Antimicrobial susceptibility and molecular characterisation using whole-genome sequencing of Clostridioides difficile collected in 82 hospitals in Japan between 2014 and 2016

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We studied the antimicrobial susceptibility and the molecular characteristics using draft whole-genome sequencing of Clostridioides (Clostridium) difficile strains before and after treatment in adults with C. difficile infection (CDI) enrolled in a Phase III, randomised, nationwide study of fidaxomicin versus vancomycin in Japan (NCT02179658). C. difficile strains were cultured from stool samples collected before and after standard treatment with either fidaxomicin or vancomycin. Overall, 285 C. difficile strains were recovered, with 188 derived from CDI cases at baseline (87 patients received fidaxomicin and 101 received vancomycin). No strains isolated from episodes of CDI at baseline were shown to have reduced susceptibilities to fidaxomicin (MIC ≥1 mg/L), or resistance to vancomycin and metronidazole. Thirty-three sequence types (STs) were identified, the most common being ST17 (n=61; 32.4%), ST8 (n=26; 13.8%) and ST2 (n=21; 11.2%). Core-genome single nucleotide polymorphism analysis showed that outbreaks of C. difficile were unlikely to have occurred at each hospital. The predominant toxin gene profile was tcdA⁺tcdB⁺cdtA/cdtB⁻ (n=149; 79.3%). Six of 87 patients who received fidaxomicin harboured C. difficile isolates with reduced
fidaxomicin susceptibilities conferred by previously described mutations,

Val1143Leu/Gly/Asp in RpoB or Arg89Gly in RpoC or putative mutations,

Gln1149Pro in RpoB or Arg326Cys in RpoC. Allelic exchange studies of these putative mutations were not performed. Prior to fidaxomicin use, we found no C. difficile strains with reduced fidaxomicin susceptibilities causing CDI in Japan; however, mutant strains with reduced fidaxomicin susceptibilities were detected after fidaxomicin treatment.
45 **Introduction**

46 *Clostridioides (Clostridium) difficile* is a prominent cause of antimicrobial-associated diarrhoea (1). Recurrent *C. difficile* infection (CDI) occurs in 20–30% of cases (2, 3) and is associated with significant morbidity, mortality and excess economic healthcare burden (4, 5). Fidaxomicin (FDX), a narrow-spectrum antibiotic, is associated with improved sustained clinical cure and reduced recurrence of CDI compared with vancomycin (VCM) (6, 7).

51 Whole-genome sequencing of *C. difficile* provides a valuable tool for profiling strains and evaluating their genetic diversity, enabling assessment of the epidemiology of strains implicated in infection recurrences and outbreaks (8). Data generated from whole-genome sequencing is of particular relevance to evaluate the presence of genes and genetic mutations linked to antibiotic resistance. Reduced susceptibility to FDX among *C. difficile* strains has been associated with single, non-synonymous mutations of genes encoding RNA polymerase subunit β (RpoB) and β’ (RpoC) (9–11). Only one strain isolated from a patient cured of CDI had an elevated FDX minimum inhibitory concentration (MIC) of 16 mg/L at the time of recurrence in
two Phase III randomised, double-blind trials at sites in North America and seven European countries (12). This strain’s mechanism of reduced susceptibility to FDX was not analysed.

Few studies have evaluated clinically relevant strains of *C. difficile* in Japan with a view to examine their antibiotic susceptibilities and resistance mechanisms. Such information may help to tailor the choice of treatment for CDI. In this study, we performed molecular characterisation using whole-genome sequencing of *C. difficile* strains isolated from 188 samples from patients with CDI, enrolled in a Phase III study in Japan.

**Results**

**Participants and *C. difficile* strains**

In all, 212 participants were randomised in the Phase III study: 106 to FDX and 109 to VCM (Figure 1). A total of 285 *C. difficile* strains were recovered from the stool samples of 188 patients (87/106 [82.1%] who received FDX and 101/109 [92.7%] who...
received VCM). Of these, 188 were non-duplicate *C. difficile* strains recovered from patients with CDI at baseline (before receiving FDX or VCM; Figure 1). Secondary strains were recovered from 42/87 (48.3%) patients after receiving FDX and 55/101 (54.5%) patients after receiving VCM (Figure 1).

*Whole-genome sequence analysis of *C. difficile* strains*

We performed whole-genome sequencing of all 285 *C. difficile* isolates, with a mean (standard deviation) map read depth of 76.7 (30.0) (Data set 1). Assembled genomes had an average number of 218.7 (128.6) contigs and an N50 value of 53,033 bp (39,128 bp).

*Multilocus sequence typing, phylogenetic analysis and toxin gene patterns*

Using multilocus sequence typing (MLST), 188 *C. difficile* strains isolated before treatment were classified into 33 sequence types (STs) (Table 1). ST470 was a type not
previously detected. Strains belonging to ST17 were the most common, accounting for 32.4% (n=61) of all isolates from CDI cases at baseline, followed by those belonging to ST8 (n=26; 13.8%), ST2 (n=21; 11.2%), ST81 (n=19; 10.1%) and ST183 (n=13; 6.9%)

(Table 1). No single ST was found to accumulate in one centre. Core-genome single nucleotide polymorphism analysis suggested that outbreaks of *C. difficile* were unlikely to have occurred at each hospital (Table 2 and Figure 2). The nucleotide substitution rate of *C. difficile* ST17, as representative of *C. difficile*, was estimated based on draft whole genome sequence data. The average substitution in the core genome was estimated to be 5.2 SNPs/core-genome/year (95% highest posterior density interval, 0.1, 15.3).

The toxin-encoding gene profiles of each ST are included in Table 2. ST and toxin gene profiles, excluding *tcdC*, were consistent. *tcdA*+/*tcdB*+/*cdtA/cdtB* was the dominant profile (n=149; 79.3%), followed by *tcdA*+*tcdB*/+*cdtA/cdtB* (n=23, 12.2%) and *tcdA*+*tcdB*/+*cdtA/cdtB*+ (n=5; 2.7%). Eleven (5.9%) non-toxigenic strains were recovered. The nucleotide deletion in *tcdC* at position 10 to frame shift mutation was found in one of three ST5 strains. The nucleotide deletion in *tcdC* at position 117 to frame shift
A nonsense nucleotide mutation (from cytosine to thymine) at nucleotide position 184 was found in two ST5 strains and one ST11 strain. The amino acid insertion of lysine-alanine-glycine-glycine-alanine-lysine at position 114 in TcdC was detected in one strain each belonging to ST8, ST17, ST47, ST54 and ST82.

**Antibiotic susceptibilities of C. difficile strains isolated from CDI episodes at baseline**

No strains having reduced susceptibilities to FDX (MIC ≥1 mg/L) or with resistance to VCM and metronidazole (MNZ) were isolated from episodes of CDI at baseline. All strains belonging to ST17, ST81 and ST183 showed resistance to moxifloxacin (MFLX) and clindamycin (CLDM) (Table 3). The number of strains having resistance to MFLX and CLDM among ST8 were 18 (69.2%) and 21 (80.8%) of 26 strains, respectively, and among ST2, the respective number of strains were three (14.3%) and 18 (85.7%) of 21 strains. One ST1 strain was resistant to MFLX (Table 3).
Antibiotic resistant determinants of *C. difficile* strains

The amino acid substitutions Gly91Asp, Ser94Ile and Pro115Ser in RNA polymerase subunits α (RpoA) were detected in seven *C. difficile* strains. Asp492Val, His502Asn, Arg505Lys, Ile548Met, Ile750Met/Val, Glu1037Gln, Asp1160Glu, Ala1205Val, Asp1232Glu in RNA polymerase subunit β (RpoB) were detected in 68 *C. difficile* strains. Thr543Ile, Asn564Lys, Ala617Ser, Ile788Val, Ile833Leu and Pro1084Thr in RNA polymerase subunit β' (RpoC) were detected in 10 *C. difficile* strains. Furthermore, *C. difficile* strains in which any RpoA, RpoB or RpoC amino acid mutations were detected showed no reduced susceptibility to FDX (Table S1). Of 124 MFLX-resistant *C. difficile* strains, 117 had amino acid substitutions from threonine to isoleucine or valine at GyrA position 82 (GyrA Thr82Ile/Val) or from aspartate to alanine, asparagine or valine at GyrB position 426 (GyrB Asp426Ala/Asn/Val) (Table 3 and Table S1). All strains belonging to ST17, ST81 and ST183 had GyrA Thr82Ile/Val or GyrB Asp426Ala/Asn/Val substitutions, while few strains belonging to other STs, except ST8, had these substitutions. Of 172
CLDM-resistant strains, 67 were carrying erm(B). No strains showing CLDM susceptibility had erm(B).

Antibiotic susceptibilities and mutational analysis of C. difficile strains isolated before and after antibiotic treatment

Of 188 CDI cases, C. difficile strains were recovered from the stool samples of 97 patients at the follow-up appointment within 4 weeks after treatment with FDX (n=42) or VCM (n=55; Figure 1). Paired C. difficile strains isolated in 78 of 97 cases belonged to the same ST. ST17 was the most common type, occurring in 32.1% (n=25) of patients, followed by strains belonging to ST2 (n=12), ST8 (n=11), ST183 (n=6) and ST81 (n=5). In seven cases in the FDX treatment group and 12 cases in the VCM group, strains isolated at baseline and during a secondary episode of CDI belonged to different STs.

The FDX, MNZ and MFLX MICs had increased by more than 4-fold compared with the MICs before treatment in six, three and one case, respectively (Table
4). *C. difficile* strains with substantially reduced susceptibility to FDX were obtained only in the patient group that had received FDX treatment (six isolates with 30–2000-fold reduced susceptibility), and not from the VCM group. No isolate from the VCM group showed reduced VCM susceptibility (Table 4). There was no substantial increase in VCM and CLDM MICs. Of the three cases from whom the isolated *C. difficile* strains had 4-fold reduced susceptibility to MNZ after VCM treatment, only one case (patient no. 8) had received MNZ prior to VCM treatment. One *C. difficile* isolate with a 4-fold reduced susceptibility to MFLX was isolated from patient no. 157, who had not received any fluoroquinolone prior to VCM treatment. Six of ten paired strains had reduced susceptibilities to FDX after FDX treatment (MICs ranged from 0.25 to 64 mg/L); all six patients who harboured these strains were reported to be cured after treatment.

Identified single nucleotide polymorphisms (SNPs) with amino acid substitutions were from valine to leucine, glycine, or aspartate at position 1143 in RpoB (Val1143Leu/Gly/Asp), and from arginine to glycine at position 89 (Arg89Gly) in RpoC, which are previously described mutations associated with decreased FDX
sensitivity (Table 4). Amino acid substitutions from glutamine to proline at position 1149 in RpoB (Glu1149Pro) and from arginine to cysteine at position 326 in RpoC (Arg326Cys) were putative mutations (Table 4). No other SNPs were detected in the C. difficile strains with reduced susceptibilities to FDX. SNPs were not detected in paired strains with reduced susceptibilities to MNZ or MFLX (Table 4).

Discussion

In a previous study, ST17 (RT018/smz) was the most common type isolated (frequency, 21.5% to 61.6%) from patients with CDI between 1999 and 2013 in Japan (13–18). ST17 was also the most predominant isolate in the present study, conducted from 2014 to 2016 in Japan, suggesting that the molecular epidemiology of C. difficile strains causing infections in Japan was relatively unchanged over time. Additionally, ST17 was a frequently isolated lineage implicated in CDI in Korea and Italy (19–22) however, the genetic relationship to ST17 in Japan is unknown. Strains belonging to ST8 (RT002; detection frequency 13.8%) and ST2 (RT014; detection frequency 11.2%) were also common in our study: RT002 was detected at a frequency of 4–6% and RT014 was detected at a frequency of around 10% in European countries (23). ST81
frequency 10.1%) was toxin A-negative, toxin B-positive (A B+) and belonged to MLST clade 4, along with ST37 (RT017): these are dominant lineages in China and South Africa (17, 24–27).

Regarding *C. difficile* ST17 isolated in this study, there were nine pairs of strains that had a genetic distance of $\leq$ 2 SNPs and while the possibility of a small number of transmission events could not be ruled out, there was no evidence of infection outbreaks (Figure 2A). The transmission cutoff value based on the nucleotide substitution rate of *C. difficile* ST17 estimated in this study was evaluated as acceptable. The threshold of $\leq$ 2 SNPs was also adopted as the direct transmission cutoff value for STs other than ST17 (RT037) in previous reports (28, 29). For ST2, ST8, ST81 and ST183, when the transmission threshold of $\leq$ 2 SNPs was applied, no outbreak was reported involving any of these STs.

After antibiotic susceptibility testing of isolated *C. difficile* strains, SNP analysis of whole-genome sequences from the strains with reduced susceptibilities isolated before and after treatment with FDX or VCM suggested that there was reduced susceptibility to FDX in six strains after FDX treatment. Mutations at positions 1143 of
RpoB (Val1143Leu/Gly/Asp) and 89 of RpoC (Arg89Gly) have been reported to be determinants of reduced susceptibility to FDX (9). RpoB Gln1149Pro and RpoC Arg326Cys mutations had not been reported in previous studies. In a previous *in-vitro* study, the frequency of detection of *C. difficile* strains with reduced susceptibility to FDX at 4-fold the MIC ranged from $1.28 \times 10^8$ to $<1.41 \times 10^9$ (30). Also, the Val1143Gly/Asp mutation in RpoB appears to be associated with a fitness cost *in vitro* and reduced virulence *in vivo* (10). In this study, the RpoB and RpoC mutants that appeared at low frequency may have been selected for by FDX, because these mutants were recovered only in FDX-treated patients. However, it is not clear what the clinical implications are of using FDX to treat CDI caused by strains having reduced susceptibility to FDX. In practice, six cases of CDI from whom *C. difficile* strains with reduced susceptibilities to FDX were isolated were deemed to be clinically cured at the end of treatment with FDX (Table 4). Despite infection with strains having reduced susceptibilities to FDX, clinical response appears to reflect the high concentrations of FDX in stool (mean 1225 mg/kg) achieved with a standard regimen of FDX (6).
A potential limitation of this study was that *C. difficile* strains recovered from patients after antibiotic treatment were isolated at different times: culture of stool was performed after 10 days of antibiotic treatment, but isolates were also included from specimens collected during the follow-up period, i.e. between days 11 and 28. In a previous report, the same *C. difficile* ribotypes isolated at baseline were also isolated over a long period of time (14–56 days) in recurrent CDI cases (31). Therefore, the differences in the timing of sampling for analysis of *C. difficile* isolates in the present study appear acceptable. Another potential limitation was that the mutations not previously reported, RpoB Gln1149Pro and RpoC Arg326Cys, in *C. difficile* strains with reduced FDX susceptibility were not analysed by allelic exchange methods, which are often used to examine the fitness cost of such mutations (32). Because RpoB Gln1149Pro and RpoC Arg326Cys mutations were only detected by comparing the core genomes of FDX-susceptible strains with those from strains having reduced susceptibilities to FDX, which were isolated from the same patient, we suggest that the mutations could contribute to reduced susceptibility of *C. difficile* to FDX.
In conclusion, our findings showed that no *C. difficile* strain with reduced FDX susceptibility was isolated from patients with CDI in hospitals nationwide in Japan before FDX administration. However, mutant *C. difficile* strains with reduced FDX susceptibilities may have been selected for in the gut of patients treated with FDX.

Future studies should assess the potential emergence of CDI caused by *C. difficile* strains having reduced susceptibility to FDX after the widespread introduction of FDX as a treatment for CDI in Japan.

**Materials and Methods**

**Ethics**

Procedures completed at LSI Medience Corporation were conducted according to Astellas Research Ethics Committee (AREC) standards established at Astellas Pharma, Inc. Procedures completed at Toho University were deemed to be beyond the scope of examination by the AREC. The study was conducted with approval from the Institutional Review Board of the Toho University Omori Medical Centre (no. 2810-CL-3002). Written informed consent was obtained from patients prior to the start.
of any study-related procedures using the written information for patients and informed
consent form that was approved by the institutional review board of each study site.

Summary of the clinical trial

A Phase III, VCM-controlled, double-blind, parallel-group study of FDX was
carried out in 82 hospitals in Japan between June 2014 and September 2016 (33). The study was registered at ClinicalTrials.gov with the identifier NCT02179658. Briefly,
participants were ≥20 years of age with a diagnosis of CDI, defined by the presence of
diarrhoea (with more than four unformed bowel movements in the 24-h period before
randomisation) and C. difficile toxin A, B, or both in a stool specimen obtained within
96 h before randomisation. Patients could have received up to four doses of MNZ or
VCM before randomisation, but no other potentially effective concurrent treatment for
CDI. Enrolled patients were randomised to receive either oral FDX (200 mg every 12 h
with intervening placebo given 6 h after FDX) or oral VCM at the clinically
recommended dose (34) (125 mg every 6 h with intervening placebo given 6 h after
VCM) for 10 days. Patients were assessed every day during the 10-day treatment period
and for 2 days afterwards, and at least weekly during the 28-day follow-up.
were assessed at an end-of-treatment visit for clinical cure and at an end-of-study visit when recurrence had not been reported.

Bacterial isolation and species identification

Stool samples collected within 96 h before study randomisation, within 24 h after completing treatment with FDX or VCM (days 10–11) and within 28 days (±3 days) after completing treatment with FDX or VCM (days 11–31), were sent to a central laboratory (LSI Medience Corporation) and were plated directly onto chromID® C. difficile agar (bioMérieux, France). Cultures were incubated in an anaerobic chamber (5% CO₂, 10% H₂, and 85% N₂) at 35±2°C for 24 h. Identification of bacterial species was performed using Rapid ID 32A API system (bioMérieux). Frozen, stored C. difficile isolates were sent to the Department of Microbiology and Infectious Diseases, Toho University School of Medicine.

Whole-genome sequencing and data analysis
To determine the draft whole genome sequence of *C. difficile* isolates, bacterial DNA was extracted using a standard achromopeptidase and phenol/chloroform method (35). We used the Nextera XT DNA Library Preparation Kit (Illumina Inc., CA, USA) to prepare DNA libraries for sequencing. Libraries were sequenced on the MiSeq system with the MiSeq Reagent Kit V3-600 cycles (300 bp paired-end reads; Illumina Inc.). Draft genomes (contigs) were assembled using the CLC Genomics Workbench software (version 11, Qiagen Bioinformatics). Identification and alignment of the following genes was performed using BLASTn (36) and Jalview (version 2) (37) tpi for species identification of *C. difficile*; *tcdA* encoding toxin A (TcdA); *tcdB* encoding (TcdB); *cdtA* encoding binary toxin A (CdtA); *cdtB* encoding binary toxin B (CdtB); *tcdC* encoding the negative regulator of the *tcdA* and *tcdB* genes; *gyrA* encoding DNA gyrase subunit A (GyrA) and *gyrB* encoding DNA gyrase subunit B (GyrB) for analysis of quinolone resistance-determining regions; *rpoA*, *rpoB*, and *rpoC* encoding the respective RpoA, RpoB and RpoC for analysis of the mechanism of reduced susceptibility to FDX. Genetic variations in the *C. difficile* toxin gene sequences were identified in BLASTn databases including *C. difficile* strain 630 (*tcdA*<sup>+</sup>*tcdB*<sup>+</sup>*cdtA/cdtB<sup>-</sup>), accession no.
NC_009089) and *C. difficile* strain CD196 (*tcdA*+*tcdB*+*cdtA/cdtB*+, accession no. NC_013315) (27). MLST was performed using *C. difficile* MLST databases in PubMLST.org (https://pubmlst.org/cdifficile/) and the Center for Genomic Epidemiology MLST 2.0 web tool (https://cge.cbs.dtu.dk/services/MLST/). Acquired antibiotic resistance genes were identified using the Center for Genomic Epidemiology ResFinder version 2.1 database (https://cge.cbs.dtu.dk/services/ResFinder/). The BioProject ID of this study is PRJDB7714. Draft genome sequences were deposited at the DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp/ddbj/index-e.html; accession number BIMY01000000- BIXW01000000 (Data set 1).

Core genome single nucleotide polymorphism analysis

Core genome SNP-based phylogenetic analysis was performed with whole-genome sequencing data. MiSeq sequencing data were aligned to the genomic sequence of the reference isolate, *C. difficile* 630, using the Burrows-Wheeler Aligner with ‘SW’ algorithm (38). We aligned the core genome sequences using the Sequence...
Alignment/Map software (SAMtools mpileup, version 1.1) (39), which were read using VarScan (version 2.3.7) mpileup2cns (40) and a maximum-likelihood phylogenetic tree was constructed using PhyML (41). Using this as the starting tree, we inferred homologous recombination events that imported DNA fragments from beyond the phylogenetic clade and constructed a clonal phylogeny with corrected branch lengths using ClonalFrameML (42). The core genome, excluding homologous recombination sequences estimated using ClonalFrameML, was subjected to SNP detection.

**Antibiotic susceptibility testing**

Antibiotic susceptibility testing was performed at LSI Medience Corporation using the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) M11-A8 2012 guidelines. The antibiotic agents used were FDX, MNZ, VCM, MFLX and CLDM. The resistance breakpoints for FDX have not been established, while the breakpoints for the remaining drugs were based on CLSI M100-ED28 (MNZ, ≥32 mg/L; MFLX, ≥8 mg/L; CLDM, ≥8 mg/L) (43) and European Committee on
Antimicrobial Susceptibility Testing clinical breakpoints

(http://www.eucast.org/clinical_breakpoints/) version 8.1 (VCM, ≥4 mg/L). *C. difficile*

ATCC 700057 was used for susceptibility testing quality control.

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**Author contributions**

All authors met the following criteria for authorship: substantial contributions to the acquisition, analysis and interpretation of data for the work; contribution to drafting the work and revising it critically; giving the final approval of the version submitted; and agreeing to be accountable for all aspects of the work.

**Transparency declarations**

ST and TM are employees of Astellas Pharma, Inc. KA, YI and KT have no conflicts of interest to disclose.
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Figure 1. Patient flow in the Phase III, randomised, nationwide study of fidaxomicin versus vancomycin in Japan.

aPatients diagnosed with *Clostridioides difficile* infection (CDI) were positive for *C. difficile* toxin A, B, or both in a stool specimen obtained within 96 h before randomisation.

bDays 1–10.

cIf a stool specimen was obtained from the patient within 24 h of completing the treatment (days 10–11), *C. difficile* culture was performed.

dIf diarrhoea occurred within 28 days (± 3 days) after treatment, *C. difficile* culture was performed.

eOne secondary *C. difficile* strain was isolated unexpectedly during fidaxomicin administration.

fTwo secondary *C. difficile* strains were isolated unexpectedly during vancomycin administration.
Figure 2.

Phylogenetic tree of *Clostridioides difficile* strains belonging to each sequence type (ST) constructed with maximum-likelihood phylogenetic analysis based on single-nucleotide polymorphisms (SNPs) in the core genome, excluding estimated homologous recombination. The scale of the distances corresponds to the average number of substitutions per site and SNP.

2A.

Phylogenetic tree of 61 strains of *C. difficile* ST17. A core genome region, amounting to 58.4% (2,505,609/4,290,252 bp), was shared with the genome of a reference strain, *C. difficile* 630 ST54.

2B.

Phylogenetic tree of 26 strains of *C. difficile* ST8. A core genome region, amounting to 32.0% (1,374,263/4,290,252 bp), was shared with the genome of a reference strain, *C. difficile* 630 ST54.

2C.
Phylogenetic tree of 22 strains of \textit{C. difficile} ST2. A core genome region, amounting to 34.1\% (1,461,002/4,290,252 bp), was shared with the genome of a reference strain, \textit{C. difficile} 630 ST54.

Phylogenetic tree of 19 strains of \textit{C. difficile} ST81. A core genome region, amounting to 72.6\% (3,114,637/4,290,252 bp), was shared with the genome of a reference strain, \textit{C. difficile} 630 ST54.

Phylogenetic tree of 13 strains of \textit{C. difficile} ST183. A core genome region, amounting to 41.8\% (1,795,356/4,290,252 bp), was shared with the genome of a reference strain, \textit{C. difficile} 630 ST54.
Table 1. Multilocus sequence typing (MLST) and estimated PCR ribotype of *Clostridioides difficile* strains recovered from patients with *C. difficile* infection

| Sequence type (ST) | Clonal complex (CC) | MLST clade | Estimated ribotype (RT)  | Strains, n (%) | No. centres | Antibiotic treatment | Fidaxomicin Strains, n | Vancomycin Strains, n |
|-------------------|---------------------|------------|--------------------------|----------------|-------------|--------------------|------------------------|------------------------|
| ST17              | CC3                 | 1          | RT018                    | 61 (32.4)      | 43          |                    | 28                     | 33                     |
| ST8               | CC3                 | 1          | RT002                    | 26 (13.8)      | 20          |                    | 12                     | 14                     |
| ST2               | CC3                 | 1          | RT014/020/076/220        | 21 (11.2)      | 10          |                    | 5                      | 16                     |
| ST81              | CC37                | 4          | NA                       | 19 (10.1)      | 17          |                    | 9                      | 10                     |
| ST183             | CC3                 | 1          | NA                       | 13 (6.9)       | 13          |                    | 6                      | 7                      |
| ST55              | CC99                | 1          | NA                       | 5 (2.7)        | 5           |                    | 5                      | 0                      |
| ST37              | CC37                | 4          | RT017                    | 4 (2.1)        | 4           |                    | 3                      | 1                      |
| ST5               | CC5                 | 3          | RT023/063                | 3 (1.6)        | 3           |                    | 2                      | 1                      |
| ST14              | CC3                 | 1          | RT014                    | 3 (1.6)        | 3           |                    | 0                      | 3                      |
| ST15              | CC3                 | 1          | RT010                    | 3 (1.6)        | 3           |                    | 1                      | 2                      |
| ST103             | CC103               | 1          | NA                       | 3 (1.6)        | 3           |                    | 0                      | 3                      |
| ST3               | CC3                 | 1          | RT001/009/072/220        | 2 (1.1)        | 2           |                    | 1                      | 1                      |
| ST27              | CC3                 | 1          | RT067                    | 2 (1.1)        | 2           |                    | 0                      | 2                      |
| ST48              | CC3                 | 1          | NA                       | 2 (1.1)        | 2           |                    | 2                      | 0                      |
| ST58              | CC3                 | 1          | NA                       | 2 (1.1)        | 2           |                    | 1                      | 1                      |
| ST109             | CC238               | 4          | NA                       | 2 (1.1)        | 2           |                    | 1                      | 1                      |
| ST1               | CC3                 | 2          | RT027                    | 1 (0.5)        | 1           |                    | 1                      | 0                      |
| ST6               | CC3                 | 1          | RT005                    | 1 (0.5)        | 1           |                    | 0                      | 1                      |
| ST11              | CC11                | 5          | RT078                    | 1 (0.5)        | 1           |                    | 1                      | 0                      |
| ST  | CC  | RT          | N (%) | 1  | 1  | 0  |
|-----|-----|-------------|-------|----|----|----|
| ST13| CC3 | RT129       | 1 (0.5)| 1  | 1  | 0  |
| ST26| CC3 | RT039/140   | 1 (0.5)| 2  | 0  | 1  |
| ST35| CC3 | RT046       | 1 (0.5)| 1  | 0  | 1  |
| ST42| CC3 | RT0106/118/174 | 1 (0.5)| 1  | 1  | 0  |
| ST47| CC3 | NA          | 1 (0.5)| 1  | 0  | 1  |
| ST53| CC3 | RT0103      | 1 (0.5)| 1  | 1  | 0  |
| ST54| CC3 | RT0102      | 1 (0.5)| 1  | 1  | 0  |
| ST59| ND  | NA          | 1 (0.5)| 1  | 1  | 0  |
| ST67| CC3 | RT019       | 1 (0.5)| 1  | 0  | 1  |
| ST82| CC3 | NA          | 1 (0.5)| 1  | 0  | 1  |
| ST98| CC3 | NA          | 1 (0.5)| 1  | 1  | 0  |
| ST100| ND | NA          | 1 (0.5)| 1  | 1  | 0  |
| ST182| CC3 | NA          | 1 (0.5)| 1  | 1  | 0  |
| ST470| CC3 | NA         | 1 (0.5)| 1  | 1  | 0  |

**Total**

188 87 101

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*Not assigned to any CC.

1According to the PubMLST web site (https://pubmlst.org/cdifficile/).

2NA, not assigned as information on the relationship between ST and RT was not available.
Table 2. Toxin-encoding gene profiles of the *Clostridioides difficile* strains isolated from *C. difficile* infection episodes at baseline

| ST   | No. strains | tcdA\(^+\) | tcdB\(^+\) | cdtA/cdtB\(^+\) | tcdA\(^-\) | tcdB\(^-\) | cdtA/cdtB\(^-\) | Non-toxigenic |
|------|-------------|------------|------------|----------------|------------|------------|----------------|--------------|
| ST17 | 61          | 61         | 0          | 0              | 0          | 0          | 0              | 0            |
| ST8  | 26          | 26         | 0          | 0              | 0          | 0          | 0              | 0            |
| ST2  | 21          | 21         | 0          | 0              | 0          | 0          | 0              | 0            |
| ST81 | 19          | 0          | 19         | 0              | 0          | 0          | 0              | 0            |
| ST183| 13          | 13         | 0          | 0              | 0          | 0          | 0              | 0            |
| ST55 | 5           | 5          | 0          | 0              | 0          | 0          | 0              | 0            |
| ST37 | 4           | 0          | 4          | 0              | 0          | 0          | 0              | 0            |
| ST5  | 3           | 0          | 0          | 3              | 0          | 0          | 0              | 0            |
| ST14 | 3           | 3          | 0          | 0              | 0          | 0          | 0              | 0            |
| ST15 | 3           | 0          | 0          | 0              | 0          | 0          | 3              | 0            |
| ST103| 3           | 3          | 0          | 0              | 0          | 0          | 0              | 0            |
| Other\(^b\) | 27        | 17         | 23         | 5              | 11         |

Total 188 149 23 5 11

\(^a\) *tcdA* encoding Toxin A; *tcdB* encoding Toxin B, *cdtA* encoding binary toxin A; *cdtB* encoding binary toxin B;

\(^b\) Other STs with fewer than two assigned strains: ST1, ST3, ST6, ST11, ST13, ST26, ST27, ST35, ST42, ST47, ST48, ST53, ST54, ST58, ST59, ST67, ST82, ST98, ST100, ST103, ST109, ST182, ST188, ST223, ST470.
| ST     | Fidaxomicin | Vancomycin | Metronidazole | Moxifloxacin | Clindamycin |
|--------|-------------|------------|---------------|--------------|-------------|
|        | MIC range   | MIC range  | MIC range     | MIC range    | MIC range   |
|        | (μg/mL)     | (μg/mL)    | (μg/mL)       | (μg/mL)      | (μg/mL)     |
| 17     | 0.008-0.12  | 0.03-0.12  | 0.012-0.025   | 0.012-0.025  | 0.012-0.025 |
| 8      | 0.015-0.12  | 0.03-0.12  | 0.012-0.025   | 0.012-0.025  | 0.012-0.025 |
| 2      | 0.03-0.5    | 0.06-0.25  | 0.25-0.25     | 0.012-0.025  | 0.012-0.025 |
| 81     | 0.015-0.25  | 0.12-0.25  | 0.012-0.025   | 0.012-0.025  | 0.012-0.025 |
| 183    | 0.015-0.12  | 0.06-0.12  | 0.012-0.025   | 0.012-0.025  | 0.012-0.025 |
| 55     | 0.008-0.06  | NA         | NA            | NA           | NA          |
| 37     | 0.015-0.25  | NA         | NA            | NA           | NA          |
| 5      | 0.004-0.12  | NA         | NA            | NA           | NA          |
| 14     | 0.06-0.25   | NA         | NA            | NA           | NA          |
| 13     | 0.015-0.03  | NA         | NA            | NA           | NA          |
| Other  | 0.004-0.25  | 0.06-0.12  | 0.012-0.025   | 0.012-0.025  | 0.012-0.025 |
| Total  | 0.004-0.5   | 0.06-0.12  | 0.012-0.025   | 0.012-0.025  | 0.012-0.025 |

MIC, minimum inhibitory concentration; NA, not applicable (owing to the number of strains being too small); ND, not determined; ST, strain type. MICs and range shown in μg/mL.

*Other, STs with fewer than two assigned strains: ST1, ST3, ST6, ST11, ST13, ST26, ST27, ST35, ST42, ST47, ST48, ST53, ST54, ST58, ST59, ST67, ST82, ST98, ST100, ST103, ST109, ST182, ST188, ST223, ST470.
Table 4. Single nucleotide polymorphism (SNP) analysis and antibiotic susceptibilities of 10 paired strains of *Clostridioides difficile* with MIC increases of more than 4-fold for any antibiotic tested.

| Mutation category | Strain ID (Isolation date: mo/day/yr) | Patient no. | Antibiotic treatment | Before treatment | After treatment | MIC (mg/L, strain isolated before / after FDX or VCM treatment) |
|-------------------|--------------------------------------|-------------|---------------------|-----------------|----------------|----------------------------------------------------------------|
| FDX sensitivity   |                                      |             | MLST | Gene coding | SNP (Amino acid substitution) | FDX | VCM | MNZ | MFLX | CLDM |
| Already described | FD070 (3/1/2015) | 80 | FDX | FD077 (3/16/2015) | ST17 | RpoB | G3427C (Val1143Leu) | 0.12 | 8 | 0.25 | 0.25 | 0.5 | 0.5 | 32 | 16 | >128 | >128 |
|                   | FD080 (6/21/2014) | 92 | FDX | FD070 (3/16/2015) | ST17 | RpoC | A265G (Arg89Gly) | 0.12 | 4 | 0.25 | 0.5 | 0.5 | 0.5 | 16 | 16 | 128 | 128 |
|                   | FD128 (6/15/2015) | 155 | FDX | FD132 (7/20/2015) | ST17 | RpoB | T3428G (Val1143Gly) | 0.03 | 16 | 0.25 | 0.5 | 0.25 | 0.12 | 32 | 16 | >128 | >128 |
|                   | FD282 (6/1/2016) | 194 | FDX | FD282 (6/6/2016) | ST8 | RpoB | T3428A (Val1143Asp) | 0.06 | >64 | 0.5 | 1 | 0.5 | 0.12 | 32 | 32 | 16 | 16 |
|                   | FD105 (5/12/2015) | 174 | FDX | FD113 (6/6/2015) | ST17 | RpoC | A260G (Glu89Gly) | 0.03 | 64 | 0.25 | 0.5 | 1 | 0.25 | 32 | 32 | >128 | >128 |
| Putative          | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| None              | FD199 (10/26/2015) | 7 | FDX | FD219 (11/30/2015) | ST17 | RpoB | A3446C (Gln1149Pro) | 0.03 | 0.25 | 0.5 | 0.25 | 0.25 | 0.25 | 32 | 32 | >128 | >128 |
|                   | FD268 (5/29/2016) | 8 | VCM | FD279 (6/29/2016) | ST103 | ND | ND | ND | 0.03 | 0.03 | 0.5 | 0.1 | 0.25 | 0.1 | 32 | 32 | 0.5 | 0.5 |
|                   | FD265 (4/19/2016) | 33 | VCM | FD277 (5/24/2016) | ST2 | ND | ND | ND | 0.12 | 0.06 | 0.5 | 1 | 0.25 | 1 | 2 | 2 | 16 | 16 |
|                   | FD159 (8/6/2015) | 74 | VCM | FD163 (9/17/2015) | ST18 | ND | ND | ND | 0.12 | 0.06 | 0.25 | 0.5 | 0.12 | 0.5 | 16 | 16 | >128 | >128 |
|                   | FD904 (4/14/2015) | 157 | VCM | FD109 (5/19/2015) | ST67 | ND | ND | ND | 0.12 | 0.06 | 0.5 | 0.5 | 1 | 1 | 2 | 8 | 16 | 16 |

The 10 *C. difficile* infection cases shown above were deemed to be clinically cured with FDX or VCM treatment. FDX, fidaxomicin; GyrA, DNA gyrase subunit A; GyrB, DNA gyrase subunit B; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; ND, not determined; RpoB, DNA-directed RNA polymerase subunit β encoded by *rpoB*; RpoC, DNA-directed RNA polymerase subunit βʹ encoded by *rpoC*; SNP, single nucleotide polymorphism; VCM, vancomycin. *C. difficile* isolate MICs shown in bold are those for which the MIC increased 4-fold or more between pre- and post-treatment with FDX or VCM.
Informed consent obtained 227 patients
Randomised 215 patients

Fidaxomicin group 106 patients
Baseline C. difficile 87 strains
Study treatment 104 patients
Completion of study treatment 92 patients
Follow-up 83 patients
Secondary C. difficile 42 strains
Completion of the study 68 patients

Vancomycin group 109 patients
Baseline C. difficile 101 strains
Study treatment 108 patients
Completion of study treatment 100 patients
Follow-up 93 patients
Secondary C. difficile 55 strains
Completion of the study 70 patients

Dropout before randomisation 12 patients
Withdrawal before treatment 2 patients
Withdrawal before treatment 1 patients
Withdrawal during the treatment period 12 patients
Withdrawal during the treatment period 8 patients
Withdrawal during the follow-up period 15 patients
Withdrawal during the follow-up period 23 patients
Lack of efficacy 9 patients
Lack of efficacy 7 patients

Baseline C. difficile 87 strains
Secondary C. difficile 42 strains
Baseline C. difficile 101 strains
Secondary C. difficile 55 strains

Figure 1
Figure 2B

| Strain ID | Hospital ID | mo/day/yr |
|-----------|-------------|-----------|
| FD019     | 60          | 9/12/2014 |
| FD017     | 53          | 9/22/2014 |
| FD074     | 42          | 3/7/2015  |
| FD075     | 42          | 3/10/2015 |
| FD175     | 53          | 8/21/2015 |
| FD220     | 55          | 12/3/2015 |
| FD200     | 51          | 10/24/2015|
| FD209     | 52          | 11/18/2015|
| FD227     | 59          | 12/15/2015|
| FD040     | 8           | 11/17/2014|
| FD130     | 40          | 6/15/2015 |
| FD018     | 26          | 5/30/2015 |
| FD124     | 45          | 6/11/2015 |
| FD151     | 46          | 7/21/2015 |
| FD305     | 4           | 7/28/2016 |
| FD239     | 35          | 1/21/2016 |
| FD013     | 7           | 8/28/2014 |
| FD299     | 12          | 7/8/2016  |
| FD078     | 2           | 3/15/2015 |
| FD088     | 8           | 4/6/2015  |
| FD154     | 6           | 7/31/2015 |
| FD288     | 33          | 6/20/2016 |
| FD276     | 13          | 5/20/2016 |
| FD024     | 42          | 9/25/2014 |
| FD286     | 4           | 6/7/2016  |

4 SNPs
Figure 2C

| Strain ID | Hospital ID | mo/day/yr   |
|-----------|-------------|-------------|
| FD042     | 35          | 11/17/2014  |
| FD149     | 56          | 7/16/2015   |
| FD012     | 31          | 8/27/2014   |
| FD065     | 9           | 4/19/2016   |
| FD082     | 54          | 3/27/2015   |
| FD024     | 15          | 7/24/2016   |
| FD216     | 24          | 11/28/2015  |
| FD284     | 52          | 6/3/2016    |
| FD115     | 7           | 5/26/2015   |
| FD145     | 42          | 7/8/2015    |
| FD136     | 4           | 6/24/2015   |
| FD177     | 6           | 8/27/2015   |
| FD244     | 46          | 2/4/2016    |
| FD011     | 31          | 8/26/2014   |
| FD191     | 52          | 10/6/2015   |
| FD221     | 9           | 12/8/2015   |
| FD270     | 13          | 5/10/2016   |
| FD281     | 50          | 5/31/2016   |
| FD043     | 39          | 12/5/2014   |
| FD121     | 7           | 6/7/2015    |
| FD297     | 15          | 7/4/2016    |

5 SNPs
| Strain ID | Hospital ID | mo/day/yr   |
|-----------|-------------|-------------|
| FD140     | 25          | 7/2/2015    |
| FD194     | 34          | 10/14/2015  |
| FD253     | 66          | 3/4/2016    |
| FD027     | 61          | 9/30/2014   |
| FD179     | 67          | 8/31/2015   |
| FD159     | 29          | 8/6/2015    |
| FD006     | 45          | 8/11/2014   |
| FD085     | 60          | 3/30/2015   |
| FD181     | 31          | 9/10/2015   |
| FD126     | 24          | 6/12/2015   |
| FD160     | 61          | 8/6/2015    |
| FD054     | 11          | 1/9/2015    |
| FD065     | 26          | 2/25/2015   |
| FD086     | 3           | 4/1/2015    |
| FD106     | 47          | 5/15/2015   |
| FD062     | 54          | 2/18/2015   |
| FD210     | 35          | 11/18/2015  |
| FD007     | 60          | 8/14/2014   |
| FD195     | 17          | 10/16/2015  |
Figure 2E

| Strain ID | Hospital ID | mo/day/yr |
|-----------|-------------|-----------|
| FD240     | 52          | 1/26/2016 |
| FD141     | 10          | 6/30/2015 |
| FD285     | 21          | 6/9/2016  |
| FD132     | 10          | 6/7/2015  |
| FD046     | 36          | 12/15/2014|
| FD032     | 55          | 10/7/2014 |
| FD256     | 18          | 3/7/2016  |
| FD192     | 12          | 10/6/2015 |
| FD180     | 11          | 9/3/2015  |
| FD021     | 31          | 9/19/2014 |
| FD033     | 31          | 10/14/2014|
| FD201     | 38          | 10/26/2015|
| FD272     | 47          | 3/22/2016 |

1 SNP