Emergence of a Ribotype 244 Strain of
Clostridium difficile Associated With Severe
Disease and Related to the Epidemic Ribotype 027 Strain

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Background. We identified 12 patients with Clostridium difficile infection between July 2011 and March 2012 from whom an unusual C. difficile strain was isolated. This strain had a single-nucleotide deletion of the tcdC gene at position 117 and binary toxin genes, which are characteristic of the hypervirulent ribotype (RT) 027 strain.

Methods. A retrospective cohort study of 12 patients infected with C. difficile RT244 and 24 patients infected with non-RT244/non-RT027 strains matched for place of diagnosis and time of collection of specimen was performed. We performed whole-genome sequencing to understand the relationship of the RT244 strain to other C. difficile strains and further understand its virulence potential.

Results. Clostridium difficile RT244 was associated with more severe disease and a higher mortality rate. Phylogenomic analysis using core genome single-nucleotide polymorphisms showed that RT244 is in the same genetic clade (clade 2) as RT027 but is distinct from all RT027 strains. The pathogenicity locus of the RT244 strain encodes a variant toxin B, and this was confirmed by demonstration of Clostridium sordellii–like cytopathic effect on Vero cells. Toxin B production in culture supernatants was lower than that seen with a RT027 strain.

Conclusions. Our findings demonstrate the pathogenic potential of this RT244 C. difficile strain and emphasize the importance of ongoing surveillance for emergent strains.

Keywords. Clostridium difficile; virulence; toxin B; whole-genome sequencing.

Over the last decade, there has been a dramatic increase in the incidence and severity of Clostridium difficile infection (CDI) in North America, the United Kingdom, and Europe associated with the emergence of a fluoroquinolone-resistant C. difficile clone known as restriction endonuclease type BI/pulsed-field type NAP1, toxinotype III, or polymerase chain reaction (PCR) ribotype (RT) 027 C. difficile [1–4]. The RT027 strain harbors the toxin genes tcdA and tcdB (encoding the main C. difficile toxins, toxin A and toxin B, respectively), binary toxin genes, and an 18 base-pair (bp) deletion in the toxin regulatory gene tcdC, which also contains a deletion at position 117, resulting in an inactivating frame-shift mutation in the gene product TcdC.
TcdC is an anti-sigma factor that negatively regulates toxin A and B production, and the truncating mutation of TcdC present in RT027 strains has been shown to increase production of toxins A and B and to contribute to its virulence [6]. Other emergent strains of Clostridium difficile, such as ribotypes 078 [7], 017 [8], 014/020, 015, and 002 [9], have been identified in human disease, contributing to the increasing burden of disease but also changing the pattern of CDI. Clostridium difficile RT078, for example, appears to share the same genetic virulence characteristics as RT027 and causes severe disease at a similar rate, but has also been associated with community-acquired infection [7]. The description of RT027 and RT078 as hypervirulent has been called into question [10], although a recent analysis appeared to confirm the association of both ribotypes with more severe disease [11].

Phylogenomic studies using multilocus sequence typing and whole-genomic comparison of Clostridium difficile strains have identified 5 major clades, with RT027 isolates all within clade 2 [12, 13]. Human disease–causing strains are found within all clades, including highly divergent strains such as RT078, indicating that despite extensive genetic diversity there exists a large pool of potentially pathogenic Clostridium difficile strains containing virulence loci. Surveillance for changing strain epidemiology is limited by the fact that routine diagnostics are based on toxin detection, and isolates are not routinely cultured. Since 2009, our laboratory has used the Xpert Clostridium difficile PCR (Cepheid, Sunnyvale, California) as the confirmatory diagnostic test for CDI. This assay includes a detection probe for the tcdC117 deletion (tcdCA117) characteristic of ribotype 027 strains but which can also be found in other non-RT027 strains [6, 14]. Although there have been reports of local acquisition of Clostridium difficile RT027 [15], recent surveys indicate that RT027 is not currently endemic within Australia and that the PCR ribotypes 014, 002, and 020 predominate [16].

In 2011, we detected patients with a clinically severe CDI identified as presumptive RT027 by Xpert Clostridium difficile PCR. However, subsequent PCR ribotyping showed these strains to be RT244 and moxifloxacin susceptible on phenotypic antimicrobial resistance testing. We performed a matched retrospective cohort study to investigate the epidemiology, risk factors, and clinical outcomes of CDI caused by this newly recognized strain. Whole-genome sequencing was undertaken to determine its relatedness to a local Clostridium difficile RT027 isolate.

METHODS

Study Design and Participants
The study was a single-center, retrospective cohort study in subjects with CDI. Monash Health includes 5 major hospitals with >2100 beds, serviced by a single diagnostic laboratory that processes all fecal specimens. The study was approved by the Human Research Ethics Committee of Monash Health in Victoria, Australia (approval number 12024A). Participants were adults (aged >18 years) with laboratory-confirmed CDI between June 2011 and May 2012. An episode of CDI was defined as a clinical illness compatible with CDI and microbiological evidence of toxin-producing Clostridium difficile in stool without evidence of another cause of diarrhea or pseudomembranous colitis as diagnosed during endoscopy, after colectomy, or on autopsy [17, 18].

For every case patient with CDI caused by Clostridium difficile RT244, we selected 2 patients with positive Xpert Clostridium difficile PCR but tcdCA117 negative (and who therefore did not have Clostridium difficile RT244 or RT027), matched to the presenting hospital site and within 4 weeks of each case patient. A standardized data collection tool was used to collect demographic information from the medical record, disease severity, treatment course, length of hospital stay, and clinical outcome. Data were also collected on potential risk factors for developing CDI, including exposure to antibiotics, gastric acid suppressive agents, and chemotherapy. Comorbidity was established according to the International Classification of Diseases, Tenth Revision (ICD-10).

Each episode was classified as either healthcare facility–associated CDI, community-associated CDI, or community-onset, healthcare facility–associated CDI according to standard criteria [18]. Recurrence was defined as diarrhea within 8 weeks of resolution of initial episode of CDI and microbiological evidence of toxin-producing Clostridium difficile. The severity of each episode of CDI was assessed using 2 different criteria as proposed by the Infectious Diseases Society of America (IDSA)/Society for Healthcare Epidemiology of America (SHEA) [17, 18] and Zar et al [19]. Mortality was considered to be attributable to CDI when a patient died as a consequence of CDI as reported in the medical notes or on the death certificate. Response to treatment was defined as resolution of diarrhea and/or clinical signs during the course of therapy.

Statistical Analysis
All data were analyzed using SAS software, version 9.2 (SAS Institute, Cary, North Carolina). The risk factors for CDI were assessed using conditional logistic regression, taking into account the matched design. Exact logistic regression was used when some of the cells formed by the outcome and categorical predictor variables had no observations. Results from the logistic regression models were reported as odds ratios with 95% confidence intervals for each risk factor. Continuous variables were presented as mean ± standard deviation and categorical variables as proportions. Statistical significance was set at a 2-sided P value of .05.

Microbiological Diagnosis of CDI
During the period of the study, all unformed fecal specimens in patients aged >2 years, regardless of the period of
hospitalization, were screened for the presence of *C. difficile* by C.DIFF CHEK-60 (Techlab, Blacksburg, Virginia) enzyme immunoassay for the detection of glutamate dehydrogenase. Positive samples were then tested for the presence of toxigenic *C. difficile* by real-time multiplex Xpert *C. difficile* PCR (Cepheid). Samples positive for tcdCA117 were identified as presumptive RT027 and cultured on ChromID *C. difficile* agar (bioMérieux, Lyon, France) at 37°C for 24 hours [20]. Black colonies that were gram-positive bacilli were provisionally identified as *C. difficile* and confirmed by multiplex PCR detecting the tpi ( triose phosphate isomerase), tcdA, and tcdB genes [21], and moxifloxacin susceptibility was tested by Etest (bioMérieux).

**Molecular Biology and PCR Techniques**

Genomic DNA was isolated from *C. difficile* as outlined in O’Connor et al [22]. The genes for binary toxin cdtA and cdtB were detected by multiplex PCR, and a monoplex PCR was used to detect the tcdC gene [23], [24]. Nucleotide sequencing was carried out using a PRISM BigDye Terminator cycle sequencing kit measured on a 3730 Genetic Analyser (Applied Biosystems).

Toxinotyping classifies strains based on restriction fragment length polymorphisms in the toxin genes encoded in the Pathogenicity Locus (PaLoc) and was performed using the method of Rupnik et al [25]. PCR ribotyping of isolates was performed as previously described [26]. PCR ribotyping reaction products were concentrated using the Qiagen MinElute PCR Purification kit (Ambion, Austin, Texas) and run on the QIAxcel capillary electrophoresis platform (Ambion). Ribotypes were compared with a known reference library, containing prevalent PCR ribotypes currently circulating in Australia and a selection of binary toxin-positive strains using BioNumerics version 6.5 (Applied Maths, Saint-Martens-Latem, Belgium).

**Whole-Genome Sequencing and Assembly**

Ribotype 244 *C. difficile* strain DLL3110 was obtained from a patient of our hospital who died of severe CDI, and DLL3109 was a local RT027 strain. Isolates were sequenced on an Ion Torrent Personal Genome Machine (Life Technologies, Guilford, Connecticut) machine using 316 chips and 100 bp chemistry according to manufacturer protocols [27].

De novo assembly was performed using Newbler version 2.6 (Roche), and reads were aligned to reference genomes, and single-nucleotide polymorphisms (SNPs) were identified using SHRiMP version 2.2. and Nesoni, an in-house Python utility, against 9 existing publicly available *C. difficile* genomes: 630 (RT012), ATCC_43255 (RT087), B19 (RT001), CD196 (RT027), CIP_107932 ( Montreal RT027), R20291 (United Kingdom RT027), NAP07 (GenBank accession number ADVM00000000), NAP08 (GenBank accession number ADNX00000000), and QCD_23m63 (all RT078 human isolates). Genome annotation was performed with Prokka (Victorian Bioinformatics Consortium, Monash University, Australia), and further analysis of coding sequences was performed using Artemis version 14.0 (Sanger Institute, Oxford, United Kingdom). Phylogenetic relationships were inferred by neighbor joining and split decomposition analysis using SplitsTree version 4.10 (University of Tubingen, Germany), using a distance matrix based on pairwise SNP alignments among all isolates and reference genomes.

**Vero Cell Cytotoxicity Assays**

Toxin B from *C. difficile* culture supernatants was detected using a Vero cell cytotoxicity assay, as described previously [6]. Morphological changes of the Vero cells were observed and scored by microscopy after 24 hours. For quantification, serial doubling dilutions of culture supernatants were made, and the toxin titer is the reciprocal of the endpoint (no cytopathic effect) dilution.

**RESULTS**

From June 2011 to May 2012, screening for *C. difficile* was performed on 3626 stool specimens. The diagnosis of CDI was confirmed on 385 specimens, 16 (4.2%) of which also had tcdCA117 and binary toxin by Xpert *C. difficile* PCR. We successfully cultured *C. difficile* from 14 of these 16 stool specimens, and 12 (3.1% of all *C. difficile* positive stools) were identified as RT244. The timing of the identification of RT244 positive specimens is shown in Figure 2.

**Clinical Analysis**

**Demographics and Risk Factors**

There were no significant differences in age and the distribution of comorbidities between the 2 cohorts (Table 1). Most patients had documented antibiotic exposure (83.3% in both cohorts). Community-acquired CDI was common in both RT244 (33%) and non-RT244 (25%) patients.

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**Figure 1.** Polymerase chain reaction ribotyping pattern of strain DLL3110 (lane 1) and another ribotype 244 strain (lane 2) compared with the ribotype 027 type strain (lane 3).

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Disease Severity and Clinical Outcome

Patients infected with the C. difficile RT244 strain were significantly more likely to develop severe disease, renal impairment, and hypoaalbuminemia (Table 1). Using the IDSA/SHEA criteria, 58% of RT244 patients and 25% of non-RT244 patients met the definition of severe disease. Applying the Zar criteria, 75% of RT244 patients had severe disease, compared with 33.3% of non-RT244 patients (odds ratio [OR], 8.11 [95% confidence interval, 9.8–67.21]; P = .05). They were also more likely to die, with a 30-day mortality of 42%; 4 of the 5 deaths in the RT244 cohort were considered attributable to CDI. There were no deaths in the non-RT244 cohort. Using the exact logistic regression analysis, death was 13 times more likely in patients infected with the C. difficile RT244 isolate compared with non-RT244 (OR, 13.5; P = .008). Patients with RT244 CDI were more likely to receive vancomycin than metronidazole, in line with treatment recommendations favoring oral vancomycin for severe CDI [18]. Among the 8 patients with RT244 CDI who received vancomycin, 3 were initially started on metronidazole and then switched to vancomycin. Treating clinicians were informed of a possible hypervirulent strain based on the Xpert C. difficile result, which may have influenced the choice of treatment. Two cases required intensive care unit (ICU) admission. One of these cases required colectomy and later died. There were no ICU admissions or colectomies in the non-RT244 cohort.

Comparing in-hospital mortality of the RT244 to those of the total non-RT244 detected during the study period showed mortality rates of 25% (3/12) and 1.6% (6/373), respectively (P = .004).

Microbiological Analysis

No C. difficile RT244 isolates were resistant to moxifloxacin, and all were found to be toxigenotype IX although in silico inspection of the PaLoc sequence indicates the strain may be a closely related novel toxigenotype (data not shown). PCR sequencing of toxin loci of the RT244 isolates confirmed a deletion at base position 117 of the tcdC gene, resulting in a truncating frame-shift mutation but no other major deletions.
strains are ST1. DLL3110 matched to sequence type (ST) 41 [30]. All RT027 previously described clade structure of DLL3110 was separate from DLL3109, which forms a clonal RT027 strain. Phylogenomic analysis showed that strain and by DLL3109 was confirmed as producing toxin A, toxin B, and binary toxin CDT (binary toxin) (data not shown).

Whole-Genome Sequencing and Phylogenomic Comparison
We obtained in excess of 200 Mb of sequence from strains DLL3110 and DLL3109, with a mean read length of 110 bp, sufficient for 30–50 times coverage of the approximately 4.1-Mb genome. Ribotype 244 C. difficile strain DLL3110 was obtained from a patient who died of severe CDI, and DLL3109 was a local RT027 strain. Phylogenomic analysis showed that strain DLL3110 was separate from DLL3109, which forms a clonal complex with other sequenced RT027 isolates (Figure 3). The 244 ribotype was more closely related to the RT027 cluster than were the other strains included in the analysis. There were 10,803 SNPs between the RT027 cluster and DLL3110, which indicates substantial genetic difference. A recent study estimated that up to 3 SNPs might be expected for an isolate obtained from the same source over a 1-year period [28].

The SNP tree generated in this study was concordant with the previously described clade structure of C. difficile strains [12], and placed strain DLL3110 within clade 2 (Figure 3). Our whole-genome sequencing findings confirmed other previous whole-genome microarray hybridization studies that included a single RT244 strain (labeled BI-14 in [29]) and also placed it as an outlier within the RT027 clade 2 [29]. In silico analysis of the loci adk, atp, dxr, glyA, recA, sodA, and tpi of strain DLL3110 matched to sequence type (ST) 41 [30]. All RT027 strains are ST1.

No fluorquinolone resistance mutations were found in the DNA gyrase subunits encoding genes gyrA or gyrB of C. difficile DLL3110, in agreement with its moxifloxacin-susceptible phenotype [31].

Toxin Analysis
Production of toxin A, toxin B, and binary toxin by DLL3110 and by DLL3109 was confirmed by Western blotting (data not shown). Toxinotype IX strains have previously been characterized as producing toxin A, toxin B, and binary toxin CDT and causing a Clostridium sordellii–like cytopathic effect (CPE) observed on Vero cells [32], [33]. Vero cell cytotoxicity assays, which primarily measure toxin B activity, showed that supernatants from isolate DLL3110 produced clumping and cell rounding, consistent with C. sordellii–like CPE (Figure 4A). Toxin titers produced on Vero cells were not significantly different from the control 630 (Figure 4B). Toxins A and B are members of a family of large clostridial toxins including C. sordellii lethal toxin (TcsL) that cause cytotoxicity through inactivation of Rho/Ras small GTPases, and the target specificity of toxin B is determined by amino acids 365–516 [34]. The amino acid homology of this region in TcdB (TcdB365–516) in DLL3110 showed 98% identity (99% similarity) to C. difficile 8864, a previously described toxinotype X/toxin A negative strain, isolated from an asymptomatic human source, that also produced C. sordellii–like CPE on Vero cells [35], but only 51% identity (70% similarity) to TcdB365–516 of RT027 strains.

DISCUSSION
We have identified and characterized a C. difficile strain associated with a severe clinical phenotype that genetic analysis showed to be ST41/RT244/toxinotype IX. Compared with non-RT244, patients with RT244 CDI more frequently had severe disease and a 13-fold increased risk of mortality. Our findings accord with those of a case-control investigation undertaken in New Zealand, showing an OR for severe disease in cases with RT244 CDI of 9.33 compared with controls [36].

Clostridium difficile RT244 produced binary toxin, toxin A and toxin B, but did not produce elevated toxin B levels in vitro and caused atypical cytotoxicity associated with a variant N-terminal catalytic domain of toxin B that alters its catalytic target. Clostridium difficile strains previously described with this phenotype have been of uncertain clinical significance [35]. Toxin B variants resulting from sequence polymorphism of TcdB have been found to alter the virulence of encoding strains, including the hypervirulent C. difficile RT027 [37]. The production of binary toxin may also be an important virulence factor, although its role is less well established [38]. The C. sordellii–like toxin B from C. difficile strain 8864 was more cytotoxic and lethal in mice than control toxin B but equally potent as TcsL [33], and so we postulated that the coexistence of a more potent toxin B with an inactivating mutation of tcdC could contribute to the virulence of strain DLL3110 we identified. The exact role of tcdC mutation in the virulence of C. difficile is complex. Restoration of tcdC function in C. difficile RT027 represses toxin production by 16- to 32-fold and reduces virulence in a hamster model [6]; however, comparison of different C. difficile genotypes has not shown a consistent correlation between inactivating tcdC mutations and toxin production in vitro [6] or clinical virulence [39]. In keeping with this latter
observation, *C. difficile* DLL3110 produced toxin B at a lower level than the toxin-hyperproducing RT027 strain M7404. It is possible that the in vitro toxin production does not accurately predict that occurring in vivo, and future studies using an animal model could examine the virulence potential of strain DLL3110.

The epidemic curve from our study shows the emergence of *C. difficile* RT244 at our laboratory from July 2011 but then a decrease in cases from early 2012. Increasing use of broad-spectrum fluoroquinolones has been correlated with the emergence of moxifloxacin-resistant RT027 CDI; however, *C. difficile* RT244 is moxifloxacin susceptible and factors other than the use of broad-spectrum antibiotics may play a part in driving the emergence of new hypervirulent strains [8]. The sudden appearance of our RT244 *C. difficile* strain and the frequency of community-acquired disease suggest a previously unrecognized community origin, noting that the non-RT244 CDI in our sample also frequently had community disease. We can find no reports of RT244 or toxinotype IX strains in animals or foods [40], but further studies on the epidemiology of these strains and the source of community-associated CDI is warranted.

A survey conducted in 2009–2010 showed that whereas 60% of Australian and New Zealand laboratories performed toxigenic culture, only a small number carried out further genotyping, hampering our ability to detect the emergence of novel strains [16]. In this report, the detection of the *tcdC*Δ117 deletion by Xpert *C. difficile* PCR led to the further typing and identification of the strain, but future emergent strains will of course not necessarily be detected in this way. Clearly, future surveillance must include coordinated sampling through diagnostic laboratories as well as the capacity to investigate CDI clusters of increased prevalence and/or severity.

Our study was limited by the small number of cases, insufficient to allow a multivariate analysis to be performed. It was conducted in a single health network in one state of Australia and may not reflect the epidemiology or severity of CDI in other populations. We were unable to use other cases of *C. difficile* RT244 infection identified at other laboratories, because these isolates may have been submitted because of clinically severe disease, and their inclusion would have produced a bias toward severe cases. Because our laboratory used Xpert PCR throughout the outbreak period, we are confident there was no bias in identification of included cases. Although molecular
typing was not carried out on the non-RT244 cohort, it is unlikely that these strains were caused by a single low-virulence strain from the results of previous strain surveys [16]. As diagnostic algorithms differ between laboratories in Australia, it is difficult to ascertain the true prevalence and incidence of RT244. It is unknown if strains with heterogeneous virulence are constantly emerging and receding in incidence. In light of these uncertainties, the overall clinical significance of RT244 cannot be clearly determined at present. At our laboratory, C. difficile RT244 has spontaneously declined, and the epidemiological data available to us provided no clear evidence for the source of the strain, how it disseminated in our community, or why its incidence has declined. Further studies are required to answer these questions, and to better understand the virulence of the strain and its potential to become endemic and to cause further outbreaks.

Notes

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