

Phenotypic and Genotypic Analysis of *Clostridium difficile* Isolates: a Single-Center Study

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Clostridium difficile infections (CDI) are a growing concern in North America, because of their increasing incidence and severity. Using integrated approaches, we correlated pathogen genotypes and host clinical characteristics for 46 *C. difficile* infections in a tertiary care medical center during a 6-month interval from January to June 2010. Multilocus sequence typing (MLST) demonstrated 21 known and 2 novel sequence types (STs), suggesting that the institution's *C. difficile* strains are genetically diverse. ST-1 (which corresponds to pulsed-field gel electrophoresis strain type NAP1/ribotype 027) was the most prevalent (32.6%); 43.5% of the isolates were binary toxin gene positive, of which 75% were ST-1. All strains were ciprofloxacin resistant and metronidazole susceptible, and 8.3% and 13.0% of the isolates were resistant to clindamycin and tetracycline, respectively. The corresponding resistance loci, including potential novel mutations, were identified from the whole-genome sequencing (WGS) of the resistant strains. Core genome single nucleotide polymorphisms (SNPs) determining the phylogenetic relatedness of the 46 strains recapitulated MLST types and provided greater interstrain differentiation. The disease severity was greatest in patients infected with ST-1 and/or binary gene-positive strains, but genome-wide SNP analysis failed to provide additional associations with CDI severity within the same STs. We conclude that MLST and core genome SNP typing result in the same phylogenetic grouping of the 46 *C. difficile* strains collected in a single hospital. WGS also has the capacity to differentiate those strains within STs and allows the comparison of strains at the individual gene level and at the whole-genome level.

Clostridium difficile infections (CDI) are the most common infectious antibiotic-associated gastrointestinal disorders. *C. difficile* colonization of the intestine results in a range of clinical states, ranging from asymptomatic carriage to self-limited diarrhea to life-threatening colitis. CDI was the leading cause of gastroenteritis- and gastrointestinal tract infection-associated deaths between 1999 and 2007 in the United States (1). Risk factors for CDI include antibiotic exposures (especially fluoroquinolones [FQ] and cephalosporins), advanced age, and the severity of the underlying illness (2, 3, 4).

The most common *C. difficile* strain that has emerged in the past decade in North America and some areas in Europe has been classified as 027 by ribotyping, NAP1 by pulsed-field gel electrophoresis (PFGE), BI by restriction endonuclease analysis, and ST-1 by multilocus sequence typing (MLST). ST-1 strains account for half of the sporadic hospital-associated CDI in some settings (5). Some studies have reported that ST-1 strains elaborate *C. difficile* toxins (TCDs) at high concentrations; its purported hypervirulence is plausibly related to this trait. This strain has single and 18-bp deletions of *tcdC*, a negative regulator of *tcdA* and *tcdB*. These mutations cause premature stops, and this truncation is believed to cause toxin overproduction (6, 7). However, this assumption was challenged by recent studies showing no significant difference in toxin production between hypervirulent and nonhypervirulent *C. difficile* strains, and no association of the *tcdC* genotype and toxin production (8, 9).

C. difficile strains containing *cdtA* and *cdtB* binary toxin genes are associated with greater mortality in their hosts than strains in which these genes are absent (10). However, it is not clear if the binary toxin genes increase the virulence of ST-1 or if they are

simply epidemiologic markers of *C. difficile* strains with increased virulence (i.e., guilt by association). It is also notable that other ribotypes with binary toxin, such as 078 (ST-11), can also cause severe CDI, especially in young adults. These *C. difficile* ribotype 078 strains were highly related to animals and food-borne *C. difficile* strains (11). It is concerning that 078 strains have increased in prevalence from 3% (2008) to 13% (2011) (1). CDI caused by both 027/ST-1 and 078/ST-11 are associated with an increased risk of death (12).

Our understanding of the pathogenesis of *C. difficile* is based largely on studies in outbreak strains. While the epidemiology of CDI is changing, analysis of *C. difficile*, especially the strains causing severe CDI, in a nonoutbreak setting might shed light on the mechanism of the pathogenicity of sporadic *C. difficile* and possibly produce more generalizable data. The objective of this study, therefore, was to characterize the phenotypes and genotypes of 46 nonoutbreak *C. difficile* isolates from a large academic medical center using conventional microbiological analyses and whole-

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genome sequencing and to investigate the associations between strain phenotypes and genotypes and clinical outcomes.

MATERIALS AND METHODS

CDI severity, bacterial strains, and ribotyping and binary toxin characterization. This study was approved by the Washington University Human Research Protection Office. All subjects were prospectively interviewed and examined as part of a *C. difficile* assay comparison evaluation (13). The presence of clinically significant diarrhea and the severity of CDI were determined. Patients without clinically significant diarrhea or those who were colonized with a nontoxicogenic strain of *C. difficile* were not classified as having CDI. Severe CDI was defined according to the clinical practice guidelines for CDI in adults (14): subjects with a white blood cell count of $\geq 15,000$ cells/mm³ and/or serum creatinine of ≥ 1.5 times the premorbid level at the time of CDI diagnosis. Specimens were collected, and *C. difficile* strains were isolated and characterized as part of a *C. difficile* laboratory method study (13). Ribotyping (15) and detection of the binary toxin genes from the isolates were performed by PCR as previously described (16, 17).

Antibiotic susceptibility testing. *C. difficile* strains were tested for antibiotic susceptibility using a gradient diffusion method according to the manufacturer's recommendations. Isolates of *C. difficile* were grown in an anaerobic environment on prerduced sheep blood agar (BBL; BD, Sparks, MD). A bacterial suspension was prepared in 0.9% saline to a 1 McFarland standard and then applied as a lawn of growth to *Brucella* agar with hemin and vitamin K (Hardy Diagnostics, Santa Maria, CA). Etest strips for metronidazole, clindamycin, moxifloxacin, ciprofloxacin, and tetracycline (bioMérieux) were applied to the agar and incubated with quality control strains according to the manufacturer's recommendations. The resulting MIC values were interpreted according to the Clinical and Laboratory Standards Institute guidelines (18).

Whole-genome sequencing and analysis. Genomic DNA was extracted from the 46 isolates by a BiOstic bacteremia DNA isolation kit (MO BIO Laboratories). A genome paired-end library was constructed with average insert lengths from 150 to 200 bp, following the Illumina library construction protocol. The libraries were sequenced at an Illumina 2 × 100 bp platform. The genome assembly was performed by Velvet (version 1.1.04-64) (19). All assemblies were subjected to host contamination screening and met the criterion for draft genomes used in the Human Microbiome Project (20). Gene annotation employed the online RAST annotation pipeline with manual inspection (<http://www.nmpdr.org/FIG/wiki/view.cgi/FIG/RapidAnnotationServer>). The open reading frames (ORFs) were at least 300 bp long. Core gene sets were determined by pan-genome analysis pipeline (PGAP) with default parameters using all of the 46 *C. difficile* strains and reference strain 630 (21). The contigs from the draft genomes were aligned to the *C. difficile* MLST database (<http://pubmlst.org/cdifficile/>) to determine the sequence type (ST) using Mummer (22). The mutations from the novel ST types, regulatory genes in the pathogenicity locus (PaLoc), binary genes, and resistance genes were verified by manual inspection of the read alignment to reference alleles. Targeted PCR was performed to close the gaps in specific genes such as *tcdE* that were not fully covered in a subset of isolates.

Single nucleotide polymorphisms (SNPs) were identified with the SNP detection pipeline developed at the Washington University Genome Institute (TGI) by aligning the reads to the *C. difficile* reference strain 630 using BWA aligner (version 0.5.9) and SAMtools (version 0.1.12). SNP calling was performed as previously described but with increased stringency (23) as follows: (i) the coverage of a SNP is at least 10 reads and (ii) the number of reads supporting a SNP calling/the number of reads not supporting a SNP calling is ≥ 10 (≥ 12 bp between two SNP sites). A phylogenetic tree based on the SNPs from the core gene sets was constructed using the neighbor-joining algorithms in Phylip (<http://evolution.genetics.washington.edu/phylip.html>).

Statistical analysis. Fisher's exact test or the chi-square test was used to assess whether ST-1 and the presence of binary genes in *C. difficile*

isolates are associated with CDI severity. *P* values < 0.05 were considered statistically significant.

RESULTS

Isolate characterization. The 46 *C. difficile* isolates represented 23 STs and 20 ribotypes. ST-1 (which corresponds to NAP1/027) accounted for 32.6% of the isolates. Other STs with at least two representatives in the collection were STs 2, 6, and 8 (Table 1). Three strains were not assignable to any STs in the current MLST database. One was a new allele profile (*adk* 3, *atpA* 7, *dxr* 14, *glyA* 8, *recA* 6, *sodA* 25, *tpi* 10), and the other two had an identical SNP at nucleotide position 198 in *sodA*. The correlation of ribotypes and STs was observed: ST-1 corresponded with ribotypes 027 and WU42, ST-2 with ribotypes 001/VPI/77/87 and 014/020, ST-6 with ribotype 005, and ST-8 with ribotypes WU22 and WU25. In addition, ribotypes 027 and WU42 were exclusively found in isolates belonging to ST-1, whereas ribotypes 001/VPI/77/87 were found among four STs (Table 1, Fig. 1). Thus, the ribotypes and ST types did not have a 1:1 correlation.

We next studied the toxin-related genes, including *tcdA*, *tcdB*, and binary genes *cdtA* and *cdtB*. *tcdA* and *tcdB* genes were detected by conventional PCR and were further validated by sequencing. PCR results were perfectly correlated with the sequence data for detecting *tcdA* and *tcdB*. Binary toxin genes were also detected by PCR and successfully reconstructed from whole-genome sequencing. Isolates were grouped into three categories based on the presence of the toxin genes: (i) positive for *tcdA*, *tcdB*, *cdtA*, and *cdtB*, which comprised 43.5% (20 of 46) of the *C. difficile* isolates; (ii) positive for *tcdA* and *tcdB* + and negative for *cdtA* and *cdtB*, which comprised 50.0% of the strains (23 of 46); and (iii) negative for *tcdA*, *tcdB*, *cdtA*, and *cdtB*, which accounted for only three isolates (Fig. 1). Of the 20 *tcdA*-, *tcdB*-, *cdtA*-, and *cdtB*-positive strains, 15 belonged to ST-1. Other STs containing *tcdA*, *tcdB* and binary toxin genes were ST-11, ST-41, ST-67, and the two novel STs.

All 46 isolates were susceptible to metronidazole (MICs from 0.032 to 4 μ g/ml) and resistant to ciprofloxacin (MICs of > 32 μ g/ml). The 46 strains had MICs between 2 and > 32 μ g/ml to moxifloxacin (overall MIC₅₀ and MIC₉₀ of 8 and > 32 μ g/ml, respectively), and 36, 8, and 2 isolates were resistant, intermediate, or susceptible to moxifloxacin, respectively. Of the isolates, 8.3% and 13.0% were resistant to clindamycin and tetracycline, respectively. Two (from ST35 and ST48) of the 46 isolates were resistant to all tested antibiotics except metronidazole; these two isolates were binary toxin gene negative and one was *tcdA* and *tcdB* negative. Five isolates were resistant to at least three antibiotics, including ciprofloxacin, tetracycline, and moxifloxacin.

Phylogenetic concordance between ST and WGS SNP typing. WGS was performed on an Illumina HiSeq platform with 2 × 100 bp read lengths at 100× coverage on average. Read assemblies yielded 193 ± 53 contigs per genome. The contigs were annotated to provide the gene calling for each isolate. The gene content ranged from 3,612 to 4,054 ORFs per genome, indicating significant genetic variations across *C. difficile* strains. ST-1 isolates had between 3,700 and 3,768 genes, corresponding to 121 fewer genes on average than the other ST types in this study (see Table S1 in the supplemental material).

To determine the core genes used for phylogeny inference, we computed the shared genes from the 46 *C. difficile* strains and the *C. difficile* reference strain 630 (an ST-54 strain first isolated from

TABLE 1 Phenotypic and genotypic characterization of 46 *C. difficile* strains^a

Strain	Ribotype	ST	Presence (+) or absence (-) of:		Strain response to ^b :			
			<i>tcdA</i> and <i>tcdB</i>	<i>cdtA</i> and <i>cdtB</i>	Clindamycin	Tetracycline	Moxifloxacin	Disease severity ^c
e01	5	6	+	-	S	S	R	0
a02	5	6	+	-	S	S	R	0
b09	5	6	+	-	S	S	R	1
b01	27	1	+	+	R	S	R	1
a01	27	1	+	+	S	S	R	0
a08	27	1	+	+	S	S	R	0
b10	27	1	+	+	S	S	R	1
d09	27	1	+	+	S	S	R	1
b07	27	1	+	+	S	S	R	1
d02	53	103	+	-	S	S	R	0
d01	77	3	+	-	S	S	I	0
a12	78	11	+	+	S	R	R	1
d10	2/75	55	+	-	S	S	R	0
e09	001/VPI/77/87	46	+	-	S	S	I	0
a11	001/VPI/77/87	48	-	-	R	R	R	0
b04	001/VPI/77/87	7	-	-	S	R	R	0
c04	001/VPI/77/87	2	+	-	S	S	S	0
a09	106/174	36	+	-	S	S	R	0
a03	106/174	42	+	-	S	S	R	0
c12	014/020	2	+	-	S	S	R	0
d04	014/020	2	+	-	S	S	R	0
d03	014/020	2	+	-	S	S	R	1
d08	014/020	14	+	-	S	S	R	0
b08	014/020	110	+	-	S	S	R	0
b05	15/46	10	+	-	S	S	I	1
c11	WU1	53	+	-	S	S	I	0
d12	WU22	35	+	-	R	R	I	0
c03	WU22	8	+	-	S	S	R	0
a04	WU22	8	+	-	S	S	S	0
a05	WU24	NA ^d	+	+	S	S	R	1
e04	WU25	8	+	-	S	S	R	0
b11	WU26	41	+	+	S	S	I	1
b12	WU26	10	+	-	S	S	R	0
e07	WU40	67	+	+	S	S	I	0
d11	WU42	1	+	+	S	I	R	0
c05	WU42	1	+	+	S	S	R	0
c08	WU42	1	+	+	S	S	R	0
c10	WU42	1	+	+	S	S	R	0
e03	WU42	1	+	+	S	S	R	0
d05	WU42	1	+	+	S	S	R	0
c01	WU42	1	+	+	S	S	R	1
e08	WU42	1	+	+	S	S	R	1
c09	WU42	1	+	+	S	S	R	1
e02	WU54	54	+	-	S	R	I	0
d07	WU60	NA ^d	+	+	S	S	R	1
a07	WU63	NA ^e	-	-	S	S	R	0

^a Organized by the ribotypes in alphabetical order.

^b S, susceptible; R, resistant; I, intermediate resistant.

^c 0, not severe CDI; 1, severe CDI.

^d Mutation in *sodA* gene.

^e New allele profile.

a patient with pseudomembranous colitis). The *C. difficile* strain 630 was chosen as the reference because its genome was well annotated, and it has been widely used as the reference for SNP identification. We identified a total of 2,871 core genes across the isolates in this study, accounting for 64.0% of their gene content.

Between 1,096 and 44,935 SNPs were identified from the whole

genomes of these isolates, of which 60.4% to 80.5% were distributed among the core genes. The median (interquartile range [IQR]) number of core genome SNPs was 6,926 per strain. No strains were identical at the SNP level, and 46 SNPs were identified between the two closest strains. The phylogenetic tree constructed using the SNPs from core genes from the 46 strains and the *C.*

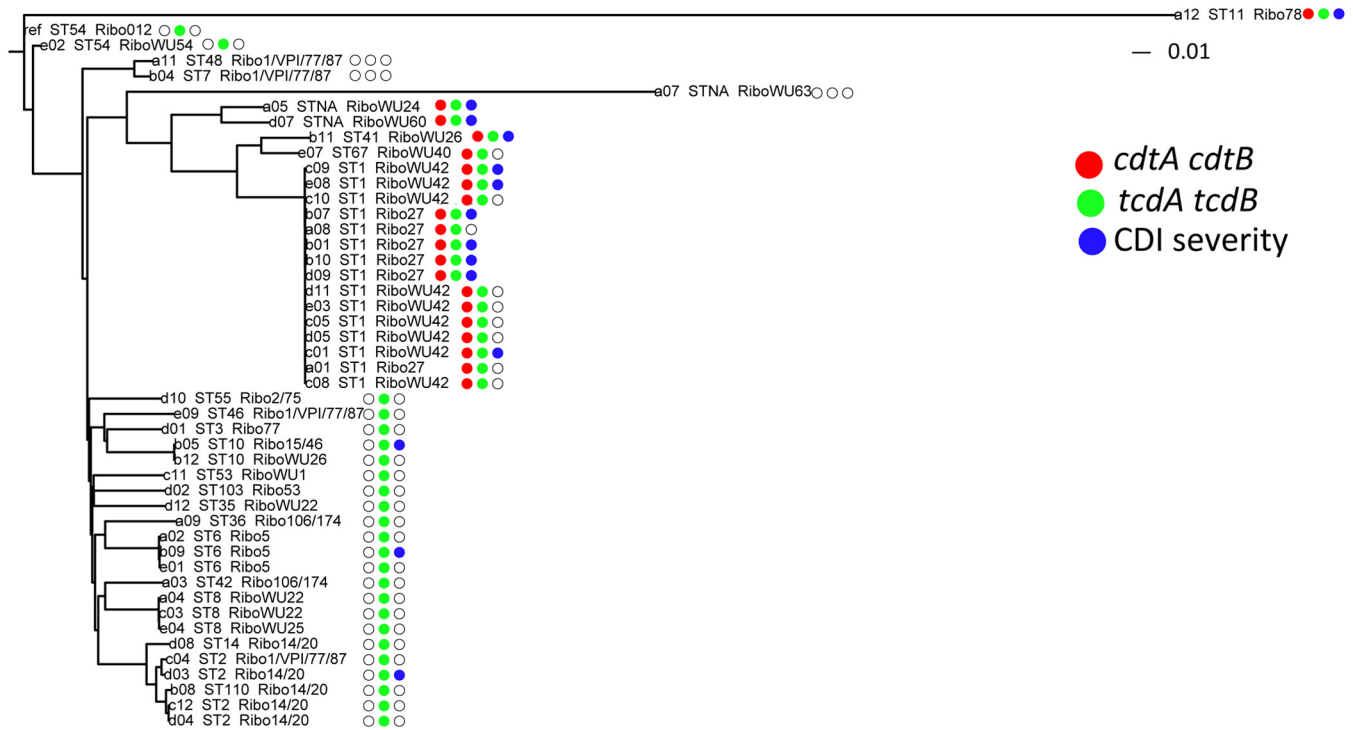


FIG 1 Phylogenetic tree of the 46 *C. difficile* strains based on the genome-wide SNPs. The neighbor-joining phylogenetic tree was constructed based on the SNPs from the core gene sets. *C. difficile* strain 630 is used as the reference strain for SNP calling. The STs and ribotypes are appended after the strain labels. The tree is annotated by the presence of the *tcdA* and *tcdB* genes (green), binary genes (red), and disease severity (blue) at the right side of the dendrogram. The white circles represent the absence of the toxin genes or nonsevere CDI.

difficile reference strain 630 demonstrated a heterogeneous genetic nature of *C. difficile* strains in this collection (Fig. 1). Clades 1, 2, and 5 from a previous study (24) were identified. Clade 1 was composed of several STs. The ST-11 strain from clade 5 was most distant from the rest of lineages with 36,039 SNPs compared to the reference strain 630. The number of SNPs identified among the ST-11 strains was 3.3-fold higher than those of the other STs on average. Clade 2 was dominated by the ST-1/NAP1 strains. Two novel ST strains were genetically most similar to the ST-1 strains, and the third (from a nontoxic isolate) was distantly related to all other strains. The cluster of the SNPs from the core gene sets recapitulated the ST phylogeny, indicating the correspondence of the ST type with WGS (25).

WGS can offer an improved resolution compared to MLST characterization of isolates. For example, 99 to 656 SNP differences were detected within ST-1 strains, representing 1.3% to 4.4% of the differences in core gene sets. Within STs 6, 8, and 10, we identified 26 to 112 SNPs in the core genes, while 1,016 SNPs were detected for ST-2. The numbers of SNPs between ST types ranged from 1,568 to 44,204. However, the number of strains within a single ST type can change the degree of divergence within a ST type and may affect the pattern we observed here.

Genetic heterogeneity of toxin-related and antibiotic resistance genes. Contigs were mapped to the six genes spanning across the PaLoc, two binary toxin genes, and resistance genes to determine the genetic variation of these regions.

Compared to the *C. difficile* reference strain 630, 3 strains lacked *tcdA*, *tcdB*, *tcdC*, *tcdE*, and *tcdR* genes and CD630_06620 (coding for a hypothetical protein). All PaLoc genes were present in the

remaining 43 strains. A phylogenetic tree constructed from SNPs across the PaLoc revealed that diversity at this locus recapitulated ST typing, except for the PaLoc associated with ST-2, which appeared to be mixed with other STs (see Fig. S1 in the supplemental material). *tcdA*, *tcdB*, and *tcdC* genes had the greatest degrees of conservation within the same ST and high variation between STs as indicated in the circular plot (see Fig. S2 in the supplemental material). Other genes, such as *tcdR* and *tcdE*, were conserved even between STs, indicating different evolutionary changes in the PaLoc. The *tcdC* genes in these isolates were 92.7% to 100% identical to those in reference strain 630. Sequence alignment of the 43 *tcdC* genes demonstrated 12 *tcdC* variants. Based on the deletion pattern, we categorized the *tcdC* gene variants into 5 groups: (i) a single base pair deletion at nucleotide position 117 and an 18-bp deletion at nucleotide positions 330 to 347, which included all of the ST-1 strains; (ii) a single base pair deletion at nucleotide position 117 and no accompanying 18-bp deletion, which included only the ST-41 strain; (iii) a single base pair deletion at 117 bp, an insertion of T at 213 bp, and an 18-bp deletion, which included the two novel ST strains and has not been previously reported; (iv) a 39-bp deletion at nucleotide position 333, which included the ST-11 strain; and (v) single mutations without deletions, which occurred among heterogeneous STs.

Because binary toxin genes are not present in the *C. difficile* reference strain 630, the gene variation was determined by aligning the contigs to the binary genes in *C. difficile* strain CD196, which is an epidemic ST-1 strain harboring both type of toxin genes (26). In 15 of 21 strains, the sequences of *cdtA* and *cdtB* were identical to those of the CD196 strains. A transition from T to C at

nucleotide position 813 of the *cdtA* gene was discovered in the remaining six strains containing binary toxin genes. Two ST-11 strains exhibited significant polymorphisms (21 SNPs) in comparison to those for CD196 in the 1,391-bp *cdtA* gene. Similarly, the relation of the *cdtB* gene in ST-11 strains was distant compared to those of other STs as indicated by 49 SNPs in this 2,630-bp region (see Table S2 in the supplemental material).

Fluoroquinolone (FQ) resistance is typically attributed to mutations in *gyrA* and *gyrB*, encoding DNA gyrase subunits. Among the 46 ciprofloxacin-resistant strains, 17 (36.9%) had a mutation at nucleotide position 82 (substitution T→I), as in a prototype FQ-resistant strain R20291 (ST-1). This mutation was common to all study ST-1 strains and to one ST-54 and one ST-55 strain. This substitution at position 82 in *gyrA* is the cause of FQ resistance in most European strains (27). We also identified eight other non-synonymous mutations in *gyrA* from other ST strains, and these mutations tended to be conserved in the same clades. In some cases, these mutations are also ST specific. *gyrB* was intact in most (64.6%) strains. The precise contributions to ciprofloxacin resistance at additional mutations (see Table S2 in the supplemental material) are not yet known, but the *gyrA* and *gyrB* genes from two moxifloxacin-susceptible strains were identical to these loci in some of the moxifloxacin- and ciprofloxacin-resistant strains.

tetM and *ermB* are the genetic determinants of resistance to tetracycline and clindamycin, respectively. Multiple polymorphisms were observed in *tetM* genes compared to those in the reference strain 630. *tetM* genes displayed different degrees of heterogeneity between STs but were conserved within STs. Compared to the rest of the STs, in the ST-11 strains *tetM* contained an additional amino acid substitution at position 490 (M→T). We did not detect any other *tet* genes in the tetracycline-resistant strains. Three strains were resistant to clindamycin. The *ermB* gene of one strain (ST48) was 100% identical to the reference strain 630. The other two strains were from ST-1 and ST-35, sharing four nonsynonymous mutations at 454 (K→Q), 511 (A→V), 649 (Y→H), and 664 (D→N).

No link between SNPs and disease severity within ST-1. Severe CDI accounted for 30.4% (14 of 46) of the CDI cases. The strains associated with severe CDI were from 7 different STs with ST-1 being predominant (46.7%). ST-6, ST-41, ST-11, ST-2, a novel ST with a mutation in the *sodA* gene, and ST-10 accounted for the remaining severe CDI cases (Fig. 1). ST-1 strains were not significantly different in their association with severe CDI compared to that of non-ST-1 strains ($\chi^2 = 1.2$, $df = 1$, $P = 0.28$). Among ST-1 strains, regulatory PaLoc genes (i.e., *tcdC*, *tcdR*, and *tcdE*) were identical in the seven and eight isolates from patients with severe and nonsevere CDI, respectively. Because of the highly repeated sequences in *tcdA* and *tcdB*, full ORFs could not be constructed with accuracy. Thus, we compared the SNPs identified in those two genes between ST-1 strains causing severe CDI and nonsevere CDI. The majority of the SNPs (88.5%) were shared for all ST-1 strains independent of disease severity in the patients from whom they were isolated. The rest of the SNPs were shared in either all ST-1 strains causing severe CDI/nonsevere CDI or a proportion of the ST-1 strains causing nonsevere CDI/severe CDI. Thus, genetic heterogeneity of the PaLoc did not distinguish disease severity within ST-1 strains (see Fig. S1 in the supplemental material). Similarly, SNPs within the PaLoc between non-ST-1 strains (ST-6 and ST-10) failed to identify associations with CDI

severity, indicating no strong role for the genetic composition of pathogenic loci and disease phenotype.

Interestingly, 11 of 14 (78.6%) strains causing severe CDI were binary gene positive. Eleven of 20 (55%) binary toxin gene-positive strains were recovered from patients with severe CDI. The chi-square test showed that binary toxin gene-positive strains were significantly associated with severe CDI compared to binary toxin gene-negative strains (Fisher's exact test, $P = 0.003$). Thus, the presence of binary genes might be a marker for strains that cause severe CDI, but the current genetic data do not support the role of binary genes in causing severe disease, as within the STs, binary gene sequences were identical in strains causing severe CDI and nonsevere CDI.

Whole-genome-wide SNP analysis showed that 92.3% and 92.7% of the SNPs were conserved within all of the ST-1 strains causing severe CDI and nonsevere CDI, respectively. However, no single SNP distinguished the ST-1 strains causing severe CDI from those causing nonsevere CDI.

DISCUSSION

Using integrated approaches, we delineated the phenotypes and genotypes of *C. difficile* strains causing CDI from a single institution over a 6-month interval in 2010. Our data most notably indicate that ST-1 strains remain predominant in nonoutbreak settings, comparable to a statewide strain collection (548 strains) conducted from 2006 to 2009 (5). The vast majority of the strains were *tcdA*- and *tcdB* positive, and no *tcdB*-positive and *tcdA*-negative strains were detected in our data. This is likely in line with the low prevalence (~2%) of this type of strain in the United States, despite the high prevalence rates in Japan, Israel, and Argentina (28).

Our data suggest that MLST and ribotyping are robust approaches for identifying phylogenetic relationships in *C. difficile* strains (25, 29), but the most precise resolution requires WGS. WGS allows single nucleotide-level resolution for strain comparisons, thus serving as a powerful tool for outbreak investigations and clarifying institutional versus community acquisition. As demonstrated by outbreaks of *Escherichia coli* O104:H4 in Europe, the resolution of single nucleotide differences using WGS data led to the distinction of lineages from German and French isolates, which standard tests failed to distinguish (30). Along the same line, whole-genome sequencing of methicillin-resistant *Staphylococcus aureus* (MRSA) in a special care nursery unit successfully tracked the transmission within the unit (31). Recent studies have proved the feasibility of using WGS to track *C. difficile* transmission (32). Importantly, more efforts are needed to conduct prospective epidemiologic studies since the current studies are retrospective in response to a perceived outbreak, and, thus, early shifts in local epidemiology may not be detected (5). With the dropping of sequencing cost and establishment of streamlined analysis pipeline, WGS is becoming an advantageous approach in real-time pathogen surveillance and outbreak detection (33). Close surveillance and the prospective epidemiology of the *C. difficile* strains, especially those associated with severe CDI such as ST-1, ST-11, and ST-6, might prevent future *C. difficile* outbreaks.

In addition, the antibiotic resistance genes identified from WGS data matched well with the antibiotic susceptibility testing, providing another application for WGS data in clinical settings. However, correlation of antibiotic resistance by WGS relies on a database of well-curated resistance genes. With the potential an-

timicrobial resistance suggested by novel mutations identified from WGS, phenotypic testing and genetic engineering are still indispensable for validating the potential resistance from the novel mutations identified from WGS. The novel mutations in *gyrA* and *gyrB* from non-ST-1 strains revealed diverse genetic compositions in conferring potential resistance. The mutations require further verification to determine their role in fluoroquinolone resistance. The virulence genes reconstructed from WGS reads were consistent with the toxin gene testing in the clinic, demonstrating the versatile potentials of WGS in the clinic.

Interestingly, the phylogenetic topology of *C. difficile* is reflected in the PaLoc. Specifically, *tcdC* exhibited identical mutations across all of the ST-1 strains, as indicated by 1- and 18-bp deletions. Along the same line, two binary toxin genes, fluoroquinolone resistance genes (especially *gyrA*), also had identical sequences in all of the ST-1 strains studied and differed from those found in other STs. These findings suggest coevolution of the MLST genes with toxin genes and *gyrA*.

Although the clonal nature of the *C. difficile* strains might provide genetic insights into pathogenicity and antibiotic resistance, it did not shed light on the likelihood of causing severe disease. Previous reports suggested a poor correlation between the presence of *tcdC* or binary genes with clinical outcomes in nonepidemic settings (34, 35). These studies were based on comparisons of NAP1 strains in different severity groups without a genetic comparison, or the genetic comparison was limited to *tcdC* and binary toxin genes. In our study, we found that the presence of binary genes in *C. difficile* significantly increased the risk of severe CDI, but we could not attribute such increased risk to binary gene sequence differences in the strains that caused severe CDI and nonsevere CDI. Moreover, among the 40,000+ SNPs throughout these strains, no single nucleotide variation correlated with disease severity. However, larger sample sizes and future multicenter studies are needed to validate these findings. Disease presentation and outcome are determined by multiple aspects of the host-pathogen interaction, including toxin production, intestinal microbial ecology, host immune response, and timing and selection of treatment. The lack of association between *C. difficile* genetics and disease severity suggests that the bacterial genome itself only partly contributes to disease severity. Future RNA sequence analysis might provide more insight into the *C. difficile* virulence at a bacterial gene expression level. Also, investigation of the role of gut microbiota in CID severity will add an ecological perspective for understanding the disease, especially in the antibiotic-disturbed ecological niche that predominates in patients with CDI.

Although this study demonstrated the potential for application of WGS in clinical diagnosis and linked the genotype, phenotype, and clinical outcome of a collection of *C. difficile* strains, the major limitations of the study were small sample size and a single-center cohort. Future work with a larger sample size will allow a more complete picture of *C. difficile* epidemiology and confirm our findings of genotypic and clinical data. Additional evaluations of a regional or national *C. difficile* network will provide collective resources and both facilitate epidemiological surveillance and inform genetic determinants linked to pathogenesis and specific-disease phenotypes in *C. difficile*.

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