuates the association between hypervirulent ribotypes and severe CDI and again implied that white blood cell count (leukocytosis/leukopenia) and albumin level are significant predictors of a severe clinical outcome.

Complete information was available regarding age, sex, and CDI surveillance definition (HO-HCFA, etc) for all but one patient in the derivation data set and all patients in the validation data set. We were unable to obtain complete laboratory data on all patients because the decision of which tests to order was made during the course of routine clinical care by the clinician at the time of diagnosis. To assess the association between hypervirulent ribotype and severe CDI with the most complete data (n = 309 and 433), we adjusted for only age, sex, and CDI surveillance definition in a secondary analysis. This yielded nonsignificant adjusted odds ratios for hypervirulent ribotype and severe CDI of 1.92 (95% confidence interval [CI]: .82–4.32) in the derivation set and 1.73 (95% CI: .83–3.48) in the validation set. This analysis also yielded a
Table 2. Odds Ratios for Predictors of Severe *Clostridium difficile* Infection Based on Unadjusted Analysis of the Derivation Data Set (n = 310)

<table>
<thead>
<tr>
<th>Predictorsa</th>
<th>OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiologic</td>
<td></td>
<td></td>
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<tr>
<td>Hypervirulent ribotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>027/078 vs other (reference)</td>
<td>2.33 (1.03–5.02)</td>
<td>.035</td>
</tr>
<tr>
<td>non-027/078 (reference)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdtAB (binary toxin) vs</td>
<td>1.66 (.75–3.53)</td>
<td>.195</td>
</tr>
<tr>
<td>tcdC allele with any deletion vs full length (reference)</td>
<td>2.64 (1.24–5.60)</td>
<td>.011</td>
</tr>
<tr>
<td>Epidemiologic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.02 (1.00–1.04)</td>
<td>.047</td>
</tr>
<tr>
<td>Sex</td>
<td>Male vs female (reference)</td>
<td>0.90 (.44–1.84)</td>
</tr>
<tr>
<td>CDI surveillance definition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO-HCFA</td>
<td>23.16 (4.75–417.99)</td>
<td>.003</td>
</tr>
<tr>
<td>CO-HCFA</td>
<td>8.30 (1.29–161.36)</td>
<td>.056</td>
</tr>
<tr>
<td>IND</td>
<td>6.77 (.84–139.21)</td>
<td>.102</td>
</tr>
<tr>
<td>CA (reference)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charleson comorbidity index</td>
<td>1.19 (1.03–1.38)</td>
<td>.022</td>
</tr>
<tr>
<td>ARC score</td>
<td>1.19 (.99–1.42)</td>
<td>.060</td>
</tr>
<tr>
<td>Laboratory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cell count</td>
<td>Abnormal (&gt;12 000 or &lt;4000) vs normal (reference)</td>
<td>2.89 (1.36–6.46)</td>
</tr>
<tr>
<td>Albumin levels (g/dL)</td>
<td>0.22 (.10–0.45)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Creatinine levels (mg/dL)</td>
<td>1.22 (.97–1.52)</td>
<td>.072</td>
</tr>
<tr>
<td>Hematocrit levels (%)</td>
<td>0.88 (.81–94)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Platelet count (×1000/µL)</td>
<td>0.995 (.992–999)</td>
<td>.014</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>1.16 (1.06–1.33)</td>
<td>.005</td>
</tr>
<tr>
<td>Urea nitrogen levels (mmol/L)</td>
<td>1.018 (1.005–1.032)</td>
<td>.007</td>
</tr>
</tbody>
</table>

Abbreviations: ARC, age, renal function, and history of cancer comorbidity score; CA, community acquired; CDI, *Clostridium difficile* infection; CI, confidence interval; CO-HCFA, community-onset, healthcare facility associated; HO-HCFA, healthcare facility-onset, healthcare facility-associated; IND, indeterminate; OR, odds ratio.

* Significant (α < 0.05) predictors are indicated with bold values.

The global incidence of CDI has increased markedly [27, 28] and coincided with the emergence of a previously less common and sporadically encountered *C. difficile* genotype, known as ribotype 027 [29]. An increase in the severity of CDI cases was reported in Quebec, where the 30-day CDI-associated mortality rose from 4.7% in 1991–1992 to 13.8% in 2003 [1]. When patients were examined in 2004, 12 of 13 cases (92%) were found to be caused by 027 isolates [1]. Similarly, a CDI outbreak investigation of US healthcare facilities between 2001 and 2003 found ≥50% of isolates at 5 of 8 facilities belonged to ribotype 027 [2]. However, it was unclear from the clinical data whether these isolates necessarily caused more severe disease. For example, results from 2 facilities suggested that 027 isolates were hypervirulent, but results from 2 other facilities did not [2]. Evidence that ribotype 078 isolates are hypervirulent [3] is similarly inconclusive. For example, cases of severe diarrhea caused by 078 isolates were more common than cases caused by non-078 isolates [3]. However, this observation was marginally significant at an α level of 0.10.

A number of hypotheses have been proposed to explain the apparent increase in virulence of 027 and 078 isolates. It was suggested that elevated production of *C. difficile* toxins (TcdA and TcdB) due to a dysfunctional negative regulatory protein (TcdC) was important [2]. However, recent evidence suggests that 027 isolates, at least, do not necessarily produce more toxins in vitro than isolates of other lineages [7]. Similarly, the

significant adjusted odds ratio for HO-HCFA (vs CA; CDI surveillance definition) and severe CDI of 19.96 (4.03–361.83) in the derivation data set, although it did not reach significance in the validation data set.

Unadjusted and adjusted analyses of a combined data set (derivation + validation) are shown in supplemental Table 1. These analyses yielded a nonsignificant odds ratio (OR, 1.06; 95% CI: .49–2.20) for *C. difficile* ribotype upon adjustment for other covariates. White blood cell count and albumin were significant predictors after adjustment, as was a marginal increase in blood urea nitrogen level (OR, 1.02; 95% CI: 1.01–1.04).
functional status of TcdC based on nucleotide sequencing does not necessarily correlate with disease severity [9, 12]. Our results offer additional support to these studies, as we found no association between mutations in tcdC and severe disease after adjusting for other covariates.

A 2-component binary toxin (CdtA/CdtB) has also been implicated in CDI severity [2, 30], although its role in disease pathogenesis is uncertain and binary toxin-carrying isolates that do not express TcdA/TcdB have been isolated from asymptomatic patients [31]. A recent study failed to identify an association between binary toxin and case severity [9]. Our results similarly suggest that binary toxin is not involved in the development of a severe clinical outcome within 30 days of diagnosis.

In general, our results support the findings of at least 5 other studies that failed to detect a ribotype association with CDI case severity [8, 10, 11, 32, 33]. Notably, all of these studies used different criteria to define cases of severe CDI; they considered the 027 ribotype only; and only 2 of them used a similar logistic regression approach where adjustments were made for the influence of other covariates [8, 10]. The most similar severity definition to ours [8] considered the same clinical measures (intensive care unit admission, interventional surgery, or death) but was based on 60-day post-diagnosis and not 30 days as recommended by McDonald et al [13]. We recognize that the McDonald et al definition is not without faults. For example, we only considered outcomes where C. difficile was listed in patients’ charts as a contributing factor, and it is possible that CDI was not the attributable cause of all of these outcomes. Similarly, we cannot rule out the possibility that severe cases were missed due to omission of contributing factors in patient charts. In light of these limitations, it remains possible that a link between ribotype and severity will be found if alternative definitions are used.

Our results illustrate the importance of adjustment for the effects of covariates when considering the clinical importance of pathogenic C. difficile ribotypes. Isolate characterization at the ribotype level is time consuming and costly compared to evaluating laboratory values that are often routinely collected on patients at risk of severe CDI. In addition, other adjusted analyses of CDI cases support our hypothesis that an abnormal white blood cell count [8, 10] and hypoalbuminemia [34] are significant predictors of a poor clinical outcome even after adjustment for other covariates.

An advantage of our study design is a validated model, which suggested reliability of the results. Moreover, when the data were combined to minimize type 2 error, the main finding of no significance remained for hypervirulent ribotype. A limitation of the models presented here is that they were generated from CDI cases at a single institution. Studies that incorporate broader geographic, temporal, and socioeconomic heterogeneity are needed to assess the generalizability of the results.

The percentage of HO-HCFA cases in this study is somewhat lower than other reports. For example, the mean percentages of HO-HCFA and CA cases in a 5-institution study of 6906 cases were 65.5% and 7.6%, respectively [35]. The percentages observed in our study were quite different (HO-HCFA = 38.7% in the derivation set and 42.3% in the validation set; CA = 28.7% in the derivation set and 29.6% in the validation set). A number of differences between our institution and the others may account for this discrepancy, including differences in clinical practice (eg, more patients treated in the outpatient setting), the number of outpatient clinics that are serviced by our diagnostic laboratory (eg, more outpatient clinics), regional differences in patient populations (eg, northern vs central US), and the influence of time when the studies took place (ie, 2000–2006 vs 2010–2012). However, a significant proportion of CDI cases occurred in the community in a recent US population-based cohort study [36]. It is tempting to speculate that HO-HCFA cases are decreasing while CA cases are increasing, although more data are needed to verify this trend.

It is interesting that patient CDI surveillance definition was not a significant predictor in the final model. This model was

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Derivation OR (95% CI)</th>
<th>P Value</th>
<th>Validation OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypervirulent ribotype:</td>
<td></td>
<td></td>
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<tr>
<td>027/078 vs non-027/078 (reference)</td>
<td>0.82 (.07–10.0)</td>
<td>.874</td>
<td>1.34 (.53–3.16)</td>
<td>.516</td>
</tr>
<tr>
<td>White blood cell count:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Leukocytosis (&gt;12 000 cells/mL) or leukopenia</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;4000 cells/mL) vs normal (reference)</td>
<td>4.27 (1.14–19.46)</td>
<td>.041</td>
<td>2.32 (1.07–5.18)</td>
<td>.035</td>
</tr>
<tr>
<td>Albumin level (g/dL)</td>
<td>0.25 (.07–.77)</td>
<td>.025</td>
<td>0.47 (.25–.87)</td>
<td>.018</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; OR, odds ratio.

* Significant values are shown in bold.
based on less than half of all cases (48%), so it is possible that these patients were not representative. Results from a secondary analysis of complete data were not consistent between data sets; therefore, no clear hypothesis postadjustment was supported for this covariate. These results suggest that similarly powered studies (n \sim 300) reporting marginal associations should be interpreted with caution. Because the model presented here was validated, we feel it would be useful for others to consider as they begin assessing the importance of clinical factors in the prediction of severe CDI cases at other institutions.

Although previous studies found that CCI and ARC score were predictive of severe CDI [15, 37, 38], neither factor was significant in the present study upon adjustment. Differences in definitions used for severity (eg, diffuse diarrhea, shock index, and 90-day mortality) may explain differences in study results. However, it seems worthwhile to identify new CDI-specific comorbidity criteria that better reflect CDI outcomes. Further assessment and refinement of surveillance groups (HO-HCFA, CO-HCFA, and IND) is required to determine whether they are predictive of severe disease.

Another limitation of this study was the consideration of a single C. difficile isolate per patient stool sample as it is possible that an individual patient is infected with multiple ribotypes concurrently. We recently addressed this limitation directly, and although the data are part of a forthcoming manuscript (unpublished), the co-occurrence of multiple toxigenic ribotypes appears to be no more than that of a previous report (approximately 13% of patients with >1 genotype) [39].

Our results add support to the hypothesis that 027/078 ribotypes are not more virulent than other C. difficile ribotypes. These data should temper enthusiasm for using ribotype, or the presence of binary toxin or tcdC mutations, to influence patient care. Gaps in our knowledge of the determinants of C. difficile pathogenesis limit our capacity to predict clinical behavior based solely on microbial characteristics. It is important to recognize, however, that there may be other attributes of the C. difficile genome that can significantly influence virulence. More discriminant characterizations are needed to address this hypothesis. Until these data become available, patient rather than pathogen characteristics should be used to guide treatment strategies.

**Supplementary Data**

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are notcopied. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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