Co-infection and emergence of rifamycin resistance during a recurrent *Clostridium difficile* infection

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Running Title: Recurrent *C. difficile* infection
Abstract

*Clostridium difficile* (*Peptoclostridium difficile*) is a common health care associated infection with a disproportionately high incidence in elderly patients. Disease symptoms range from mild diarrhoea through to life threatening pseudomembranous colitis. Around 20% of patients may suffer recurrent disease which often requires re-hospitalisation 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 characterise 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.
Introduction

Clostridium 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 prior history of CDI (2). Recurring CDI not only causes distress to patients, but is also a substantial burden on the healthcare system due to the increased cost (3) associated with possible prolonged stay or re-admission of a patient to hospital and the re-administration of diagnostic tests and antibiotics (4). Collaborations between clinicians and researchers are contributing to the knowledge of how both the host and bacterium are affecting recurrent disease, in order to reduce recurrence rates, through personalised patient care regimens (5).

In this study multiple stool samples from a patient enrolled in a clinical trial (see case information) were collected and C. difficile was isolated. The strains isolated from these samples were then phenotypically and genotypically characterised 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 who had a history of chronic kidney disease and was on multiple long term medication including, bumetanide, candesartan, digoxin, simvastatin, doxazosin, ferrous fumarate and prednisolone. Three months prior to trial enrolment the participant received cefuroxime and gentamicin for a urinary tract infection. One month prior to trial enrolment he was admitted to the hospital, where he received clindamycin (both orally and intravenously) for an infected leg ulcer.
In June 2013, after being re-admitted to hospital, he was diagnosed with CDI (Table 1; sample A), was treated with metronidazole (MET) and discharged. He was diagnosed again with CDI in July (Table 1; sample B) and was again treated with MET.

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

The participant successfully provided one trial stool sample (Table 1; sample C). However within 10 days, suffered another diarrhoeal 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 8-10 weeks provided two more trial samples (Table 1; samples F & 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 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 randomised 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.
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 ~ 300mg of stool using a previously published protocol (6). 1-20 C. difficile colonies were isolated per sample (Table 2).

C. difficile typing

After 48 hours (hrs) 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 hrs in an anaerobic workstation (Don Whitley) (CO₂:H₂:N₂ (80:10:10 vol:vol:vol)). After 24 hrs 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 (Supplementary data). PCR-ribotype profiles were analysed with a QIAxcel capillary Electrophoresis machine (Qiagen) using the OL400 programme with the QX 15bp-1Kb alignment marker and the QX Size Marker 50 bp –800 bp. Individual profiles were assessed, and then one isolate from each distinct typing profile
that had been obtained from each stool sample, was re-cultured 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 characterisation.

**Phenotypic Characterisation**

Growth, sporulation and toxin A and B ELISAs were performed on all isolates.

**Antibiotic Susceptibility**

Isolated strains were tested for antibiotic resistance to metronidazole (MET) and vancomycin
(VAN), using the E-test method (Oxoid) and Rifampicin (RIF) and Rifaximin (RFX)
resistance by broth dilution (see supplementary data) using a 2-fold antibiotic dilution range
from 512 - 0.5 µg/ml. *C. difficile* strains 630Δerm, 630 wild type (WT) and R20291 were
used as controls. Guideline MIC breakpoints for RIF and RFX were taken from (7), where
isolates with MICs ≥32 µg/ml were considered resistant. Intermediate resistance was defined
as an MIC of 0.003–32 µg/mL (8).

**Genotypic Characterisation**

DNA was extracted from strains using a Phenol:Chloroform:Isoamyl alcohol (25:24:1)
saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma) extraction method adapted from
(9)(See supplementary data).

**Whole Genome comparison**

Genomic DNA was sent for Illumina sequencing using MisSeq 250-PE technology,
(DeepSeq, University of Nottingham). DNA from one of the isolates from sample A (earliest
RT002 isolate), and sample E (earliest RT014 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 filters process. The minimum frequency setting was changed to 50% to try and 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. Co-infection 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 low number of colonies obtained from the stools of these samples. The RT014 isolate, may therefore, 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 titre 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 4,330,205bp. Contigs were identified by means of Blast searches using the dc-megablast option against the nt database. BLAST analysis of the one contig from isolate E2 suggests that it shares sequence similarity with the Peptoclostridium difficile genome assembly CD630DERM, chromosome : 1 (LN614756.1).

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 & 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 the RT002 genomes and twelve 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 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-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. Isolate D and E1 both contained the same four additional SNVs compared to isolate A, two of which were non-synonymous 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 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 frequency in poorly mapped regions and were probably not real. Thus, this isolate had six SNV differences compared to isolate 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 *flkK*, but which is not actually part of the flagellar operon. Only two of these five SNVs were non-synonymous and occurred at a frequency <52% and with an average quality of <22. Closer inspection of this region revealed that the sequence quality was poor most likely due to it being repetitive, suggesting that these SNVs were not likely to
be 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 bp, 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 as resistant ≥2 µg/ml) or VAN (breakpoint considered as resistant ≥4 µg/ml) (data not shown). Early RT002 isolates A, B and C, showed complete susceptibility to rifampicin (RIF) and rifaximin (RFX) (Figs. S1a, S1b, S2a & S2b). However, RT002 isolates D (from the relapse sample) and E1 (post relapse sample) showed high resistance (≥ 256 µg/ml) to RIF and RFX (≥128 µg/ml), whilst 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). (Figs. S1a, S1b, S2a & S2b).

**Frequency of rpoB SNVs in all cultured A to G RT002 isolate samples**

To try and 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 the QIAxcel (Table 1). The PCR amplified DNA fragment was sent for Sanger sequencing and the sequences were checked for the above SNVs. The frequency of these SNVs in each isolate is detailed in Table 5.

**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 co-infection of two different PCR-ribotypes. At one
particular point during the infection the participant also, either acquired a RT002 isolate which was resistant to rifamycins (re-infection), 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 four weeks during which stool samples are collected. The participant is then followed up for another eight weeks during which more stool is collected. As this was a randomised 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 enrolment, 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 pre-enrolment 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 (re-infection) with resistance to RIF and RFX, or the original RT002 isolate evolved to become RIF and RFX resistant (≥256 µg/ml and ≥128 µg/ml respectively) relapse. Prior to sample D all 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 colonised with one population of non-resistant RT002 isolates at that 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 isolate 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-H revealed that the probable cause of RIF and RFX
resistance in isolate D, was a C>A SNV at position 1465bp 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 multi-drug resistant strains of *Mycobacterium
tuberculosis* (MTB) (15) within a small 23 amino acid region from position 511-533 (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 > 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 rifampicin (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 a genetically identical RT002 isolate (E1) to
isolate D, which also shared the same SNV change in *rpoB* and was also RIF and RFX
resistant (≥256 µg/ml and ≥128 µg/ml respectively ). The sample also contained another
ribotype (RT014 - 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 pre-relapse 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 a 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 (amongst 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, E1 and all other isolates. However, this 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). SNVs in this region may, therefore, 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 isolate A, B and C. Four of the SNVs were completely different to the previous isolate (E1) and isolate D. However, one SNV was again located in rpoB, at a different position (1475bp), resulting in an A>T change that caused an amino acid change from aspartic acid > valine at position 492 of the peptide. The substitution 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(≥ 4 µg/ml) and RFX (≥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 a 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 co-existed 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 fifteen 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 sub-populations of RT002 isolates could have potentially co-existed at this point. Amongst the sample G isolates, there were equal population 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 poignant 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 on the RAPID trial, as it may prompt clinicians to alter dosing regimen if the therapy was approved for use in patients suffering recurrence.
Conclusion

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 this 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 healthcare system.

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**Conflict of Interest**

The authors declare that there is no conflict of interest.
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References


identifies transmission events among patients with recurrent *Clostridium difficile* infections.


Table 1- Description and date of sample collection for participant 01008 on the RAPID trial

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Table 2 - PCR-ribotype of isolates obtained from participant 01008 stools samples

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*Samples obtained from Queens Medical Centre microbiology department
Table 3: PacBio Contig Assembly information from RT002 isolate A.
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<td>Aminobenzoyl-glutamate transport protein:c.210T&gt;A</td>
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<td>149</td>
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<td>32.74</td>
<td>Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1):c.479delA</td>
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<td>C</td>
<td>A</td>
<td>161</td>
<td>161</td>
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<td>DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1465C&gt;A p.Gln489Lys</td>
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<td>F</td>
<td>1181867</td>
<td>SNV</td>
<td>G</td>
<td>T</td>
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<td>174</td>
<td>98.85</td>
<td>33.01</td>
<td>FIG00512976: hypothetical protein:c.722C&gt;A p.Thr241Asn</td>
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<td>1861424</td>
<td>SNV</td>
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<td>SNV</td>
<td>A</td>
<td>T</td>
<td>204</td>
<td>207</td>
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<td>34.08</td>
<td>DNA-directed RNA polymerase beta subunit (EC 2.7.7.6):c.1475A&gt;T p.Asp492Val</td>
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Table 4 - Additional SNVs in genome of sequential RT002 isolates.

<table>
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<th>ID</th>
<th>SNV</th>
<th>G</th>
<th>T</th>
<th>Cnt</th>
<th>Typ</th>
<th>SNV freq</th>
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<tr>
<td>A</td>
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<td>D</td>
<td>T</td>
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<tr>
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<td>63.64</td>
<td>38.24</td>
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</table>

* (-): absence of SNV, (+): presence of SNV. Numbers in brackets are the frequency of the isolates with or with the SNV. Square brackets identify which SNV was present.

Table 5. Frequency of all cultured RT002 isolates with and without rpoB SNVs

<table>
<thead>
<tr>
<th>ID</th>
<th>Specimen</th>
<th>Number of Colonies typed</th>
<th>SNV present*</th>
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<tr>
<td>A</td>
<td>1st diagnostic sample</td>
<td>8</td>
<td>-</td>
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<td>B</td>
<td>2nd diagnostic sample</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>Week 0 (visit 1) RAPID sample</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>Relapse sample 1</td>
<td>5</td>
<td>+ (4) [C&gt;A]</td>
</tr>
<tr>
<td>E</td>
<td>Diagnostic sample post relapse</td>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>Week 4 (visit 3) RAPID sample</td>
<td>14</td>
<td>+ [A&gt;T (13)]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ [C&gt;A (1)]</td>
</tr>
<tr>
<td>G</td>
<td>Week 12 (Visit 5) RAPID sample</td>
<td>20</td>
<td>+ (10) [A&gt;T]</td>
</tr>
</tbody>
</table>

* (-): absence of SNV, (+): presence of SNV. Numbers in brackets are the frequency of the isolates with or with the SNV. Square brackets identify which SNV was present.
Figure 1- A simplified study timeline indicating the collection of stool samples for the participants involved in the RAPID trial.
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).

End of chaser therapy

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

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

Trial Enrolment/Randomisation

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).

End of chaser therapy

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

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

Trial Enrolment/Randomisation