Plasmid-mediated metronidazole resistance in *Clostridioides difficile*

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Metronidazole was until recently used as a first-line treatment for potentially life-threatening *Clostridioides difficile* (CD) infection. Although cases of metronidazole resistance have been documented, no clear mechanism for metronidazole resistance or a role for plasmids in antimicrobial resistance has been described for CD. Here, we report genome sequences of seven susceptible and sixteen resistant CD isolates from human and animal sources, including isolates from a patient with recurrent CD infection by a PCR ribotype (RT) 020 strain, which developed resistance to metronidazole over the course of treatment (minimal inhibitory concentration [MIC] = 8 mg L−1). Metronidazole resistance correlates with the presence of a 7-kb plasmid, pCD-METRO. pCD-METRO is present in toxigenic and non-toxigenic resistant (*n* = 23), but not susceptible (*n* = 563), isolates from multiple countries. Introduction of a pCD-METRO-derived vector into a susceptible strain increases the MIC 25-fold. Our finding of plasmid-mediated resistance can impact diagnostics and treatment of CD infections.
Clostridioides difficile (Clostridium difficile) is a Gram-positive obligate anaerobe capable of causing Clostridioides difficile Infection (CDI) upon disruption of the normal intestinal microbiota. Although it is one of the major causes of nosocomial infectious diarrhea, community-acquired CDI is becoming more frequent. CDI infection poses a significant economic burden with an estimated cost at €3 billion per year in the European Union and impairs the quality of life in infected individuals. The incidence of CDI has increased over the last two decades with outbreaks caused by epidemic types such as PCR ribotype (RT) 027 (NAP1/BI). CDI is not restricted to this type, however, as infections caused by RT001, RT002, RT014/020, and RT078 are frequently reported in both Europe and the United States. Metronidazole is used for the treatment of mild-to-moderate infections and vancomycin for severe infections, though vancomycin is increasingly indicated as a general first-line treatment. Fidaxomicin has recently also been approved for CDI treatment, but its use is limited by high costs. Fecal microbiota transplantation (FMT) is effective at treating recurrent CDI (rCDI) that is refractory to antimicrobial therapy. Reduced susceptibility and resistance to clinically used antimicrobials, including metronidazole, has been reported and this, combined with the intrinsic multiple drug-resistant nature of C. difficile, stresses the importance for the development of better diagnostics and new effective treatment modalities.

Routine antimicrobial susceptibility testing is generally not performed for C. difficile and consequently, reports of resistance to metronidazole are rare. Longitudinal surveillance in Europe found that 0.2% of clinical isolates investigated were resistant to metronidazole, but reported rates from other studies vary from 0 to 18.3%. These differences may reflect geographic distributions in resistant strains, or differences in testing methodology and breakpoints used. Moreover, metronidazole resistance can be unstable, inducible and heterogeneous. Finally, metronidazole resistance appears to be more frequent in non-toxigenic strains such as those belonging to RT010, which have a 7–9-fold increase in Minimal Inhibitory Concentration (MIC) values compared to RT001, RT027 and RT078.

Metronidazole is a 5-nitroimidazole prodrug that upon intracellular reductive activation induces cellular damage through nitro-radicals. It is not only used in the treatment of CDI, but also an important drug for treating parasitic infections and as prophylactic antimicrobial in for instance abdominal surgery. Mechanisms associated with metronidazole resistance described in other organisms include the presence of 5-nitroimidazole reductases (nim genes), altered pyruvate-ferredoxin oxidoreductase (PFOR) activity and adaptations to (oxidative) stress. The knowledge on resistance mechanisms in C. difficile is very limited, but may involve modulation of core metabolic and stress pathways as well.

Here, we present a case of a patient with rCDI due to an initially metronidazole susceptible (MTZS) RT020 strain, which developed resistance to metronidazole over time. We analyze the genome sequences of these toxigenic MTZs and metronidazole-resistant (MTZR) strains, together with 5 MTZs and 11 MTZR non-toxigenic RT010 strains. We identify pCD-METRO, a 7-kb plasmid conferring metronidazole resistance. This plasmid is internationally disseminated and also occurs in epidemic types. We thus report a clinically relevant phenotype associated with plasmid carriage in C. difficile.

Results

In-patient development of a metronidazole-resistant strain. A 54-year-old kidney–pancreas transplant patient with a medical history of Type I diabetes mellitus, vascular disease and a double lower-leg amputation was on hemodialysis when developing diarrhea. The patient was subsequently diagnosed with CDI and a toxigenic metronidazole sensitive (MIC = 0.25 mg L⁻¹) RT020 strain was isolated from the fecal material of the patient. Treatment with metronidazole was started, leading to initial resolution of the symptoms (Fig. 1). Two more episodes of CDI occurred during which the patient was treated primarily with vancomycin (but also metronidazole) prior to an FMT provided by the Netherlands Donor Feces Bank. At the start of the second episode a MTZs RT020 strain was once more isolated.

Three months after the first FMT, the patient once again developed bloody diarrhea and two more episodes of rCDI were diagnosed, which were treated with a vancomycin and a

Fig. 1 Timeline of the course of the antibiotic treatment and rCDI in the patient. Dates and timepoints on which treatment was initiated or stopped and C. difficile isolates were recovered are indicated above the timeline. (r)CDI was diagnosed when both a toxin enzyme-immune assay and nucleic acid amplification test were positive for C. difficile in combination with a physician’s assessment of symptoms consistent with CDI. Yellow highlighting indicates the time where pCD-METRO-positive MTZR C. difficile was isolated. Source data are provided as a Source Data file.
fidaxomicin regime. At two instances, RT020 strains were again isolated from the fecal material of the patient. Strikingly, these two clinical isolates were now phenotypically resistant to metronidazole (MIC = 8 mg L\(^{-1}\) as determined by agar dilution). Ultimately the patient was cured by a second FMT.

We hypothesized that the 1C1D episodes were due to clonal RT020 strains that persisted despite antimicrobial therapy and an FMT. Clonal MTZR\(^*\) and MTZS\(^*\) strains would allow us to determine the underlying genetic changes that resulted in metronidazole resistance. To determine the relatedness between these RT020 isolates whole-genome sequencing (WGS) was performed (Table 1). We also included two more MTZR\(^*\) RT020 strains and a non-related RT078 strain isolated from the same patient and 4 MTZS\(^*\) and 8 MTZR\(^*\) RT010 strains from our laboratory collection (Supplementary Data 1) to perform single-nucleotide polymorphism (SNP) analyses. Strains were considered resistant to metronidazole with MIC values >2 mg L\(^{-1}\). According to the EUCART epidemiological cutoff value\(^{25}\). All strains resistant to metronidazole (n = 12) showed cross-resistance to the nitroimidazole drug tinidazole.

Assembly of the MTZR\(^*\) RT020 strain IB136 (Supplementary Data 2) resulted in a genome of 4166362 bp with 57 contigs, and an average G + C-content of 28.5% (N50 = 263391 bp, mapping rate 98.97%). A BLAST comparison between this genome and the NCBI nr database showed that the genome is closest to the genome of strain LEM1\(^{33}\). As expected, 5/6 strains isolated from the patient (all RT020) showed 100% identity over the majority of all contigs, suggesting they are highly similar. All RT020 strains were found to be of multi-locus sequence type (ST) 2, consistent with data from others\(^{34}\). The sixth strain (IB137), was a clear outlier and was identified as being closest to the RT078 reference strain M12035\(^{35}\). This is consistent with another ribotype (RT078) and sequence type (ST11) assignment. All RT010 strains belonged to ST15.

Resistance does not correlate with a SNP. Previous studies analyzing the mechanism behind metronidazole resistance in C. difficile only studied a single isolate each\(^{29,36}\). We performed a core genome SNP analysis on selected strains (n = 18; Table 1), comparing MTZS\(^*\) (n = 6) and MTZR\(^*\) (n = 12) strains within and between the different PCR ribotypes (RT010, RT020 and RT078). The evolutionary rate of C. difficile has been estimated at 0–2 SNPs per genome per year, but might vary based on intrinsic (strain type) and extrinsic (selective pressure) factors\(^{37}\). Our analysis identified a single SNP in the MTZS\(^*\) RT020 (IB136), compared to the MTZR\(^*\) RT020 strains derived from the same patient, conclusively demonstrating that these strains are clonal. Considering the time of isolation of the susceptible and resistant isolates, this implies the MTZS\(^*\) RT020 strain most likely acquired metronidazole resistance. In contrast, between the MTZS\(^*\) and MTZR\(^*\) RT010 isolates (which come from diverse human and animal sources) 457 SNPs were detected. Moreover, RT010 and RT020 were separated by >25,000 SNPs.

The SNP identified in the RT020 strains discriminating the MTZS\(^*\) from the MTZR\(^*\) isolates is located in a conserved putative cobalt transporter (CbiN, IPR0035705). However, the SNP is not observed in the MTZS\(^*\) RT010 strains. Thus, metronidazole resistance is either multifactorial or not contained within the core genome. We did not investigate the contribution of this SNP to metronidazole resistance further.

MTZR\(^*\) C. difficile strains contain a 7-kb plasmid. Next, we investigated extrachromosomal elements (ECEs), which can include plasmids. Although plasmids containing antimicrobial resistance determinants have been described in Gram-positive bacteria, they appear to be more common in Gram-negatives\(^{38}\). Plasmids in C. difficile are known to exist, but no phenotypic consequences of plasmid carriage have been described to date\(^{39}\). The investigation of the pan-genome of all sequenced strains, including a prediction of ECEs predicted by an in-house pipeline similar to PLACNET\(^{40,41}\), showed a single contig that was present in all MTZS\(^*\) strains (4.6–19.27% of reads mapped, with a minimum of 479497), but absent from MTZS\(^*\) strains, of both RT010 and RT020 (0% of reads mapped with a maximum 327 reads). Circularization based on terminal repeats yielded a putative plasmid of 7056 bp with a G + C-content of 41.6% (Fig. 2a). Correct assembly was confirmed by PCR (Fig. 2b) and Sanger sequencing.

To confirm the circular nature of the contig, total DNA isolated from the MTZS\(^*\) RT010 strain IB136 before and after PlasmidSafe DNase (PSD, Epicenter)\(^{39}\) treatment was analyzed by PCR using primers specific for chromosomal DNA (gluD) and the putative plasmid (Fig. 2c). A positive signal for gluD was only observed in samples that had not been treated with PSD, demonstrating that PSD treatment degrades chromosomal DNA to below the detection limit of the PCR. By contrast, a signal specific for the putative plasmid was visible both before and after PSD treatment. Consequently, we conclude that our whole-genome sequence identified a legitimate 7-kb plasmid.

A total of eight open-reading frames (ORFs) were annotated on the plasmid (Fig. 2a). ORF1-5 encode a hypothetical protein (ORF1), a MobC-like relaxase/Arc-type ribbon-helix-helix (ORF2; PF05713), a Moba/VirD2 family endonuclease relaxase protein (ORF3; PF003432), a hypothetical protein with a MutS2 signature (ORF4), and a predicted replication protein (ORF5), respectively. ORF6 is a small ORF that is likely a pseudogene, and the remaining ORFs encode a metallohydrolase/oxidoreductase protein (ORF7; IPR001279) and a Tn5-like transposase gene (ORF8; PF13701). Intriguingly, ORF6 showed homology on the protein level to the N-5-nitroimidazole reductase (nim) gene nimB (33% identity, 54% positives over 61 amino acids) described in Bacteroides fragilis (CAA50578.1) and found in both metronidazole-resistant and susceptible isolates of anaerobic Gram-positive cocci\(^{42,43}\). The ORF lacks the region encoding the N-terminal part of the Nim protein, and the Phyre2-predicted protein structure shows it lacks the catalytic site residues. Of note, the plasmid sequences from all strains are highly similar. Compared to the plasmid of strain IB136, only strains IB143, IB144, and IB145 contained a single SNP resulting in a Y286S mutation within the Tn5-like transposase ORF (Supplementary Fig. 1).

Altogether, these results show that all of the MTZS\(^*\) strains, but none of the MTZS\(^*\) strains, sequenced in this study contain a plasmid, hereafter referred to as pCD-METRO (for plasmid from C. difficile associated with metronidazole resistance).

pCD-METRO is found in strains from different countries. Two clinical isolates with stable metronidazole resistance have been described and we evaluated the presence of pCD-METRO in the assembled genome sequences from these strains using BLAST\(^{29,36}\). We failed to identify pCD-METRO in the draft genome of a toxigenic NAP1 isolate that acquired stable metronidazole resistance through serial passaging under selection\(^{36}\). We did identify pCD-METRO (fragmented over multiple contigs) in the draft genome a non-toxigenic Spanish RT010 strain with stable metronidazole resistance (strain 7032989), whereas neither the reduced-susceptible strain nor the susceptible strain from the same study contained the plasmid\(^{29}\). We confirmed these results using PCR, as described for strain IB138 (Fig. 3; lanes SP), demonstrating pCD-METRO is indeed present in strain 7032989. These data show that the presence of pCD-METRO may explain
at least part of the cases of metronidazole resistance described in literature. We did not detect pCD-METRO in the sequence read archive in entries labeled as *C. difficile*, or otherwise.

Our observations above raise the question how prevalent pCD-METRO is in MTZR *C. difficile* isolates and if there is a bias towards specific types or geographic origins. As metronidazole resistance in *C. difficile* is rare, we expanded our collection of clinical isolates through our network (including the ECDC) (*n* = 76) and with selected strains from the Tolevamer (*n* = 42) and MODIFY (*n* = 46) clinical trials\(^4\)-\(^6\). To correct for interlaboratory differences in typing and antimicrobial susceptibility testing, all strains were retyped by ribotyping and tested for metronidazole resistance using agar dilution according to Clinical & Laboratory Standards Institute (CLSI) guidelines in our laboratory Brain-Heart Infusion (BHI) agar but resistant (MIC > 2 mg L\(^{-1}\)) based on the EUCAST epidemiological cutoff for metronidazole\(^3\), ND not determined by agar dilution, but only by E-test (Supplementary Fig. 2). The PCR ribotype determined at the LUMC standard PCR ribotyping.

### Table 1 Strains described in this study.

| Name | Characteristics | PCR ribotype | Toxin profile | MTZ resistance | Source | Reference |
|------|-----------------|--------------|---------------|----------------|--------|-----------|
| 630\(^a\)erm | Wild type | 012 | A + B + CDT- | 0.125 (S) | Laboratory | 49 |
| IB125 | 630\(^a\)erm pIB86 (pCD-METROshuttle); thi\(^R\) | 012 | A + B + CDT- | ≥8 (R) | Laboratory | This study |
| IB132 | pCD-METRO- | 020 | A + B + CDT- | 0.25 (S) | Human | This study |
| IB133 | pCD-METRO + | 020 | A + B + CDT- | 8 (R) | Human | This study |
| IB134 | pCD-METRO + | 020 | A + B + CDT- | 8 (R) | Human | This study |
| IB135 | pCD-METRO + | 020 | A + B + CDT- | 8 (R) | Human | This study |
| IB136 | pCD-METRO + | 020 | A + B + CDT+ | 1.25 (S) | Human | This study |
| IB137 | pCD-METRO - | 078 | A - B - CDT- | >8 (R) | Human | This study |
| IB138 | pCD-METRO - | 010 | A - B - CDT- | 1 (S) | Human | This study |
| IB139 | pCD-METRO - | 010 | A - B - CDT- | 0.25 (S) | Human | This study |
| IB140 | pCD-METRO - | 010 | A - B - CDT- | 0.125 (S) | Human | This study |
| IB141 | pCD-METRO - | 010 | A - B - CDT- | 0.125 (S) | Human | This study |
| IB142 | pCD-METRO - | 010 | A - B - CDT- | 0.125 (S) | Human | This study |
| IB143 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Animal | This study |
| IB144 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Animal | This study |
| IB145 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Animal | This study |
| IB146 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Animal | This study |
| IB147 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Animal | This study |
| IB148 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Animal | This study |
| IB149 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Animal | This study |
| IB151 (P016134) | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Unknown | 81 |
| IB30 | 630\(^a\)erm pIB20 (pCD6 replicon, P\(_{CDT}\)\(^{i}\)sucl\(_{c}\)pp); thi\(^R\) | 012 | A + B + CDT- | 0.25 (S) | Laboratory | This study |
| IB90 | 630\(^a\)erm pIB80 (pCD-METRO replicon, P\(_{CDT}\)\(^{i}\)sucl\(_{c}\)pp); thi\(^R\) | 012 | A + B + CDT- | 0.125 (S) | Laboratory | This study |
| LUMCM18 0002 | pCD-METRO | 020 | A + B + CDT- | ND (S) | Human | This study |
| LUMCM19 0348 | pCD-METRO + | 020 | A + B + CDT- | ND (R) | Human | This study |
| LUMCM19 0830 | pCD-METRO - | 010 | A - B - CDT- | 4 (R) | Unknown | This study |
| LUMCM19 0880 | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Unknown | This study |
| LUMCM19 0970 (7032989) | pCD-METRO + | 010 | A - B - CDT- | >8 (R) | Unknown | This study |
| LUMCM19 0960 | pCD-METRO + | 027 | A + B + CDT+ | >8 (R) | Human | 46 |

\(\text{MIC} > 8 \text{ mg L}^{-1}\) (Table 1). A PCR on PSD-treated chromosomal DNA isolated from these strains yielded a positive signal using primers targeting the plasmid, but not the chromosome (Fig. 3, lanes PL/CZ/DE), demonstrating all three strains contain pCD-METRO. WGS showed that pCD-METRO in strain LUMCM19 0960 was identical to that of strain IB136, whereas LUMCM19 0830 contained a single SNP resulting in a D131N substitution in the plasmid, but not the chromosome (Fig. 3, lanes PL/CZ/DE). Demonstrating all three strains contain pCD-METRO. Using E-tests, we found this strain to be susceptible to thiamphenicol resistance, 5 susceptible (MIC < 2 mg L\(^{-1}\)), R resistant (MIC > 2 mg L\(^{-1}\)) based on the EUCAST epidemiological cutoff for metronidazole\(^3\), ND not determined by agar dilution, but only by E-test (Supplementary Fig. 2).

*thi*\(^R\) thiamphenicol resistance, S susceptible (MIC < 2 mg L\(^{-1}\)), R resistant (MIC > 2 mg L\(^{-1}\)) based on the EUCAST epidemiological cutoff for metronidazole\(^3\), ND not determined by agar dilution, but only by E-test (Supplementary Fig. 2).
MIC ≥ 8 mg L⁻¹ in agar dilution (22/22). By contrast, all susceptible isolates (n = 563) lacked pCD-METRO.

Taken together, our results show that pCD-METRO is internationally disseminated and can explain metronidazole resistance in both non-toxigenic- and toxigenic isolates of C. difficile, including those belonging to epidemic ribotypes such as RT027.

pCD-METRO is likely acquired via horizontal gene transfer. Our whole-genome sequence analysis suggested the acquisition of pCD-METRO by a toxigenic RT020 strain during treatment of rCDI. We made use of longitudinal fecal samples that were stored during treatment to investigate the presence of pCD-METRO in total fecal DNA at various timepoints. Total DNA derived from the fecal sample harboring the MTZR RT020 was positive for the presence of pCD-METRO (Fig. 5). This indicates that pCD-METRO was present in the gut reservoir of the patient. Post-FMT, pCD-METRO was no longer detected in total fecal DNA, suggesting that the fecal transplant reduced levels of pCD-METRO containing C. difficile and/or the donor organism to below the limit of detection of the assay. Fecal samples were stored in the absence of cryoprotectant and as a result we were unable to reculture the possible donor organism.

Although we cannot exclude the possibility that the MTZR RT020 strain was isolated, our results indicate that pCD-METRO was most likely acquired through horizontal gene transfer between the MTZ⁰ C. difficile strain and an as-of-yet uncharacterized donor organism in the gut of the patient.

PCR-based identification of metronidazole-resistant strains. We implemented a PCR targeting pCD-METRO in our routine surveillance and ad hoc typing, as part of the Dutch National Reference Laboratory (NRL) for C. difficile. In the period February-August 2019, we characterized 721 strains by ribotyping, and identified a single pCD-METRO-positive strain (LUMCM19 0348) by PCR. These preliminary data suggest a prevalence of <0.14% in an endemic setting in the Netherlands. The identified strain belonged to RT020 and was confirmed to be MTZ⁰ in an E-test on BBA (Supplementary Fig. 2). As described for the patient case above, we were able to identify an earlier RT020 isolate from the same patient (LUMCM18 0002) that was pCD-METRO negative and MTZ⁰ (Supplementary Fig. 2). WGS revealed that the susceptible and resistant strains were identical (0 SNPs difference), but differed as expected in carriage of pCD-METRO. pCD-METRO in the MTZ⁰ isolate contained two SNPs compared to the plasmid of strain IB136; a G > A conversion located intergenically between ORF6 and ORF7, and a chromosomal locus (gluD) before and after PlasmidSafe DNase treatment. Source data are provided as a Source Data file.

Fig. 2 pCD-METRO is a 7-kb plasmid. a Structure of plasmid pCD-METRO and its ORFs. The two innermost circles represent GC content (outer circle) and GC skew (innermost circle) (both step size 5 nt and window size 500nt; average above in yellow, average below in purple). The unique HaeIII site used to construct pCD-METRO shuttle (see methods) is indicated. b Gene-specific PCR products amplifying regions of ORFs 6 (lane 1 + 2), ORF5 (lane 3), ORF7 (lane 4) and ORF3 (lane 5), and a chromosomal locus (gluD) (c) The product of plasmid-specific amplification (targeting ORF6, pCD) or chromosomal-specific amplification (gluD) before and after PlasmidSafe DNase treatment. Source data are provided as a Source Data file.

Fig. 3 pCD-METRO is internationally disseminated. PCR analysis of strains 7032989 (RT010, Spain) (SP), LUMCM190960 (RT027, Poland) (PL), LUMCM19 0880 (RT010, Czech Republic) (CZ), and P016134 (RT010, Germany) (DE). The product of plasmid-specific amplification (targeting ORF8) or chromosomal-specific amplification (gluD) before and after PlasmidSafe DNase treatment are shown. Source data are provided as a Source Data file.
Source data are provided as a Source Data file without pCD-METROshuttle to 2 mg L⁻¹ or higher upon spread onto either Brucella Blood Agar (BBA) plates, or onto BHIY/CDSS agar plates (BHI). E-tests were placed, and plates were incubated for 48 h before imaging. In all, 2 mg L⁻¹ is the EUCAST epidemiological cutoff for metronidazole that was used to define resistance in this study⁵². E-test values for the indicated strains are shown next to their respective panels. The images represent three independent repeats with a single replicate per condition. Source data are provided as a Source Data file.

Our data suggests that selection by metronidazole is crucial in the acquisition of, or selection for, pCD-METRO containing C. difficile.

**pCD-METRO confers metronidazole resistance in C. difficile.**

Above, we have clearly established a correlation between the presence of pCD-METRO and metronidazole resistance. Next, we sought to unambiguously demonstrate that acquisition of pCD-METRO, and not any secondary events, lead to metronidazole resistance. To generate isogenic strains with or without pCD-METRO, we introduced a shuttle module in the unique HaellIII restriction site of the plasmid and introduced the resulting vector, pCD-METROshuttle (pIB86; Supplementary Fig. 3), into the RT012 laboratory strain 630Δerm using standard methods.⁴⁹. Metronidazole E-tests showed a reproducible 15-to-20-fold increase in the MIC from 0.064/0.19 mg L⁻¹ for the strain without pCD-METROshuttle to 2–4 mg L⁻¹ for the strain with pCD-METROshuttle (Fig. 6). These results were confirmed using agar dilution, that showed a > 24-fold increase (>5 doubling dilutions) from 0.125–0.25 mg L⁻¹ to 8 mg L⁻¹ or higher upon introduction of pCD-METROshuttle (Table 1).

As controls, we included the MTZ⁵ (IB132) and a MTZ⁷ (IB133) RT020 strain isolated from the patient. In agreement with the MIC values determined by agar dilution (MIC = 0.25 mg L⁻¹ and MIC = 8 mg L⁻¹), these isolates showed a MIC corresponding to those observed for the MTZ⁵ and MTZ⁷ RT012 isolates, respectively (Fig. 6).

Overall, our results show that acquisition of pCD-METRO is sufficient to raise the MIC of C. difficile to values greater than the epidemiological cutoff value defined by EUCAST.

**pCD-METRO contains a high copy-number replicon.**

Read depth of pCD-METRO in our WGS data indicates an estimated copy number of 100–200, in stark contrast with the pCD6 replicon commonly used in shuttle vectors for C. difficile (copy number 4–10).⁵⁰. We wanted to establish the functionality of the predicted replicon and determine the copy number sustained by this replicon in RT012 strains.

A pRPF185-based vector (pIB80) was constructed in which the conventional pCD6 replicon was replaced by a 2-kb DNA fragment of pCD-METRO that includes ORF5, encoding the putative replication protein (Supplementary Fig. 4). Transconjugants containing this vector were readily obtained in the RT012 laboratory strain 630Δerm, demonstrating this region contains a functional replicon.

Next, we compared the relative copy number of the plasmids in overnight cultures by quantitative PCR (qPCR)⁵⁰. Based on the ratio of plasmid-locus catP to the chromosomal locus rpoB, the copy number of pCD6-replicon vector was ~4, concordant with results of others.⁶. By contrast, the copy number of vectors with the pCD-METRO replicon ranges from ~25 (for pIB80, in IB90) to 38 (pCD-METROshuttle, in IB125) (Fig. 7a). We hypothesized that a higher plasmid copy number would also lead to more copies of the resistance marker on the plasmid and thus to a possible increase of resistance to the corresponding antibiotic. Indeed, a strain harboring a catP-containing plasmid with the pCD-METRO replicon is detectable in fecal total DNA from the same sample from which a MTZ⁷ laboratory strain 630Δerm was isolated using standard methods.⁴⁹. By contrast, the copy number of vectors with the pCD6-replicon vector was ~4, concordant with results of others.⁶. Of note, the copy numbers are similar to those observed for the MTZ⁷ RT012 isolates, respectively (Fig. 6). Overall, our results show that acquisition of pCD-METRO is sufficient to raise the MIC of C. difficile to values greater than the epidemiological cutoff value defined by EUCAST.

**pCD-METRO is detectable in fecal total DNA.**

pCD-METRO is detectable in fecal total DNA from the same sample from which a MTZ⁷ RT020 C. difficile was isolated. Shown are the results from a PCR targeting ORF6, but similar results were obtained for other plasmid-specific primer sets. Source data are provided as a Source Data file.

As controls, we included the MTZ⁵ (IB132) and a MTZ⁷ (IB133) RT020 strain isolated from the patient. In agreement with the MIC values determined by agar dilution (MIC = 0.25 mg L⁻¹ and MIC = 8 mg L⁻¹), these isolates showed a MIC corresponding to those observed for the MTZ⁵ and MTZ⁷ RT012 isolates, respectively (Fig. 6).

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Overall, our results show that acquisition of pCD-METRO is sufficient to raise the MIC of C. difficile to values greater than the epidemiological cutoff value defined by EUCAST.⁵².
replicon demonstrates a growth advantage over a strain harboring a similar plasmid with the pCD6 replicon when exposed to high levels (256 mg L\(^{-1}\)) of thiamphenicol. No significant difference in growth was observed at low concentrations (20 mg L\(^{-1}\)) of thiamphenicol (Fig. 7b). As pIB80 containing strains are not MTZ\(^R\) (Fig. 6), resistance to metronidazole is not mediated by a higher copy number plasmid per se, but is dependent on a determinant specific to pCD-METRO.
A difference between the read-depth estimate and the qPCR can be explained by technical bias or differences in strain background. Nevertheless, our experiments clearly demonstrate that the pCD-METRO replicon sustains plasmid levels that are ~10-fold greater than that of currently used replicons.

We investigated whether the relatively high copy number of pCD-METRO imposes a metabolic cost, by evaluating the growth of strains with and without plasmid in the absence or presence of varying concentrations of metronidazole (Fig. 8). In the absence of metronidazole, the growth of susceptible and resistant strains of both RT012 and RT020 is indistinguishable (Fig. 8a). This was not due to loss of pCD-METRO from the resistant strain, as all colonies tested after the growth experiment had retained the plasmid. With increasing amounts of metronidazole, susceptible strains show a clear growth defect already at the lowest concentration of metronidazole tested (0.125 mg L⁻¹), whereas resistant strains do not markedly differ in growth from the control culture at concentrations below the epidemiological cutoff (2 mg L⁻¹) (Fig. 8b, c). These values are in agreement with the E-tests performed on the same media (Fig. 6). We conclude that carriage of the plasmid does not affect growth rate in the absence of metronidazole, despite the high copy number, and confers a clear growth advantage in the presence of metronidazole.

We attempted to cure metronidazole-resistant strains of pCD-METRO using serial passaging on non-selective liquid or solid medium. Despite our efforts (Supplementary Methods), we failed to obtain colonies that lacked pCD-METRO, even after non-selective culturing for >50 generations. We hypothesize that the high copy number of pCD-METRO contributes to its stability.

Altogether, these results demonstrate that pCD-METRO encodes a functional replicon that is responsible for a high copy number in C. difficile and is efficiently maintained in the absence of selection.

Discussion
In this study, we describe a plasmid linked to resistance against a clinically relevant antimicrobial in C. difficile. We show that the high copy number plasmid pCD-METRO is internationally disseminated, present in diverse PCR ribotypes—including those known to cause outbreaks—and we provide evidence for the possible horizontal transmission of the plasmid. Our data suggest a possible prevalence of <0.14% (1/721; endemic) to 3.9% (22/563; collection enriched for metronidazole-resistant strains) of the plasmid, in line with previous observations.

Although the presence of plasmids in C. difficile has been known for many years, no phenotypes associated with plasmid carriage have been described. We show that introduction of pCD-METRO in susceptible strains leads to stable and medium-independent metronidazole resistance. Plasmids may play a broader role in antimicrobial resistance of C. difficile. A putative plasmid containing the aminoglycoside/linezolid resistance gene cfrC was recently identified in silico, but in contrast to our work no experiments were presented to verify the contig was in fact a plasmid conferring resistance. The presence of an antimicrobial resistance gene does not always result in resistance, and DNA-based identification of putative resistance genes without phenotypic confirmation may lead to an overestimation of the resistance frequencies.

At present, it is unknown which gene(s) on pCD-METRO are responsible for metronidazole resistance. Nitroimidazole reductase (nim) genes have been implicated in resistance to nitroimidazole type antibiotics. Although the presence of a truncated nim gene on pCD-METRO is intriguing, we do not believe this gene to be responsible for the phenotype for several reasons. Structural modeling of the predicted protein shows that it lacks the catalytic domain, and introduction of the ORF under the control of an inducible promoter (Supplementary Data 1 and Supplementary Table 2) did not confer resistance in our laboratory strain. Moreover, the RT027 strain R20291 encodes a putative 5-nitroimidazole reductase (R20291_1308) and is not resistant to metronidazole, implying the presence of a nim gene is not causally related to metronidazole resistance in C. difficile as also noted by others. Further research is necessary to determine the mechanism for metronidazole resistance in C. difficile conferred by pCD-METRO, and to investigate the contribution of the high copy number (Fig. 6) to the resistance phenotype.

Our work, combined with that of others, suggests that metronidazole resistance is multifactorial and other factors than pCD-METRO can cause or contribute to metronidazole resistance in C. difficile. For instance, pCD-METRO may not explain low level resistance, heterogeneous resistance, or stable resistance resulting from serial passaging of isolated strains under metronidazole selection. We also observed that absolute MIC values in agar dilution experiments differed between MTZ(R) isolates of different PCR ribotypes despite carriage of pCD-METRO, suggesting a contribution of chromosomal or extrachromosomal loci to absolute resistance levels. Although the SNP we identified in the RT020 strain IB136 was not found in other MTZ(R) strains of RT010/RT020/RT027, we cannot exclude that it contributes to the resistance in this particular strain. We also observed strong medium-dependent effects: the MICs obtained on BBA are generally higher than those on BHI (Fig. 4), underscoring the importance of using standard conditions for susceptibility testing. Notably, for at least one RT010 strain (LUMCM M19 0830) this led to conversion of the resistance phenotype. Clearly, medium components (possibly iron or heme) contribute to metronidazole resistance. This is in line with suggested metabolic changes in MTZ(R) strains that do not harbor pCD-METRO.

The pCD-METRO plasmid is internationally disseminated (Table 1 and Fig. 3), although further research is necessary to determine how prevalent the plasmid is in metronidazole-resistant C. difficile isolates. This study attempted to enrich for metronidazole-resistant strains as this resistance is scarce in C. difficile. We received strains that were reported to be metronidazole-resistant by the senders. However, when performing antimicrobrial susceptibility testing for these strains with agar dilution in our own laboratory, virtually all strains had MIC values below the epidemiological cutoff value from EUCAST for metronidazole and were considered susceptible. For this reason, we ended up having very few metronidazole-resistant isolates of other PCR ribotypes than RT010 (RT020 and RT027). It is not entirely clear how these differences came into existence. Depending on handling of the sample material and freeze-thawing cycles, it is possible that inducible metronidazole resistance, unrelated to pCD-METRO, was initially measured and that this was lost after storage and lack of selection. Considering the apparent stability of pCD-METRO, we do not think that the discrepancies are due to loss of the plasmid during passaging on non-selective media.

Based on the apparent stability of the pCD-METRO plasmid, we consider acquisition rather than loss of pCD-METRO a more likely explanation for the clonality of the RT020 patient isolates. As C. difficile has not been demonstrated to be naturally competent for transformation, this suggests that pCD-METRO may be transmissible. Horizontal gene transfer is consistent with the observed level of sequence conservation between the RT010, RT020, and RT027 pCD-METRO plasmids sequenced in this study (Supplementary Fig. 1). Nevertheless, we failed to demonstrate intraspecies transfer with different donor and recipient strains of C. difficile under laboratory conditions (Supplementary Methods), suggesting that the strains tested (or possibly the species) lack a determinant required for transfer. Together with its size and the presence of mobilization genes (Fig. 2a), we
therefore hypothesize that pCD-METRO is mobilizable from an uncharacterized donor organism. We screened the complete sequence read archive of the NCBI (paired-end Illumina data) for potential sources of the plasmid, but failed to identify any entries with reliable mapping (>1% of data) to pCD-METRO.

As more reports are published associating metronidazole with higher treatment failure, a shift in consensus for using metronidazole as first-line treatment for mild to moderate CDI is occurring. The reason for treatment failure is currently unknown, but no correlation between MTZ$^R$ C. difficile isolates and treatment failure seems to exist. We also observed that clinical isolates from subjects in which metronidazole treatment failed, were metronidazole susceptible and pCD-METRO negative (Supplementary Data 1). These observations, however, do not rule out a role for (other) metronidazole-resistant organisms, potentially harboring pCD-METRO, in treatment failure. Indeed, levels of metronidazole at the end of the colon and in fecal material are low (most likely due to absorption of the drug in the small intestine in the absence of diarrhea), and members of the microbiota involved in inactivation or sequestering of metronidazole may allow for growth of MTZ$^S$ species.

Our observation of a putatively transmissible plasmid associated with metronidazole resistance in C. difficile and the gut microbiome has implications for clinical practice. First, it warrants a further investigation into the role of the plasmid in metronidazole treatment failure in CDI, and—more broadly—in metronidazole resistance of organisms other than C. difficile. Second, though this work can be seen as one more argument against the use of metronidazole as a first-line treatment of CDI, detection of the plasmid in fecal material might also guide treatment decisions.

**Fig. 8 The effects of carriage of pCD-METRO on growth.**

- **a** Growth as monitored by an increase in optical density at 600 nm (OD600) of various strains in the absence of metronidazole.
- **b** Growth of RT012 strains in the presence of various concentrations of metronidazole [mtz].
- **c** Growth of RT020 strains in the presence of various concentrations of metronidazole [mtz]. Individual measurements are shown as semi-transparent dots. The median of the replicates is shown as a solid line. Source data are provided as a Source Data file.
Acrosomal sensitivity testing and ribotyping. All strains were characterized by standardized PCR ribotyping and tested multiple times for metronidazole resistance by agar dilution according to CLSI guidelines, with the inclusion of appropriate microbiological controls.\textsuperscript{74,75} No formal breakpoints have been defined for metronidazole resistance in \textit{C. difficile}; here we use the EUCAST epidemiological cutoff of 2 mg L\textsuperscript{-1} to define resistance.\textsuperscript{35} Details of all strains and their characteristics are available in Table 1 and Supplementary Table 2. F. prausnitzii (BioMerieux), bacterial suspensions corresponding to 10 McFarland turbidity were applied on BHI agar supplemented with 0.5% yeast extract (Sigma-Aldrich) and \textit{Clostridium difficile} Selective Supplement (CDSS, Oxoid) or on Brucella Blood agar plates without antimicrobials. MIC values were read after 48 h of incubation as recommended by CLSI.\textsuperscript{37}

### Methods

#### Strains

The strains sequenced as part of this study come from various sources. Twenty-one strains were isolated from a single patient at the Leiden University Medical Center (LUMC) or derived from the collection of human and animal isolates of the Dutch NRL for \textit{C. difficile}, which is hosted at the LUMC. Informed consent (approved by the Medical Ethical Committee of the LUMC) was given for the use of the patient samples for research purposes. Other clinical isolates (\( n = 567\)) were obtained through the NRL and partners in the European Center for Disease Prevention and Control (ECDC), or were derived as per Table 1 and Supplementary Table 2. F. prausnitzii (BioMerieux), bacterial suspensions corresponding to 10 McFarland turbidity were applied on BHI agar supplemented with 0.5% yeast extract (Sigma-Aldrich) and \textit{Clostridium difficile} Selective Supplement (CDSS, Oxoid) or on Brucella Blood agar plates without antimicrobials. MIC values were read after 48 h of incubation as recommended by CLSI.\textsuperscript{37}

#### Molecular biology techniques

\textit{Escherichia coli} was cultured aerobically at 37 °C in Luria–Bertani (LB) broth, supplemented with 20 mg L\textsuperscript{-1} chloramphenicol and 50 mg L\textsuperscript{-1} kanamycin when appropriate. \textit{C. difficile} was cultured in BHI supplemented with 0.5% yeast extract, CDSS and 20 mg L\textsuperscript{-1} thiamphenicol when appropriate, in a Don Whitley VA-1000 workstation (100% CO\textsubscript{2}, 10% H\textsubscript{2}O and 80% N\textsubscript{2} atmosphere). Plasmids and oligonucleotides are listed in Supplementary Tables 1 and 2, respectively. pB86 (pCDMETRO\textsubscript{shuttle}, Supplementary Fig. 3 and Supplementary File 1) was constructed using Gibson assembly using \textit{Haell}\textsubscript{I}-linearized pCD-METRO and a fragment from pRPF185 (Addgene\textsuperscript{106367}). This fragment was obtained by PCR, and contained the requirements for maintenance in, and transfer from, the restricted Escherichia coli plasmid METRO backbone using a homemade Gateway\textsuperscript{TM} Wizard Master Mix Kit (Promega, Madison, WI). \textit{Tag} Ligase (New England Biolabs), 0.025 U µL\textsuperscript{-1} Phusion polymerase (Bioké), 5 µmol L\textsuperscript{-1} polyethylene glycol (PEG-8000), 100 mM MgCl\textsubscript{2}, 100 mM Tris-Cl pH 7.5, 10 mM dithiorthreitol, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, and 1 mM β-nicotinamide adenine dinucleotide) for 30 min at 50 °C and transformed into \textit{E. coli} DH\textsubscript{50} (Supplementary Fig. 4 and Supplementary File 2) was constructed by ATUM (Newark, CA) and contains a pCD-METRO derived fragment inserted in between the \textit{KpnI} and \textit{NcoI} sites of pRPF185.\textsuperscript{76} Transfer of plasmids from \textit{E. coli CA434} to \textit{C. difficile 630Δarm}\textsuperscript{39} was done using standard methods. Routine DNA extractions were performed using the Nucleospin Plasmid EasyPure (Macherey-Nagel) and \\textit{DNAse} and Tissue (Qiagen) kits after incubating the cells in an enzymatic lysis buffer according to instructions of the manufacturers.

#### Isolation of cloning-grade pCD-METRO

Plasmid pCD-METRO was extracted from 400 mL of culture containing the MT\textsubscript{22} strain IB136 using the Macherey–Nagel Nucleospin Xtra Midi Kit. Using the \\textit{CoCl\textsubscript{2}} plasmid purification method this plasmid prep was further cleansed as summarized hereafter. pCD-METRO plasmid prep was used to TE buffer (10 mM Tris pH = 8.0, 1 mM EDTA) and \\textit{CoCl\textsubscript{2}} was added to a density of 1 g mL\textsuperscript{-1}. Approximately 220 µL mL\textsuperscript{-1} ethidium bromide was added to this solution after which samples were spun down in a Beckman Coulter Optima XE-90 ultracentrifuge for 17 h at 65 000 rpm, 20 °C. Bands were visualized with ultraviolet (UV) light and plasmid DNA was collected by withdrawing the lowest of the two resulting bands with an 18 gauge needle. To remove ethidium bromide 1x vol/vol 5 M NaCl saturated N-butanol was used to remove the upper (purple) phase after centrifugation. Samples were ethanol precipitated twice prior to resuspendng purified plasmid DNA in TE buffer.

#### Plasmid copy number determination

Real-time quantitative PCR (qPCR) experiments were performed essentially as described.\textsuperscript{35} In short, total DNA was isolated after 17 h of growth using a phenol-chloroform extraction protocol and diluted to a concentration of 10 ng µL\textsuperscript{-1}. Four microliters of the diluted DNA sample was added to 6 µL of a mixture containing SYBR Green Supermix (Bio-Rad) and gene-specific primers (0.4 µM total) for a total volume of 10 µL per well. Gene-specific primers used were targeting \textit{rpoB} (chromosome) and \textit{catB} (plasmid) and copy number was calculated using the \(ΔC_{\text{t}}\) method. Experiments were performed in triplicates on three different technical replicates. Statistical significance was calculated using two-way analysis of variance (ANOVA) and Tukey’s test for multiple comparisons (Prism 8, GraphPad)(Supplementary Table 3).

#### Preparation of figures

Agarose gel and Eth images were acquired using a Bio–Rad GelDoc XR, processed in Adobe Photoshop CC 2018 (Adobe). Plasmid maps were generated using Artemis and DNAPlotter.\textsuperscript{78,79} Graphs were generated using PloTrix (https://huygens.science.uva.nl/PloTrix/2) and PlotsOfData

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(i.e., pCD-METRO harboring patients are excluded from metronidazole treatment). And finally, screening by PCR of fecal donor material intended for FMT might be desirable to reduce the possibility of transferring pCD-METRO from hitherto uncharacterized donor organisms to \textit{C. difficile} in patients.
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Author contributions

Performed experiments: I.M.B., E.S., I.M.J.G.B.-S., J.C., E.J.K. Analyzed data: R.B., B.V.H.H., M.H., E.S., C.H., R. Peets, A. Gupta, A., E.J.K. Contributed patient samples and metadata: E.M.T., E.I.K. Contributed reagents: R.B., B.V.H.H. Drafted manuscript: I.M.B., B.V.H.H., J.C., E.J.K., W.K.S. All authors edited and approved the final version of the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Competing interests

W.K.S. has performed research for Cubist and has received speaker fees from Promega. E.J.K. has performed research for Cubist, Novartis, and Qiagen, and has participated in advisory forums of Astellas, Optimer, Actelion, Pfizer, Sanofi Pasteur, and Seres Therapeutics. E.J.K., B.V.H.H., and E.M.T. currently hold an unrestricted research grant from Vedanta Biosciences. These companies had no role in the design and execution of the experiments for this study or the decision to publish. M.B., E.S., R.B., C.H., J.C., and I. M.J.G.B.-S.: none to declare. Part of this data has been presented at the International Clostridium difficile Symposium 2018, the Scientific Spring Meeting of the KNVM/JVMM 2019, ECCMID 2019, CLOSPATH11 (2019) and Bacterial Morphogenesis, Survival and Virulence: Regulation in 4D (2019).

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-14382-1.

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