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Coinfection and Emergence of Rifamycin Resistance during a Recurrent *Clostridium difficile* Infection

AQ:
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Clostridium difficile (*Peptoclostridium difficile*) **is a common health care-associated infection with a disproportionately high incidence in elderly patients. Disease symptoms range from mild diarrhea to life-threatening pseudomembranous colitis. Around 20% of patients may suffer recurrent disease, which often requires rehospitalization of patients.** *C. difficile* **was isolated from stool samples from a patient with two recurrent** *C. difficile* **infections. PCR ribotyping, whole-genome sequencing, and phenotypic assays were used to characterize these isolates. Genotypic and phenotypic screening of** *C. difficile* **isolates revealed multiple PCR ribotypes present and the emergence of rifamycin resistance during the infection cycle. Understanding both the clinical and bacterial factors that contribute to the course of recurrent infection could inform strategies to reduce recurrence. (This study has been registered at ClinicalTrials.gov under registration no. NCT01670149.)**

AQ: A

*C*lostridium difficile (*Peptoclostridium difficile*) is a common health care-associated infection with a disproportionately high incidence in elderly patients. Recurrent *Clostridium difficile* infection (CDI) is known to occur in approximately 20% of patients following withdrawal of treatment antibiotics (1); however, this may rise to 65% if a patient has a history of CDI (2). Recurring CDI not only causes distress to patients but is also a substantial burden on the health care system due to the increased cost (3) associated with possible prolonged stay or readmission of a patient to the hospital and the readministration of diagnostic tests and antibiotics (4). Collaborations between clinicians and researchers are contributing to knowledge of how both the host and bacterium are affecting recurrent disease in order to reduce recurrence rates through personalized patient care regimens (5).

In this study, multiple stool samples from a patient enrolled in a clinical trial were collected and *C. difficile* was isolated. The strains isolated from these samples were then phenotypically and genotypically characterized to deduce if changes in the C. *difficile* strain genotype and phenotype could have contributed to recurrent infection.

Case. A male participant (01008), aged 85 years, had a history of chronic kidney disease and was on multiple long-term medications, including bumetanide, candesartan, digoxin, simvastatin, doxazosin, ferrous fumarate, and prednisolone. Three months prior to trial enrollment the participant received cefuroxime and gentamicin for a urinary tract infection. One month prior to trial enrollment he was admitted to the hospital, where he received clindamycin (both orally and intravenously) for an infected leg ulcer.

T1
In June 2013, after being readmitted to the hospital, he was diagnosed with CDI (Table 1, sample A), treated with metronidazole (MET), and discharged. He was diagnosed again with CDI in July (Table 1, sample B) and was treated again with MET.

In July 2013, within 5 days of cessation of successful treatment of CDI with MET (defined as cessation of diarrhea for 2 or more days with no loose stools assessed), he was enrolled in a randomized double-blind placebo-controlled clinical research trial aimed at using rifaximin for reducing relapse of *Clostridium*-associated diarrhea (RAPID-ClinicalTrials registration no. NCT01670149). A simplified study time line of stool sample collection is outlined in Fig. 1.

F1

The participant successfully provided one trial stool sample (Table 1, sample C). However, within 10 days he suffered another diarrheal episode (Table 1, sample D), which was initially toxin negative as confirmed using the C. Diff Quik Chek complete kit (Alere) and PCR using the BD MAX Cdiff kit (BD Molecular Diagnostics). However, 6 days after sample D, he provided another sample (Table 1, sample E) which was toxin positive and therefore defined as relapse according to the RAPID trial protocol (onset of >3 loose bowel movements per day for at least 2 consecutive days combined with a positive toxin assay [with or without a positive *C. difficile* culture]). He was treated with MET and, over 8 to 10 weeks, provided two more trial samples (Table 1, samples F and G). Participant notes revealed that during the period when he provided more RAPID samples, he was again diagnosed with CDI (September) and treated with vancomycin (VAN).

The participant was again admitted to the hospital and treated with tazocin and subsequently suffered another CDI (Table 1, sample H) around his time of death in January 2014 due to multiple organ failure. As this was a randomized double-blind, placebo-controlled clinical research trial, it was not known if participant 01008 was on placebo or rifaximin therapy at the point at which this research was undertaken.

AQ:E

TABLE 1 Description and date of sample collection for participant 01008 in the RAPID trial

Sample ID	Specimen	Sample collection date (day/mo/yr)
A	1st diagnostic sample[a]	21/6/2013
B	2nd diagnostic sample[a]	1/7/2013
C	Week 0 (visit 1) RAPID sample	9/7/2013
D	Relapse sample 1	19/7/2013
E	Diagnostic sample post relapse sample 1[a]	27/7/2013
F	Week 4 (visit 3) RAPID sample	7/8/2013
G	Week 12 (visit 5) RAPID sample	2/10/2013
H	Relapse sample after week 12 (visit 5) RAPID sample	4/1/2014

[a] Samples obtained from the Queens Medical Centre Microbiology Department.

MATERIALS AND METHODS

A total of eight stool samples were collected from the participant (Table 1). These included diagnostic specimens from the Queens Medical Centre (QMC) microbiology department, Nottingham, that were proven *C. difficile* toxin positive by using the C. Diff Quik Chek complete kit (Alere) and PCR using the BD MAX Cdiff kit (BD Molecular Diagnostics). *C. difficile* was cultured from ~300 mg of stool using a previously published protocol (6). One to 20 *C. difficile* colonies were isolated per sample (Table 2).

C. difficile typing. After 48 h of growth on cefoxitin cycloserine egg yolk (CCEY) (6) agar, up to 20 individual *C. difficile* colonies from each stool specimen were inoculated into a single well of a 96-well plate containing 200 μl anaerobic brain heart infusion (Oxoid) plus 0.1% L-cysteine (Sigma) (BHIS) broth, leaving one well blank as a control. The plate was sealed with a breathable sterile film and incubated for 24 h in an anaerobic workstation (Don Whitley) (CO$_2$:H$_2$:N$_2$ at 80:10:10, vol/vol/vol). After 24 h the wells were checked for turbidity. Overnight cultures were then diluted 10-fold with sterile PCR-grade water into a fresh 96-well plate, sealed with film, and stored at −20°C until required. One drop of 100% glycerol was added to the BHIS cultures, and the plate was stored at −80°C until required. Every colony that was isolated from each stool sample was subjected to in-house ribotyping with the diluted cultures as mentioned above. PCR amplification of the 16S rRNA intergenic spacer region was carried out according to a modified protocol obtained from the former *C. difficile* ribotyping laboratory in Cardiff (see the supplemental material). PCR-ribotype profiles were analyzed with a QIAxcel capillary electrophoresis machine (Qiagen) using the OL400 program with the QX 15-bp to 1-kb alignment marker and the QX 50-bp to 800-bp size marker. Individual profiles were assessed, and then one isolate from each distinct typing profile that had been obtained from each stool sample was recultured onto BHIS-CC agar and stored as a glycerol stock.

These stock strains were sent for official ribotyping via the *C. difficile* ribotyping network (CDRN) service in Leeds and used for downstream characterization.

Phenotypic characterization. Growth, sporulation, and toxin A and B enzyme-linked immunosorbent assays (ELISAs) were performed on all isolates (see the supplemental material).

Antibiotic susceptibility. Isolated strains were tested for antibiotic resistance to metronidazole (MET) and vancomycin (VAN), using the Etest method (Oxoid), and rifampin (RIF) and rifaximin (RFX) resistance by broth dilution (see the supplemental material) using a 2-fold antibiotic dilution range from 512 to 0.5 μg/ml. *C. difficile* 630Δ*erm*, 630 wild-type (WT), and R20291 strains were used as controls. Guideline MIC breakpoints for RIF and RFX were taken from reference 7, where isolates with MICs of ≥32 μg/ml were considered resistant. Intermediate resistance was defined as a MIC of 0.003 to 32 μg/ml (8).

Genotypic characterization. DNA was extracted from strains using an extraction method with phenol-chloroform-isoamyl alcohol (25:24:1)

saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma) (adapted from reference 9; see also the supplemental material).

Whole-genome comparison. Genomic DNA was sent for Illumina sequencing using MiSeq 250-PE technology (DeepSeq; University of Nottingham). DNA from one of the isolates from sample A (earliest RT002 isolate) and sample E (earliest RT014 isolate identified), designated E2, were also sent for Pacific Bioscience (PacBio) sequencing (McGill University and Genome Québec Innovation Centre). Paired-end reads from the MiSeq runs were mapped to PacBio contigs using CLC Genomics Workbench, version 8.0.2 (Qiagen).

Concordance of PacBio and Illumina sequencing. To demonstrate the concordance of two different sequencing methods (especially over homopolymer regions), Illumina paired-end sequencing reads from isolate A and E2 were mapped back to the PacBio reference contigs. Basic variant detection (CLC Genomics Workbench, version 8.0.2 [Qiagen]) was used to call single-nucleotide variations (SNVs), insertions, and deletions. All default parameters were kept the same, apart from the minimum frequency setting in the coverage and count filter process. The minimum frequency setting was changed to 50% to try to capture as many high-quality changes as possible.

RESULTS

Ribotype of *Clostridium difficile* isolates obtained from stool. During the course of infection, two distinct *C. difficile* PCR ribotypes were isolated (Table 2). The predominant PCR ribotype occurring during the infection was RT002. Coinfection of the participant with a second PCR ribotype (RT014) was detected in stool sample E. This sample was a diagnostic sample obtained after the participant had suffered the first relapse (Table 2). However, it cannot be deduced whether this PCR ribotype was present in earlier samples (i.e., C or D) due to the small number of colonies obtained from the stools of these samples. Therefore, the RT014 isolate may have been present at low frequency in these samples.

Isolate growth, sporulation, and toxin quantification. All isolates showed similar growth profiles in both BHIS and TY (data not shown) except isolate G. This isolate had a shorter stationary phase when grown in BHIS and reduced growth in TY broth. However, this did not affect sporulation and toxin titer, as there were no significant differences in these phenotypes between any of the isolates (data not shown).

Pacific Bioscience and Illumina MiSeq sequencing of RT002 and RT014 isolates. The PacBio sequencing was able to assemble the genome of isolate A into four contigs (Table 3) and isolate E2 into one contig of 4,330,205 bp. Contigs were identified by means of BLAST searches using the dc-megablast option against the nucleotide database. BLAST analysis of the one contig from isolate E2 suggests that it shares sequence similarity with the *Peptoclos-*
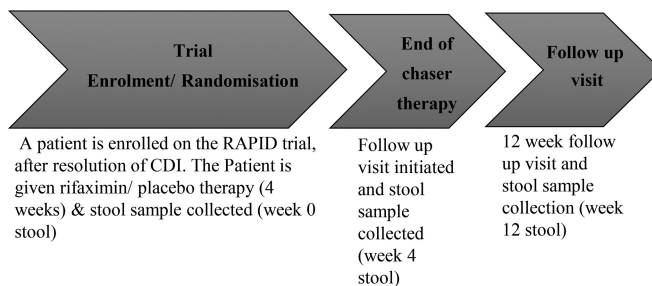

FIG 1 Simplified study timeline indicating the collection of stool samples for the participants involved in the RAPID trial.

Trial Enrolment/ Randomisation

End of chaser therapy

Follow up visit

A patient is enrolled on the RAPID trial, after resolution of CDI. The Patient is given rifaximin/ placebo therapy (4 weeks) & stool sample collected (week 0 stool)

Follow up visit initiated and stool sample collected (week 4 stool)

12 week follow up visit and stool sample collection (week 12 stool)

TABLE 2 PCR ribotype of isolates obtained from participant 01008 stool samples

Sample ID	Specimen	Sample collection date	Number of colonies obtained/typed[a]	PCR-ribotype of isolate[b]
A	1st diagnostic sample	21/6/13	20/8	002
B	2nd diagnostic sample	1/7/13	20/20	002
C	Week 0 (visit 1) RAPID sample	9/7/13	1/1	002
D	Relapse sample 1	19/7/13	8/5	002
E	Diagnostic sample post relapse sample 1	27/7/13	20/20	E1-002 (7), E2-014 (13)
F	Week 4 (visit 3) RAPID sample	7/8/13	15/14	002
G	Week 12 (visit 5) RAPID sample	2/10/13	20/20	002
H	Relapse sample after week 12 (visit 5) RAPID sample	4/1/14	20/20	014

[a] In-house capillary electrophoresis typing using a QIAxcel.
[b] Numbers in parentheses represent the frequency of that PCR-ribotype pattern.

tridium difficile genome assembly CD630DERM, chromosome 1 (LN681537.1).

The individual contigs were annotated using RAST (10) and used as reference strains for Illumina read mapping of all isolates.

Over 97% of reads from seven RT002 isolates (A, B, C, D, E1, F, and G) and >97% of reads from two RT014 isolates (E2 and H) mapped to the reference PacBio contigs for the corresponding PCR ribotype.

Concordance of PacBio and Illumina sequencing. Nine SNVs were identified in all of the RT002 genomes, and 12 SNVs were detected when Illumina MiSeq reads were mapped back to their respective PacBio contigs. All SNVs of the RT002 isolates occurred in the first contig, which represented the main *C. difficile* chromosome. All of the SNVs from both RT002 and RT014 isolates occurred in homopolymer regions of ≥4 nucleotides in length. PCR amplification and Sanger sequencing of all the regions in isolate A and E2 that contained the SNVs confirmed that they were true SNVs. Accordingly, the reference sequences A and E2 were corrected and the sequence reads from the subsequent isolates (B to G) were remapped.

SNV detection in RT002 isolates. Basic variant detection was performed on the reads to assess whether the sequential isolates contained additional changes from the original (A) isolate (Table 4). Isolate B did not contain any additional SNVs. Isolate C contained one additional SNV. The A>G nucleotide change did not result in an amino acid change. Isolates D and E1 both contained the same four additional SNVs compared to isolate A, two of which were nonsynonymous and found in *oppF* and *rpoB* (Table 4). The former encodes an oligotransport-ATP binding domain, while the latter encodes a DNA-directed RNA polymerase beta subunit. These two isolates did not contain the SNV found in isolate C. Isolate F contained five SNVs compared to the sequence of isolate A; however, these were not the same as any SNVs in previous isolates. One of the SNVs in isolate F was found in the same gene (*rpoB*) but not at the same position as in isolates D and E1. Variant detection in isolate G produced 70 SNVs. Closer inspection of these SNVs revealed that 64 were detected at low fre-

quency in poorly mapped regions and probably were not real. Thus, this isolate had six SNV differences compared to isolates A and B (Table 4). Five SNVs were in the same regions as isolate F, with two of the SNVs, in a hypothetical protein and *rpoB*, being in the exact same location as in isolate F (Table 4).

SNV detection in RT014 isolates. Six additional SNVs were found in isolate H compared to isolate E2. Five of these SNVs were in a gene annotated as *fliK*, which is not actually part of the flagellar operon. Only two of these five SNVs were nonsynonymous and occurred at a frequency of <52% and with an average quality of <22. Closer inspection of this region revealed that the sequence quality was poor most likely due to its being repetitive, suggesting that these SNVs were not real. Thus, only one additional T>C SNV was identified. The SNV was in an intergenic region of the genome of isolate H, at position 2562170, upstream of a gene encoding a small hypothetical protein that shows similarity to a putative membrane protein.

Isolate antibiotic resistance. No isolate showed resistance to MET (breakpoint considered resistant, ≥2 µg/ml) or VAN (breakpoint considered resistant, ≥4 µg/ml) (data not shown). Early RT002 isolates A, B, and C showed complete susceptibility to RIF and RFX (see Fig. S1a and b and S2a and b in the supplemental material). However, RT002 isolates D (from the relapse sample) and E1 (postrelapse sample) showed high resistance (≥256 µg/ml) to RIF and RFX (≥128 µg/ml), while RT002 isolates F and G showed intermediate resistance to both (RIF, ≥4 µg/ml; RFX, ≥16 µg/ml). The RT014 isolates (E2 and H) and control strains were fully susceptible to RIF and RFX (≥0.5 µg/ml) (see Fig. S1a and 1b and S2a and b).

Frequency of *rpoB* SNVs in all cultured A-to-G RT002 isolate samples. To discern whether there were two distinct populations of RIF- and RFX-resistant RT002 isolates, the region in *rpoB* that contained the SNVs identified in Table 4 was amplified from every isolate with a PCR ribotype banding pattern confirmed with QIAxcel (Table 1). The PCR-amplified DNA fragment was sent for Sanger sequencing, and the sequences were checked for the

TABLE 3 PacBio contig assembly information from RT002 isolate A

Contig	PacBio BLAST identification	Contig size (bp)
1	FN668944.1, *Clostridium difficile* BI9 chromosome	4,207,942
2	LN681537.1, *Clostridium* phage phiCD211, complete genome	140,450
3	FN668942.1, *Clostridium difficile* BI1 plasmid pCDBI1, complete sequence	65,380
4	GU949551.1, *Clostridium* phage phiCD6356, complete genome	52,160

TABLE 4 Additional SNVs in genome of sequential RT002 isolates

Strain	Reference position	Type	Reference	Allele	Count	Coverage	Frequency	Avg quality	Change Coding region	Amino acid
C	319864	SNV	A	G	194	201	96.52	33.44	Choline binding protein A:c.6498A>G	
D	147783	SNV	A	T	189	192	98.44	36.37	Aminobenzoyl-glutamate transport protein:c.210T>A	
D	556338	Deletion	A		177	177	100.00	31.07	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1):c.479delA	p.Gln160fs
D	2974068	SNV	G	A	210	233	90.13	36.21		
D	3762293	SNV	C	A	189	191	98.95	36.61	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1465C>A	p.Gln489Lys
E1	147783	SNV	A	T	195	198	98.48	34.89	Aminobenzoyl-glutamate transport protein:c.210T>A	
E1	556338	Deletion	A		146	149	97.99	32.74	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1):c.479delA	p.Gln160fs
E1	2974068	SNV	G	A	151	211	71.56	36.64		
E1	3762293	SNV	C	A	161	161	100.00	36.15	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1465C>A	p.Gln489Lys
F	1181867	SNV	G	T	172	174	98.85	33.01	FIG00512976: hypothetical protein:c.722C>A	p.Thr241Asn
F	1861424	SNV	A	G	158	168	94.05	31.75		
F	1861431	SNV	G	A	171	172	99.42	35.88		
F	2676955	SNV	A	C	231	233	99.14	35.90		
F	3762308	SNV	A	T	204	207	98.55	34.08	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1475A>T	p.Asp492Val
G	1181870	SNV	G	T	33	33	100	37.12	FIG00512976: hypothetical protein:c.722C>A	p.Thr241Asn
G	1861429	SNV	A	G	21	21	100	32.95		
G	1861436	SNV	G	A	22	22	100	37.64		
G	2676961	SNV	A	C	12	12	100	38.67		
G	3762308	SNV	A	T	143	174	82.18	36.96	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1475A>T	p.Asp492Val
G	3040149	SNV	C	A	21	33	63.64	38.24	FIG00534171: hypothetical protein:c.13G>T	p.Glu5*

above-described SNVs. The frequency of these SNVs in each isolate is detailed in Table 5.

T5

DISCUSSION

The recurrent infection suffered by participant 01008 is one which is endured by up to 20% of patients suffering from CDI (1). Over a 180-day period, this particular individual relapsed twice with CDI and presented with coinfection by two different PCR ribotypes. At one point during the infection, the participant also either acquired an RT002 isolate, which was resistant to rifamycins (reinfection), or the original isolate developed rifamycin resistance due to microevolution (relapse). The combination of these factors could have contributed to the persistence of this participant's infection.

The nature of the RAPID trial is to recruit participants at the end of their standard CDI therapy, when the patient has been assessed as being resolved of CDI. The participant is then started on a regimen of either RFX or placebo for 4 weeks, during which

stool samples are collected. The participant is then monitored for another 8 weeks, during which more stool is collected. As this was a randomized double-blind placebo-controlled clinical research trial, at the point at which this research was undertaken, it was not known if participant 01008 was on placebo or rifaximin therapy.

What is clear from this data is that even though this participant was deemed to have resolved symptoms of CDI prior to trial enrollment, there was still a low level of *C. difficile* in the stool (as indicated by the isolation of *C. difficile* from sample C), and in this participant it may have contributed to the recurrence of disease. There is further genetic evidence to suggest that the RT002 isolate found in the preenrollment diagnostic specimens (A and B) had persisted, at least until the next sample specimen (C). According to others (11–13), genetically identical strains differ by ≤2 SNVs, and there was only one SNV difference between isolate A/B and C. At some point during the time between sample C and sample D, participant 01008 either acquired a genetically distinct RT002 isolate (reinfection) with resistance to RIF and RFX or the original

TABLE 5 Frequency of all cultured RT002 isolates with and without *rpoB* SNVs

Sample ID	Specimen	No. of colonies typed	SNV present*
A	1st diagnostic sample	8	
B	2nd diagnostic sample	20	
C	Week 0 (visit 1) RAPID sample	1	
D	Relapse sample 1	5	+, (4), [C>A], −(1)
E	Diagnostic sample post relapse sample 1	7	+
F	Week 4 (visit 3) RAPID sample	14	+, [A>T (13)], +, [C>A, (1)]
G	Week 12 (visit 5) RAPID sample	20	+, (10), [A>T], −(10)

a Absence (−) or presence (+) of SNV is indicated. Numbers in parentheses are the frequency of the isolates with or without SNV. Brackets identify which SNV was present.

RT002 isolate evolved to become RIF and RFX resistant ($\geq$256 µg/ml and $\geq$128 µg/ml, respectively). Prior to sample D, isolates from samples A, B, and C did not contain SNVs in the *rpoB* gene (Table 5), supporting the notion that participant 01008 was probably colonized with one population of nonresistant RT002 isolates at the time of the infection. In other studies (11, 12), genetically distinct isolates are differentiated by >10 SNVs. Here, isolate D differed by only four SNVs compared to isolates A, B, and C, and within the population of isolates from sample D (Table 5) one isolate did not contain the SNV in the *rpoB* gene. Thus, it is not possible to deduce whether this was a newly acquired isolate or whether the initial strain had mutated. It is possible that the RT002 isolate in this infection was under high antibiotic selection pressure and, thus, may have mutated more rapidly than others have calculated for strains not under intense selection pressure (14).

Whole-genome sequencing of isolates A to H revealed that the probable cause of RIF and RFX resistance in isolate D was a C>A SNV at bp 1465 in the *rpoB* gene, encoding a DNA-directed RNA polymerase beta subunit (Table 4). Mutations in *rpoB* have been identified in *C. difficile* (7, 8) and also occur in multidrug-resistant strains of *Mycobacterium tuberculosis* (15) within a small 23-amino-acid region from position 511 to 533 (16). This suggests a common mechanism by which resistance to this antibiotic occurs in multiple bacterial species.

The SNV in isolate D resulted in a glutamine-to-lysine amino acid change at position 489 of the peptide. It is known that the glutamine residue at the corresponding position of RpoB in *Thermus aquaticus* directly binds to rifampin (7, 17). Thus, it is likely that this SNV is directly responsible for resistance to RIF and RFX in this isolate, and this may have played a role in the first relapse suffered by this participant. A subsequent stool specimen from participant 01008 revealed the presence of an RT002 isolate (E1) genetically identical to isolate D, which also shared the same SNV change in *rpoB* and was also RIF and RFX resistant ($\geq$256 µg/ml and $\geq$128 µg/ml, respectively). The sample also contained another ribotype (RT014; isolate E2). Isolate E2 was fully sensitive to RIF and RFX. If participant 01008 was on RFX therapy, then it is unlikely that isolate E2 contributed to the relapse, as clearly the vegetative form of the isolate is susceptible to the rifamycins and would have been killed. However, whether or not the isolate was present just prior to relapse is not discernible, as it was only possible to isolate one colony from the prerelapse sample C. The RT014 isolate (E2) may have been present in the host at low levels in the spore form, evading the effects of possible antibiotic therapy and persisting until a time when it could germinate and grow. This hypothesis is supported by the emergence of the RT014 (H) isolate in the final stool sample which was genetically indistinct from isolate E2.

Isolates D and E1 also shared an SNV in the oligotransport-ATP binding domain of *oppF* (Table 4), which belongs to an operon of oligopeptide permease (*opp*) genes that are involved in regulating sporulation (among other processes) in some species of *Bacillus* and *Clostridium* (18, 19). *In vitro* sporulation studies on all isolates revealed no significant difference (data not shown) in the rate of sporulation between isolate D and E1 and all other isolates. However, these data may not be representative of *in vivo* sporulation characteristics; therefore, it cannot be conclusively proven that this SNV had no effect. The *opp* operon is involved in other processes in other organisms, for example, competence in

Bacillus and *Streptococcus* species, plasmid transfer in *Enterococcus faecalis*, and the expression of virulence factors in *Bacillus thuringiensis* (18, 20). Therefore, SNVs in this region may have a yet-undiscovered role in *C. difficile* virulence and could present a further avenue of research.

The next specimen provided by the patient revealed the presence of an RT002 isolate (F) with five additional SNVs compared to isolates A, B, and C. Four of the SNVs were completely different from the previous isolate (E1) and isolate D. However, one SNV was again located in *rpoB*, at a different position (bp 1475), resulting in an A>T change that caused an amino acid change from aspartic acid to valine at position 492 of the peptide. The alteration of this aspartic acid residue to other amino acids, including valine, has been shown to result in RIF resistance in *Staphylococcus aureus* (7, 21). Unlike the RpoB amino acid changes in isolates D and E1, the RpoB amino acid change in isolate F apparently conferred only intermediate resistance to RIF ($\geq$4 µg/ml) and RFX ($\geq$µg/ml). The SNV at this position was also shared by the final RT002 isolate found in sample G, which showed the same level of resistance to RIF and RFX. Whether or not this level of resistance would be sufficient to contribute to the persistence of the organism in the colon is unclear, but there is clearly selective pressure for this mutation occurring either in the host or within the *C. difficile* population found at the QMC in Nottingham.

The presence of an SNV in the same gene (*rpoB*) but at different positions within the RT002 isolates supports the notion that two distinct subpopulations of RIF- and RFX-resistant RT002 isolates could have coexisted in this participant. Further sequencing of this region in sample E RT002 isolates revealed that they all shared the same C>A SNV. However, one of the 15 sample F isolates cultured (Table 5) contained the C>A SNV found in sample E isolates and not the A>T SNV found in the remaining F isolates, indicating that two subpopulations of RT002 isolates could have coexisted at this point. Among the sample G isolates, there were equal populations of isolates with and without any SNVs in *rpoB*. This either indicates the persistence of the initial susceptible isolate in the gut or that at the time sample G was taken the population was in a transient state of mutation.

It is relevant to note that other studies have documented cases of *C. difficile* rifamycin resistance after chaser therapies using rifaximin (8, 22, 23). One of these studies has linked this resistance to mutations in RpoB (8) that have been identified by others (7). The study by Curry et al. (8) indicates that in their study population, more than one-third of isolates were resistant to rifaximin, and this is something which could be looked at in a wider population of participants in the RAPID trial, as it may prompt clinicians to alter dosing regimens if the therapy was approved for use in patients suffering recurrence.

Conclusions. This case study has presented insight into the course of recurrent infection caused by *C. difficile*. In this case it was difficult to ascertain whether, in this particular individual, the *C. difficile* strain was evolving. However, it did reveal the possible presence of multiple isolates with SNVs causing distinct fitness advantages. The fact that this participant was enrolled in a trial to investigate the use of RFX to prevent recurrence suggests that we should be monitoring mutations in the *rpoB* gene more closely in the isolates from trial patients, as a side effect of this therapy could be the increased selection for RFX-resistant *C. difficile* strains. This is of importance to clinicians, as it may directly impact the antibiotic regimen they use to treat their patient.

The advent of high-throughput technologies will allow for more in-depth screening of samples to elucidate the true genetic fingerprint of the isolates found during infection. When coupled with in-depth microbiome analysis of the host, this may allow researchers to more fully comprehend the overall picture of recurrent infection and, in turn, translate this information to clinicians in order to manage at-risk patients more effectively and reduce the morbidity and economic burden of *C. difficile* within the health care system.

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FUNDING INFORMATION

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