A
s the leading cause of healthcare-associated infectious diarrhea and colitis, Clostridioides (formerly Clostridium) difficile continues to affect patients in hospitals and extended-care facilities in the United States (1–3). Among the numerous C. difficile strains, none have been more important to the healthcare community at large than the strain characterized as toxinotype III, restriction endonuclease analysis group BI, PCR ribotype (RT) 027, and sequence type (ST) 1, also known as pulsed-field gel electrophoresis type NAP1 (4,5). Because the BI/RT027 strain has been associated with numerous healthcare facility outbreaks and an increasing number of illnesses and deaths, it has been called hypervirulent (6). Factors that potentially contribute to increased virulence for this strain include increased sporulation, particular DNA gyrase mutations, the ability to survive in hostile environments, and increased toxin production (7–9).

The pathogenicity locus of BI/RT027 includes a characteristic 18-bp deletion and a single-base deletion at position 117 of the tcdC gene (4). These mutations result in a truncated TcdC protein that is rendered nonfunctional, leading to a lack of regulation of the tcdA and tcdB genes and potentially increased toxin A and B production (4). Virulence may be further affected by the binary toxin (CDT), which is coded for by genes located outside the pathogenicity locus, and its proposed role is to increase adherence of the bacterium to the epithelium (4,10).

Presumptive detection of the BI/RT027 strain by the Xpert C. difficile EPI assay (Cepheid, https://www.cepheid.com) is based on PCR amplification of targets, including the deletion at position 117 within tcdC and sequences within tcdB and the binary CDT genes (11). During the past 10 years, non-RT027 strains have emerged that test positive by this assay because they have the same or similar gene targets (12,13). Several of these non-RT027 strains also have been associated with increased numbers of illnesses and deaths, suggesting that the gene targets for this assay may be related to increased virulence (12–14).

In 2012, during a surveillance study at 2 US Veteran Affairs (VA) long-term care facilities (LTCFs) and their affiliated acute care facilities, we detected a clonal C. difficile outbreak at 1 of the LTCFs and the affiliated acute care hospital in the United States (15). The organism was initially identified as the epidemic strain BI/RT027 after it tested positive by Xpert C. difficile Epi assay
(15). However, further analysis identified this strain as REA group DQ and RT591. We report the microbiological and molecular characterization of this strain and the clinical findings of the infected patients.

**Methods**

During February 2012–August 2012, we obtained swab specimens from the perirectal area of asymptomatic LTCF patients at 2 VA facilities in Cleveland, Ohio, and Chicago, Illinois, USA, as part of a validation study comparing PCR using the Xpert C. difficile Epi assay and culture (15). In addition, fecal specimens from symptomatic patients with C. difficile infection (CDI) were cultured for C. difficile as part of a larger surveillance study of C. difficile in each LTCF and the associated acute care facility at these 2 sites (16). Specimens obtained from the perirectal area using BD BBL CultureSwabs (Becton Dickinson, https://www.bd.com) and submitted fecal specimens from symptomatic patients were cultured anaerobically on selective media as previously described (15). We reviewed medical records to obtain information about demographics, medical conditions, medications, CDI treatment, and outcomes. The severity of CDI and determination of initial versus recurrent cases were classified in accordance with the Infectious Diseases Society of America and Society for Healthcare Epidemiology of America C. difficile infection guidelines (17). The institutional review board of each hospital approved the study protocol.

**REA**

Recovered C. difficile isolates were first subjected to typing by REA. Using the methods provided by Clabots et al. (18), total cellular DNA was purified and subjected to HindIII restriction enzyme digestion and electrophoresis. The resulting banding patterns were then compared with a known database. All isolates representing a newly recognized REA type that corresponded to a presumptive BI/RT027 identified by the Xpert C. difficile Epi assay were subjected to PCR ribotyping, whole-genome sequencing (WGS), multilocus sequence typing (MLST), and PCR amplification of cdtA, cdtB, tcdA, and tcdB and an 18-bp deletion in tcdC. Sequencing of tcdC (1 isolate), toxotyping (1 isolate), toxin production in vitro (1 isolate per REA strain group), and antimicrobial susceptibility testing (6 isolates) were performed on selected isolates.

**PCR Ribotyyping**

We characterized recovered C. difficile isolates using high-resolution capillary gel electrophoresis–based PCR ribotyping. We analyzed these isolates against a library of standard profiles, as described previously in the internationally validated consensus protocol from Fawley et al. (19).

**Gene Analysis of Binary Toxin CDT, Toxins A and B, and the Negative Toxin Regulator**

We conducted PCR amplification of cdtA, cdtB, tcdA, and tcdB and an 18-bp deletion in tcdC on all isolates identified as BI/RT027 and DQ/RT591 as previously described (20). In addition, by using primers previously described by Rupnik et al. (21), we amplified cdtB and tcdB by PCR to confirm the presence of CDT and toxin B on a representative DQ/RT591 isolate followed by amplification and sequencing of the tcdC gene. A full-length tcdC PCR was performed using the following primers to produce a 910-bp product: forward primer ACTGTTTATTTGCAATTATAAAAACATCT; reverse primer TTACTTATTATTGTTAATAATTGCTTAGGG. PCR amplicons were gel purified, sequenced, and compared with BI and strain 630.

**Toxinotyping**

We conducted toxinotyping on a representative DQ/RT591 isolate by performing restriction fragment-length polymorphism PCR of the BI and A3 fragment. We assessed for variation in the first 3-kbp of tcdB and a repetitive 3-kbp fragment in tcdA (21).

**WGS and MLST**

We conducted WGS using a Nextera kit (Illumina, https://www.illumina.com) to prepare genomic DNA libraries and sequenced the RT591 organism using an Illumina MiSeq producing 2×250-bp read sets in accordance with the manufacturer’s protocols. Reads were filtered with SolexaQA++ 3.1 by dynamic trimming bases lower than Phred 30 and discarding reads <50-bp long (22). We then assembled high-fidelity filtered reads into contigs at least 500 bp long with SPAdes 3.6.2 (22,23). Isolates that met the following 5 molecular testing criteria (positive for cdtA, cdtB, tcdA, and tcdB and an 18-bp deletion in tcdC) were then analyzed by genomewide average nucleotide identity (ANI). We computed pairwise ANI values between genomes from NuCmer v3.1 alignments aided by PyANI version 0.1 (24). Pairwise identity values were sorted into a 2-dimensional matrix with pandas version 0.15.2 in Python, and a heatmap of identity values with hierarchical clustering linkages was visualized with gplots version 2.16.0 in R (25). DQ/RT591 genomes also had single-nucleotide polymorphisms (SNPs) identified with Parsnp version 1.3.
We retrieved all STs on PubMLST’s webserver (https://pubmlst.org/cdifficile) (351 as of June 28, 2016) to compare distances among STs. The database of profiles was based on the first scheme created for C. difficile, which uses the loci of adk, atpA, dxr, glyA, recA, sodA, and tpi. Of the 3,501-bp length for each ST, 524 positions were found to have a nucleotide variant among the 351 STs. The variable sites were given to RAXML version 8.1.17 with the generalized time-reversible substitution model, and the tree was visualized in Figtree version 1.4.2 (26).

In Vitro Toxin Production and Antimicrobial Susceptibility Testing
We determined toxin production on C. difficile isolate supernatants of representative isolates of 5 different REA group strains after 24, 48, and 72 hours of growth in brain heart infusion broth media (27). Toxin concentrations were determined by enzyme immunoassay (C. difficile toxA/B II EIA; TechLab, https://www.techlab.com) and interpolation from a standard curve using a toxin A standard of known concentration. Assays were performed in triplicate on a representative DQ isolate and compared with supernatants from toxigenic strains BI (RT027), J (RT001), AF (RT244), and a nontoxigenic strain, REA group T. A qualitative cytotoxin analysis was performed on the representative isolate supernatants using human fibroblast cells (Bartels cytotoxicity assay; Trinity Biotech, https://trinitylifesciences.com). We determined antimicrobial susceptibilities by Etest (bioMérieux, https://www.biomerieux-usa.com) for moxifloxacin, ceftriaxone, azithromycin, and clindamycin on taurocholate fructose agar plates (28,29). We confirmed the susceptibility results for moxifloxacin and clindamycin by testing 5 additional unique strains of BI and DQ recovered from patients at the VA site where the DQ outbreak occurred. We used Clinical and Laboratory Standards Institute guidelines for resistance cut-off values (30,31).

Statistical Analysis
We compared characteristics and outcomes of patients colonized or infected with BI/NAP1/027 and other strain types with those of patients colonized or infected with DQ/591 strains. Student t-test was used for normally distributed data and Fisher exact test for categorical data. We analyzed data using SPSS Statistics 10.0 (IBM, https://www.ibm.com).

Results
REA group BI strains were the most common strains recovered from both VA sites and accounted for 33 (40%) of the 83 isolates at the Cleveland site. In addition, 16 (19%) of the isolates from 15 Cleveland patients were identified as REA strain DQ, even though the corresponding Xpert C. difficile EPI assay results indicated the presence of the NAP1 strain (i.e., REA group BI). No DQ strains were found at the Chicago site.

We compared baseline characteristics and outcomes of the 15 patients with fecal cultures positive for the DQ strain with those of the 22 patients with BI/NAP1/027 strains and 27 with other strain types (Table). Ten (67%) of the 15 patients with the DQ strain were LTCF residents, 4 (27%) were on the spinal cord injury unit, and 1 was hospitalized on a medical ward. Of the 7 patients with CDI caused by DQ strains, 3 (43%) met criteria for severe CDI, but none had fulminating CDI. All 7 CDI cases were healthcare associated; 3 of these patients had onset in the hospital, and 4 had onset in the LTCF. In all patients with CDI caused by DQ strains, diarrhea resolved with therapy, but CDI recurred in 3. Patients colonized or infected with DQ strains were significantly more likely than those with BI/NAP1/027 or other strain types to be LTCF residents and to have received antimicrobial drugs during the past 90 days. Patients with other strain types were significantly less likely than patients with DQ strains to have a recent intensive care unit admission, to have healthcare-associated CDI, or to die within 6 months after the CDI diagnosis.

The HindIII REA banding patterns differed between the DQ and BI strains (Figure 1, panel A). The PCR ribotype patterns were more similar, except for the RT591 pattern (DQ), which was missing 2 major bands present in RT027 pattern (BI) (Figure 1, panel B).

PCR amplification of tcdB and cdtB in a representative DQ isolate indicated the presence of genes for toxin B and binary toxin CDT, consistent with the profile of the epidemic BI/RT027 strain (data not shown). In addition, amplification and sequencing of the tcdC gene showed complete alignment with tcdC from a reference BI strain, both of which contained an 18-bp deletion from positions 316 to 333 and a single base deletion at position 117 that resulted in a stop codon at position 196, unlike the reference C. difficile strain, 630 (data not shown) (32). The similarities to the BI/RT027 strain were further validated because toxinotyping indicated the DQ/591 strain was toxinotype III (data not shown).

WGS and MLST results showed that, although the strains are closely related and reside within the same clade (MLST clade 2), they form a separate cluster (Figure 2). ANI showed a clear separation of the BI/027 and DQ/591 isolates at the whole genome level.
The DQ/RT591 strain was highly resistant to clindamycin (MIC >256 μg/mL) and azithromycin (MIC >256 μg/mL) but was susceptible to moxifloxacin (MIC range 1–2 μg/mL) and ceftriaxone (MIC 16 μg/mL). BI/RT027 was resistant to moxifloxacin (MIC >32 μg/mL) and azithromycin (MIC >256 μg/mL) and variably resistant to clindamycin (MIC range 6–32 μg/mL). Furthermore, WGS revealed that all DQ/RT591 isolates contained a variant ermB sequence known to confer clindamycin resistance (33).

Discussion

During a surveillance study of C. difficile in asymptomatic LTCF patients and symptomatic patients in the affiliated acute care hospitals at 2 VA facilities (16), we detected a clonal outbreak of a newly recognized C. difficile strain at the Cleveland facility. This strain, identified as REA group DQ, ribotype 591, is closely related to the BI/RT027 strain, and a commercial PCR erroneously identified it as the epidemic NAP1 strain (i.e., BI/RT027). The strain was misidentified as NAP1 by Xpert C. difficile PCR (Cepheid) because of the similar genetic findings within the C. difficile pathogenicity locus, and the presence of the binary toxin CDT gene cdtB (33). Our findings and previous reports of other non-RT027 strains show that these genetic targets are not specific to NAP1 (12,13). Even though the outbreak of RT244 in Melbourne, Australia, was misidentified as NAP1 (RT027) by the same commercial PCR, it was associated with severe disease in the infected patients (12), suggesting the possibility of shared virulence determinants.

DQ/RT591 and BI/RT027 share several characteristics, including tcdB, cdtB, the 18-bp and position 117 tcdC deletions, and a similar cytotoxic phenotype. Despite these shared genetic and phenotypic characteristics, in vitro toxin production appeared...
Unique Clindamycin-Resistant *C. difficile* Strain

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Figure 1. Comparison of the molecular characteristics of *Clostridioides difficile* strain DQ/591 and the epidemic BI/027 strain in study of *C. difficile* at 2 US Veteran Affairs long-term care facilities and their affiliated acute care facilities. The *Hind*III REA (A) and PCR ribotype (B) banding patterns were distinct between REA strain DQ/RT591 and REA strain BI/RT027. Molecular weight markers (in kb) are shown adjacent to the REA gel pattern. An internal spiked LIZ 1200 standard was used for fragment length calibrations (in bp) of the PCR ribotype gel patterns. REA, restriction endonuclease analysis.

To be delayed and somewhat lower in the DQ/RT591 strain than in BI/RT027. The clinical manifestations of the patients colonized or infected with DQ/RT591 were not unusual and did not differ substantially from those of patients with BI/NAP1/027 and other strain types in the cohort reported here. In nearly half (47%) of the patients, symptoms developed that were consistent with CDI. Although no fulminant CDI cases or deaths directly related to *C. difficile* were recognized, in CDI that developed because of DQ/591, 43% were classified as severe CDI in accordance with Infectious Diseases Society of America and Society for Healthcare Epidemiology of America guidelines. Likewise, no fulminant CDI cases or CDI-related deaths were recognized among the patients infected with BI/RT027 during this same study. However, all-cause mortality was lower for persons with non-DQ/RT591 and non-BI/RT027 infections. All the documented transmission events occurred in the BI/RT027 patients (34). Despite the fact that all the CDI cases caused by DQ/RT591 were healthcare associated, WGS did not identify any transmission events between patients because core genome SNP differences were >8 among DQ isolates (Appendix Figure 3) (34).

All patients in whom DQ/RT591 was confirmed had received antimicrobial drugs within 90 days before testing, and all patients with a CDI were classified as having a healthcare-associated infection; nearly all cases occurred in the LTCF or spinal cord injury unit. No specific antimicrobial drug was highly associated with infection by this strain. Fluoroquinolones have been associated with CDI outbreaks with the BI/RT027 strain, which is highly resistant to fluoroquinolones in vitro (35). Among the DQ/RT591- and BI/RT027-infected patients, receipt of fluoroquinolones or...
clindamycin was limited. Despite high-level resistance to clindamycin in vitro and the presence of \textit{ermB} in DQ/RT591, only 1 patient infected with this strain had received clindamycin.

Our experience was limited, but DQ/RT591 did not appear to carry the same level of clinical severity that BI/RT027 has exhibited (6). This difference in severity might be attributable to delayed toxin production in vivo (Figure 3). Although increased toxin production has been proposed as the reason for increased virulence with the BI/RT027 strain (4,29), increased toxin production was not demonstrated for the AF/RT244 strain, which nevertheless was associated with an outbreak of increased severity in Australia (12). AF/RT244 also shows a different cytopathic effect than BI/027, which suggests the presence of a variant toxin B in AF/RT244 (4,12). Factors associated with increased virulence associated with these strains are still incompletely defined.

Because REA DQ/RT591 is closely related to BI/027, further monitoring is required to determine whether this strain carries risk for increased illness and death or has the capability of widespread dissemination. Since we completed this work, RT591 was reported as the most prevalent \textit{C. difficile} strain in 3 tertiary hospitals in Colombia. These RT591 isolates were also mostly resistant to clindamycin (85%) (36).

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About the Author
Dr. Skinner is an infectious diseases fellow at Loyola University Medical Center and the Hines Veterans Affairs Hospital. His research interests include C. difficile epidemiology, treatment, host response, and molecular characterization.

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Unique Clindamycin-Resistant C. difficile Strain

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Address for correspondence: Stuart Johnson, Edward Hines, Jr. VA Hospital, Infectious Diseases, Research Service/ 151, 5000 S 5th Ave, Hines, IL 60141, USA; email: stuart.johnson2@va.gov; Andrew M. Skinner, Loyola University Medical Center, 2160 S 1st Ave, Maywood, IL 60153, USA; email: andrew.skinner@lumc.edu

To revisit the April 2018 issue, go to: https://wwwnc.cdc.gov/eid/articles/issue/24/4/table-of-contents
Unique Clindamycin-Resistant *Clostridioides difficile* Strain Related to Fluoroquinolone-Resistant Epidemic BI/RT027 Strain

**Appendix**

**Appendix Figure 1.** Pairwise ANI comparisons demonstrate clear partitioning of DQ/RT591 and BI/RT027 isolates at the whole-genome level. Strains with a BI or DQ prefix were recovered from the outbreak (n = 23) and were PCR ribotyped but named according to our REA results, NC_ prefixed strains are public reference genomes retrieved from NCBI GenBank(n = 3), and the remaining numbered strains...
(n = 15) are genomes from our NCBI BioProject 296517 that we typed by PCR ribotyping and correspond to RT027, which cluster with the BI strains or correspond to RT591, which cluster with the DQ strains. ANI, average nucleotide identity.

Appendix Figure 2. Cytopathic effect of supernatants from toxigenic *Clostridioides difficile* strains DQ/RT591(A), BI/RT027(B), and AF/RT244 (C) on human fibroblast cells compared with the noncytopathic effect of toxin A/B negative *C. difficile* restriction endonuclease analysis (REA) group T strain (D). Note the atypical cytopathic effect of AF/RT244 with the rounding and clumping of the fibroblasts. The cytopathic effects of DQ/RT591 and BI/RT027, both of which are toxinotype III strains, are identical to the cytopathic effect of toxinotype 0 strains (data not shown). The atypical cytopathic effect seen with AF/RT244 (toxinotype IX [7]) also has been seen in toxinotype VIII strains that produce a variant toxin B (2).
Appendix Figure 3. Minimum-spanning tree of core genomic single-nucleotide polymorphism (SNP) sites for 7 DQ/RT591 isolates. Sample identifiers are in bold, and the corresponding NCBI GenBank accession numbers are in parentheses. Scale bar indicates 10 SNPs. The mean SNP distance between isolates was 38 (range 8–79).

References
