



Antimicrobial resistance in *Clostridioides difficile*

Keeley O'Grady¹ · Daniel R. Knight^{1,2} · Thomas V. Riley^{1,2,3,4}

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Abstract

Antimicrobial resistance (AMR) in *Clostridioides difficile* remains a significant threat to global healthcare systems, not just for the treatment of *C. difficile* infection (CDI), but as a reservoir of AMR genes that could be potentially transferred to other pathogens. The mechanisms of resistance for several antimicrobials such as metronidazole and MLS_B-class agents are only beginning to be elucidated, and increasingly, there is evidence that previously unconsidered mechanisms such as plasmid-mediated resistance may play an important role in AMR in this bacterium. In this review, the genetics of AMR in *C. difficile* will be described, along with a discussion of the factors contributing to the difficulty in clearly determining the true burden of AMR in *C. difficile* and how it affects the treatment of CDI.

Keywords *Clostridioides difficile* · Antimicrobial resistance · Genome · Phenotype · Genetics · Epidemiology

Introduction

Antimicrobial resistance (AMR) is a major threat to global public health that requires urgent action across all government sectors and society [1]. *Clostridioides* (*Clostridium*) *difficile* is a significant AMR pathogen, ranked by the US Centers for Disease Control and Prevention in 2013 as an urgent AMR threat costing the US healthcare system ~USD1 billion annually [2]. Despite some improvements over the last decade, *C. difficile* remains an urgent threat, with 223,900 cases of *C. difficile* infection (CDI), and 12,800 deaths in the USA in 2017 [3].

One of the features of *C. difficile* that enables it to flourish when other bacteria have perished is its extensive repertoire

of AMR, which includes resistance to lincomycin and clindamycin, aminoglycosides, tetracyclines, macrolides, cephalosporins, penicillins and fluoroquinolones [4]. The highest rates of resistance were to ciprofloxacin, levofloxacin, erythromycin and fusidic acid [5]. As with anaerobes in general, *C. difficile* typically has higher rates of resistance to older-generation antimicrobials than newer, e.g. ciprofloxacin (2nd generation, 99% resistant) vs moxifloxacin (4th generation, 36% resistance) [6]. Paradoxically, CDI is both induced by antimicrobials and then treated with antimicrobials: Overall exposure to antimicrobials was associated with a 60% increased risk of CDI [7]. Some antimicrobials are more commonly associated with inducing CDI than others, including cephalosporins, clindamycin, carbapenems, trimethoprim/sulphonamides, erythromycin, broad-spectrum β -lactams, ampicillin/amoxicillin and later generations of fluoroquinolones [4, 6–10].

In particular, antimicrobials that reduce the anaerobic microflora of the gut (e.g. *Bacteroides* spp. and *Bifidobacterium* spp.) while sparing certain facultative anaerobes (e.g. *Enterococcus* spp.) appear to be strong inducers of CDI [11]; 3rd-generation cephalosporins have the highest attributable risk, whereas clindamycin has the highest relative risk of inducing CDI [7]. Other antimicrobials such as tetracyclines and aminoglycosides are not strongly associated with the induction of CDI despite frequent resistance [7, 11]. The association of macrolides with CDI induction is unclear, with research both for [10] and against [7, 9] and their use

✉ Thomas V. Riley
thomas.riley@uwa.edu.au

¹ Centre for Biosecurity and One Health, Harry Butler Institute, Murdoch University, Murdoch, Western Australia, Australia

² Marshall Centre for Infectious Diseases Research and Training, School of Biomedical Sciences, The University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, WA 6009, Australia

³ School of Medical and Health Sciences, Edith Cowan University, Joondalup, WA, Australia

⁴ Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia

in combination therapy potentially masking associations [9, 11]. Resistance mechanisms in *C. difficile* include chromosomal resistance genes, mobile genetic elements (MGEs), alterations to metabolic pathways and antimicrobial targets, and biofilms [6], with some strains showing multidrug resistance (MDR), defined as resistance to three or more antimicrobial agents (see Tables 1 and 2). In this review, the genetics of AMR in *C. difficile* will be described, along with a discussion of the factors contributing to the difficulty in clearly determining the true burden of AMR in *C. difficile* and what it means for CDI.

Resistance to antimicrobials used to treat CDI

Of particular concern is the development of resistance to recommended treatment drugs. Since the removal of metronidazole as a recommended 1st line treatment in North America and Europe, vancomycin and fidaxomicin are now recommended for both mild and severe cases of CDI [12, 13] and, while rare, resistance or treatment failure has been reported for both [5]. In recent years, increasing rates of treatment failure, reported inferior performance compared to vancomycin and reports of neurotoxicity have relegated metronidazole to use only when vancomycin and fidaxomicin are not available, and then only for single courses [13]. However, metronidazole remains recommended in Australasia [14] and by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) for mild/moderate cases [15]. Rifaximin (a rifamycin) has proven efficacy and reduces recurrence [8] but is vulnerable to the rapid development of resistance, while a novel class antimicrobial, ridinilazole, is showing promising performance in ongoing phase III trials [16].

Vancomycin

Vancomycin is a glycopeptide antimicrobial that inhibits cell wall synthesis in Gram-positive bacteria by inhibiting peptidoglycan biosynthesis. Minimum inhibitory concentration (MIC) breakpoints vary from 2 mg/L (EUCAST) to 4 mg/L (CLSI) [17, 18]. A common vancomycin resistance mechanism in many bacteria is *van* gene clusters which alter the D-Ala-D-Ala peptidoglycan terminus and reduce its affinity for vancomycin [19]. Despite the presence of certain *van* genes and orthologs, mechanisms of vancomycin resistance in *C. difficile* remain poorly understood. The *vanG* gene cluster found in ~85% of *C. difficile* is related to the *van* operon that results in vancomycin resistance when induced in *Enterococcus faecalis* [20], but induction with vancomycin does not appear to confer resistance in *C. difficile* [11, 19]. However, a recent study demonstrated

mutations in the *VanS/R* sensor kinase/response regulator triggered constitutive expression of the previously silent *VanG*, conferring vancomycin resistance in *C. difficile* ribotype (RT) 027 strains [21]. Several transposon (Tn) elements, e.g. Tn1549, have been identified in *C. difficile*, but unlike the original *E. faecalis* elements, these transposons lack a functional *vanB* [6]. An overview of AMR transposons in *C. difficile* is provided in Table 2.

A susceptible isolate of *C. difficile* carrying a phenotypically silent *vanB* homologue has been identified, suggesting the potential for transfer of other vancomycin resistance genes [21]. The *vanB* operon was located on a ~42 kb Tn916-like conjugative Tn with significant similarity to Tn1549 from vancomycin-resistant enterococci (VRE) [22]. The *vanRB* gene, known to be involved in regulating the expression of *vanB*, was fragmented, and no in vitro effect on vancomycin resistance was observed [22]. The *vanZ* ortholog *CD1240* conferred low-level teicoplanin resistance, but not vancomycin resistance, and was not induced by glycopeptide antimicrobials, rather by host peptide LL-37 [19]. Teicoplanin is not routinely recommended, nor is it approved, for use in the USA; however, it is used in Europe and appears to result in lower recurrence rates than vancomycin [19]. Resistant isolates with mutations in other gene families have been found, including *murG*, a peptidoglycan glycosyltransferase involved in cell wall maturation [6, 21, 23]. Biofilm may also play a role, as vancomycin induces biofilm formation and biofilms are resistant to high concentrations of vancomycin [6], up to a 12-fold increase in MIC over planktonic cells [23].

Plasmids may have a role in vancomycin resistance; the acquisition of plasmid pX18-498 reduced susceptibility to vancomycin eightfold in *C. difficile* [17]. In vivo testing in mice showed decreased susceptibility to vancomycin and increased disease severity [17]. The plasmid may have a role in cell wall integrity, as cells containing the plasmid did not rupture in the presence of vancomycin while plasmid-lacking cells did. Genetic analysis identified a differentially expressed amidase gene of the family responsible for susceptibility to cell wall targeting antimicrobials in *Mycobacterium tuberculosis* [17].

Vancomycin achieves high concentrations in faeces; however, this does not appear to correlate to concentrations in mucosa nor clinical outcomes [5, 17], despite the notion that the high faecal concentration may overcome increased MICs [6]. These high concentrations may exacerbate negative outcomes as examples of the Eagle effect, where higher drug concentrations kill fewer bacteria, have been found for vancomycin against *C. difficile*. Vancomycin concentrations of up to 2048 mg/L were survivable in vitro by four strains of *C. difficile* in one study by Jarrad et al., despite a bactericidal concentration of 4 mg/L [24]. Thus, high doses may disproportionately affect the normal

Table 1 *C. difficile* AMR mechanisms

Antimicrobial(s)	Mechanism of action	Resistance mechanisms (known/putative)	Relevant loci	References
Vancomycin	Inhibit cell wall synthesis by binding to D-Ala-D-Ala portion of peptidoglycan precursors	<ul style="list-style-type: none"> - Mutations in peptidoglycan biosynthesis proteins - Biofilm formation 	<i>murG</i> , <i>vanS/R</i> and <i>vanG</i>	[4, 6, 21]
Metronidazole	Inhibits DNA synthesis after partial reduction to an active substance that triggers DNA breaks	<ul style="list-style-type: none"> - Alterations in metabolic pathways (such as nitroreductases, iron uptake, DNA repair, stress pathways) - Biofilm formation - Potentially plasmid-mediated 	<i>pfo</i> , <i>nim</i> , <i>fur</i> , <i>hemN</i> genes (putative, responsible for resistance in other species)	[4, 30, 31]
Fidaxomicin	Prevents initiation of transcription by interfering with DNA-RNA polymerase complexes	<ul style="list-style-type: none"> - Mutations in bacterial RNA polymerase 	<i>rpoB</i>	[40, 42]
Rifamycins	Inhibit bacterial DNA-dependent RNA polymerase and thus RNA synthesis	<ul style="list-style-type: none"> - Mutations in bacterial RNA polymerase 	<i>rpoB</i>	[4, 42]
Fluoroquinolones	Target DNA replication enzymes to generate ds-DNA breaks in the chromosome	<ul style="list-style-type: none"> - Mutations in Gyrase, primarily within QRDR 	<i>gyrA</i> , <i>gyrB</i>	[11, 35, 46, 49, 53–55, 58, 112–116]
Tetracyclines	Binds 30S ribosome, interfering with the binding of aminoacyl-tRNA to the mRNA-ribosome complex, thereby inhibiting protein synthesis	<ul style="list-style-type: none"> - Protection of ribosomes 	<i>Tet44</i> , <i>tetM</i> , <i>tetW</i> , <i>tetA(P)</i> , <i>tetB(P)</i> (primarily carried on transposons)	[6, 9, 54, 56, 67, 68]
MLS _B agents	Target the 50S subunit of bacterial ribosomes, preventing elongation of peptides and thus inhibiting protein synthesis	<ul style="list-style-type: none"> - Protection of ribosomes via methylation, genes often carried on transposons - Potentially mutations in 23S rRNA 	<i>erm</i> family (B/G/Q/C/FS), <i>cfiC</i> , <i>cfiB</i> , <i>msrA</i> ,	[6, 46, 49]
Linezolid	Selectively inhibits early stages in protein synthesis	<ul style="list-style-type: none"> - Mutation in ribosomal protein 3, <i>cfiC</i> 	<i>cfiC</i> , <i>rpIC</i>	[54]
Chloramphenicol	Binds 50S ribosomal subunit to block peptide bond formation	<ul style="list-style-type: none"> - Ribosomal protection via methylation, inactivation of antimicrobial via enzymes 	<i>cat(P)</i> , <i>cat(D)</i>	[6, 55]
Aminoglycosides	Inhibit protein synthesis by targeting 16S rRNA aminoacyl-tRNA recognition site	<ul style="list-style-type: none"> - Aminoglycoside-inactivating enzymes 	<i>aph</i> , <i>aac</i> , <i>ant</i> and <i>sat</i> genes as well as <i>npmA</i>	[91]
β-lactams	Inactivate cell wall synthesis enzymes	<ul style="list-style-type: none"> - Enzymatic destruction of antibiotic via beta-lactamases - Altered target, e.g. PBPs 	<i>CDD1</i> and <i>CDD2</i> , imipenem resistance mutations in <i>pbp1</i> and <i>pbp3</i>	[54, 92]

Table 2 AMR transposons in *C. difficile*

Transposon	Antimicrobial(s)	Key characteristics	Reference
Tn6189	MLS _B	A ~24 kb conjugative Tn916-like transposon carrying <i>ermB</i> . Transposition is due to excisionase (<i>xis</i>) and integrase (<i>int</i>)	GenBank accession number: MK895712.1
Tn6194	MLS _B	A 28 kb conjugative Tn916-like transposon with a single copy of <i>ermB</i> and a putative toxin-antitoxin module. It contains <i>xis</i> and <i>int</i> genes and can integrate at different sites. It is the most common <i>ermB</i> containing element in European clinical isolates	[6, 54, 117]
Tn6218	MLS _B , aminoglycoside	Related to Tn916, this 14.2 kb transposon contains <i>int</i> and <i>xis</i> genes but is not conjugative. Some variants also contain <i>ermB</i> , <i>cfr</i> , <i>matE</i> and <i>aacA-aphD</i> AMR genes	[54, 118]
Tn5398	MLS _B	This transposon integrates into the genome via the exchange of large genomic fragments and is capable of interspecies transfer. It carries two copies of <i>ermB</i>	[6, 54]
Tn6215	MLS _B	Tn6215 integrates into the genome via the exchange of large genomic fragments and can be transferred via a conjugation-like mechanism or transduction by phage phiC2. It is a 13 kb transposon and contains <i>ermB</i> as well as genes for a truncated toxin-antitoxin system	[6, 54, 80]
Tn5397	Tetracycline	A 21 kb conjugative transposon closely related to Tn916, but transposition is due to serine recombinase (<i>tniX</i>), rather than <i>int</i> and <i>xis</i> . It encodes <i>tetM</i>	[119]
Tn6944	Tetracycline, macrolide	A recently discovered transposon commonly found in clade 2. It contains <i>int</i> and <i>xis</i> genes along with <i>tetM</i> and <i>mefG</i> (Macrolide efflux) AMR genes	Dr Korakrit Imwattana, unpublished data
Unnamed Tn916-like	MLS _B , tetracycline	A non-conjugative Tn916-like element found in clinical isolate CD1911. It contains both <i>tetM</i> and <i>ermB</i>	[6]
Unnamed	MLS _B	A novel Tn916-like transposon similar to Tn6218 that encodes <i>cfr</i>	[6]
Tn916-like family	Tetracycline	Conjugative transposons found in several species that encode <i>tet(M)</i>	[6, 120]
Tn6164	Tetracycline	Found in RT078 isolates, this 106 kb transposon can carry <i>tet(44)</i> , <i>ant(9)Ia</i> and <i>ant(6)</i> genes, but this has not been shown to confer resistance to these antimicrobials. Sections of this transposon show homology to various MGEs in other species including <i>S. pneumoniae</i> , <i>C. fetus</i> , <i>Thermoanaerobacter</i> spp and <i>E. faecalis</i>	[100, 121]
Tn4453a/Tn4453b	Chloramphenicol	Mobilizable, ~7 kb transposons related to Tn4451 in <i>C. perfringens</i> ; they encode the <i>catD</i> gene (chloramphenicol acetyltransferase) for chloramphenicol resistance	[122]
Tn6190	Tetracycline	An 18 kb transposon containing <i>xis</i> and <i>int</i> genes as well as <i>tetM</i>	[117]
Tn1549-like	Vancomycin	A 42 kb transposon with homology to Tn1549. It contains <i>xis</i> and <i>int</i> genes as well as a <i>vanB2</i> operon with disrupted <i>vanRB</i> genes, resulting in a silent phenotype	[22]

gut microbiota without additional effect on *C. difficile*, promoting recurrence [24].

Reports have also found no link between clinical outcomes and vancomycin resistance [5], further clouding the causes of treatment failure. Reduced susceptibility to vancomycin has been found in several strains of *C. difficile*, RTs 027, 001, 017, 078 and 356/607 [6, 25]; however, the clinical implications of this are unclear. In one study, two isolates had reduced susceptibility to vancomycin, yet the patients responded well to vancomycin treatment [26]. In this case, reduced susceptibility was determined using the EUCAST breakpoint (> 2 mg/L), with the isolates having MICs of 3 mg/L, which would still be considered susceptible

with CLSI breakpoints [26]. This highlights the need for the standardisation of methodology.

The highest rates of vancomycin resistance have been found in North and South America [8], followed by Asia, a finding mirrored by the high levels of glycopeptide antimicrobial use in the USA and China [5]. Vancomycin was ineffective against 1.4% of isolates in a European study of 2698 isolates [27]. In a comparison of historical (1992–2014) and modern (2015–2019) isolates, resistance rates did not show a statistically significant increase [8]. However, a meta-review by Saha et al. did report a statistically significant increase in vancomycin resistance comparing pre- to post-2012, which correlated to an increase in global vancomycin usage [5].

This increase was dependant on the method of testing used, as including only studies performed with E-tests nullified the increase [5]. Vancomycin resistance was significantly higher in hypervirulent strains such as *C. difficile* RT027 than in other RTs [5], while RT018 and RT356 had increased mean MICs of vancomycin [28].

Metronidazole

Metronidazole is a nitroimidazole that inhibits DNA synthesis after its partial reduction by some anaerobic bacteria results in cytotoxicity. While metronidazole has been used as a first-line treatment for CDI for many years, it is no longer recommended by the Infectious Disease Society of America (IDSA) due to inferior cure rates for both initial and recurrent CDI compared to vancomycin [13]. Repeated or prolonged metronidazole use has also been linked to neurotoxicity [13, 29]. In addition, breakpoints for resistance vary significantly (EUCAST, 2 mg/L; CLSI, 32 mg/L) [8].

Metronidazole resistance mechanisms in *C. difficile* are just beginning to be understood but are likely multifactorial processes involving alterations to metabolism such as with nitroreductases, iron uptake, DNA repair or biofilm formation [6]. In other species, metronidazole resistance mechanisms include reduced growth and uptake rates, efflux [29], nitroimidazole reductases (encoded by *nim* genes), altered pyruvate–ferredoxin oxidoreductases (*pfo* or PFOR) and stress pathways [30], or mutations in *hemN* and ferric uptake regulator (*fur*) genes [31].

Iron plays a role in many reduction pathways associated with metronidazole, and changes in iron metabolism/homeostasis appear to be involved in resistance. PFOR (with iron–sulphur clusters) and ferredoxin are involved in the activation of metronidazole through reduction, and in *C. perfringens*, PFOR mutants were up to 100-fold more resistant [29]. Moura et al. analysed the genome of a *C. difficile* strain with stable metronidazole resistance (MIC 32 mg/L) and found that a metabolic pathway associated with *pfo* was likely altered, as the resistant isolate had a lower, more stable PFOR concentration than susceptible controls [31]. Ferritin was absent from the metronidazole-resistant strain during antimicrobial exposure, suggesting a role for iron storage [31]. A separate proteomic analysis found a reduction in iron uptake/transport proteins after metronidazole treatment and increased expression of Fur protein (regardless of metronidazole presence) in a resistant strain [32]. The resistant strain in question demonstrated fitness defects and required the inclusion of iron in media for growth [32].

Deshpande et al. constructed a highly mutable strain via deletion of DNA mismatch repair genes to study the evolution of resistance in vitro, demonstrating that resistance to metronidazole developed progressively, with MICs increasing from 2 to 16 mg/L [33]. Mutations first appeared in

feoB1 (the main iron transporter) and then *pfo*, followed by *xdh* (a putative xanthine dehydrogenase), and finally, some variants with greater resistance (MIC = 64 mg/L) also had mutations in *iscR*, an iron–sulphur cluster regulator. While changes in *nifj*, *xdh* and *iscR* genes amplified the MIC further, they did not confer resistance without *feoB1* loss, while the loss of *feoB1* alone resulted in low-level resistance [33].

Differential expression and/or increased concentrations of DNA repair protein RecA were detected in resistant strains on exposure to metronidazole [31, 32]. In other species, mutants with DNA repair defects (such as in RecA) were more susceptible to metronidazole [29]. Differential expression of oxidative stress and general stress response proteins was also found in resistant strains [32]. Mutations in thiamine biosynthesis (*thiH*) and glycerol-3-oxidoreductase (*glyC*) genes have also been associated with resistance in *C. difficile* [29], as has a C-terminal deletion at position 372 in *hsmA* in RT010 isolates [34]. Unfortunately, the role these changes may play in resistance remains elusive.

Research into plasmid-mediated metronidazole resistance has been led by the work of Boekhoud et al. with their discovery of a high-copy number 7 kb plasmid (pCD-METRO) that conferred metronidazole resistance in *C. difficile* [30]. Figure 1 shows a representation of the pCD-METRO plasmid structure. This plasmid was found only in metronidazole-resistant strains (EUCAST breakpoint > 2 mg/L) and was internationally disseminated and present in both toxigenic and non-toxigenic strains. Surveillance in the Netherlands showed a prevalence of pCD-METRO of < 0.14%; however, the exact mechanism of resistance was unclear. The plasmid contained a small pseudogene with protein homology to *nimB* from *Bacteroides fragilis*, but this lacked the catalytic domain and induction via an inducible promoter did not confer resistance in the laboratory. A metallohydrazase/oxidoreductase gene was also present but has not been further characterised. No reduction in growth rate was detected in the absence of metronidazole, and repeated passage on non-selective media failed to eliminate the plasmid from metronidazole-resistant strains. A guanine-cytosine content (GC%) of 41.6% did not match the *C. difficile* standard of ~ 28–30% and indicated acquisition via horizontal gene transfer from another unidentified organism [30].

In isolates with the pCD-METRO plasmid, MIC values differed between RTs [30]. The plasmid was found in the initial stool sample along with a susceptible isolate, which differed by one core genome single-nucleotide polymorphism (cgSNP) to the resistant isolate found later, indicating the strains were clonal. This scenario was also identified later in a second case. Introduction of the pCD-METRO plasmid into a metronidazole-susceptible strain increased the MIC > 24-fold [30]. However, metronidazole resistance was still found in isolates lacking pCD-METRO [34], indicating alternate methods of resistance exist.

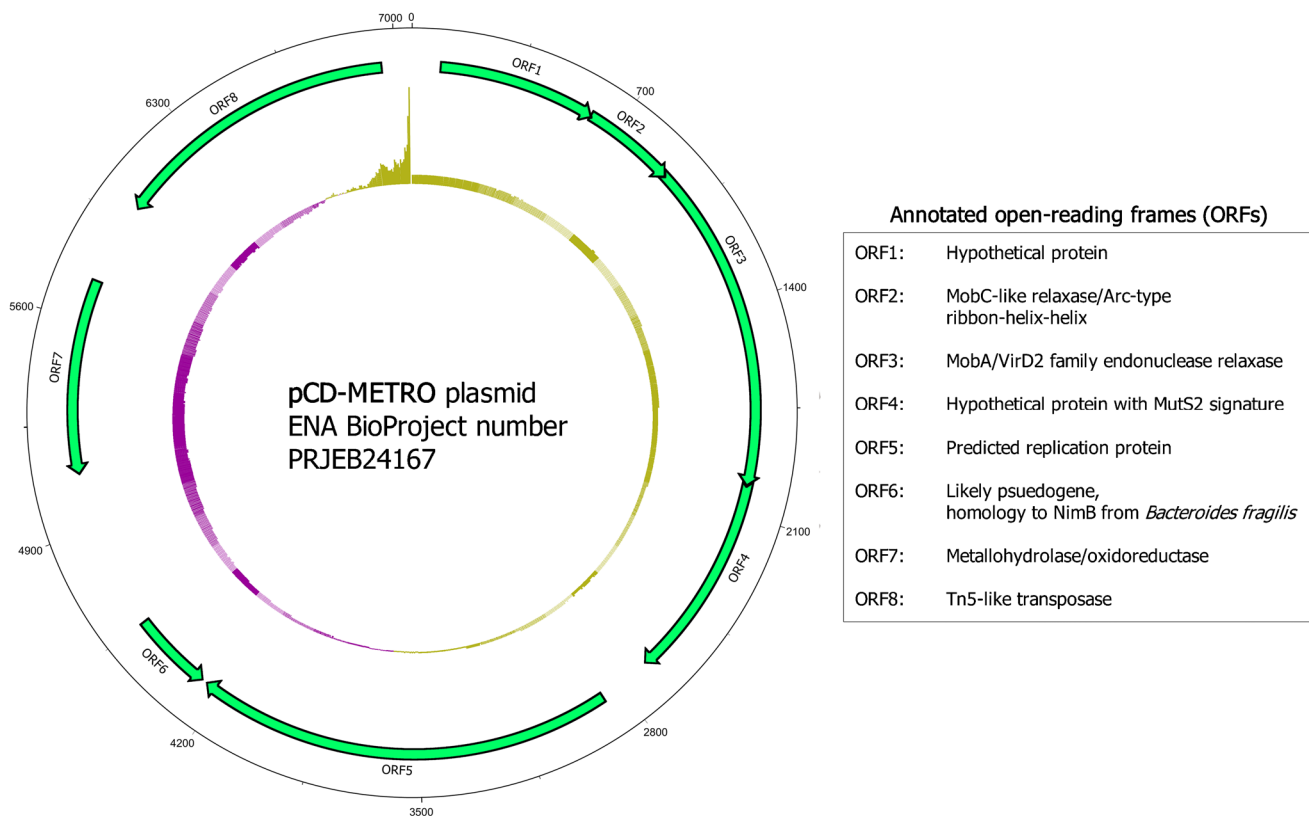


Fig. 1 Representation of the metronidazole-resistance plasmid pCD-METRO reported by Boekhoud et al. [30]. pCD-METRO plasmid with putative open reading frames (ORFs) shown in green on the

outer circle and gene products listed beside. The inner-circle represents GC content, with above average in yellow, below average in purple

Metronidazole only reaches low concentrations in the colon and faeces [30], which may promote the development of resistance and play a role in treatment failures [6, 11], as Moura et al. demonstrated subinhibitory concentrations select and maintain isolates with increased MICs [4]. Heteroresistance may also play a role in treatment failure [35], as could other metronidazole-resistant microbiota in the gut by the inactivation or sequestration of metronidazole [30]. In many cases, metronidazole resistance did not correlate to treatment failure [5] which also occurred with metronidazole-susceptible isolates [30].

Susceptibility testing for metronidazole resistance is particularly inconsistent. Boekhoud et al. took 88 strains that two other clinical trials had characterised as having altered susceptibility and retested them using agar dilution and CLSI breakpoints/guidelines; they found that most strains were metronidazole-susceptible [30]. Inducible resistance subsequently lost during freeze–thaw cycles in handling was suggested as a possible explanation [30]. This is consistent with other reports that metronidazole resistance is lost after storage at low temperatures [32, 36, 37]. As samples are usually frozen for storage or transport

before testing, metronidazole resistance may be higher than surveillance studies indicate [37].

Differences in media may also contribute to inconsistent findings, as two studies have demonstrated that heme appears to be essential for media-dependent metronidazole resistance. In one, strains classified as susceptible to metronidazole on brain–heart infusion (BHI) agar were resistant with higher MICs on Brucella blood agar (BBA) (measured by E-test) [30]. In a later study, MICs were increased up to 24-fold for strains grown on BBA/BHI blood agar and BHI agar supplemented with hemin [34]. Lynch et al. were able to generate a temperature-stable metronidazole resistant but reduced-fitness strain via a repeated passage on media with subinhibitory metronidazole concentrations [36]. This strain had mutations in the *hemN* (corporphyrinogm III oxidase) and *thiH* genes (involved in haem and thiamine biosynthesis, respectively), as well as in the *fur* gene [36]. Finally, testing methods also contribute to differences in characterisation, as the E-test may not distinguish isolates with reduced metronidazole susceptibility, with as many as 8% of metronidazole-resistant isolates misclassified [5, 38].

The highest rates of metronidazole resistance in *C. difficile* have been found in Asia, then North America [5] and Europe [8, 27]. Resistance remains rare but varied, with rates from 0 to 40% [28, 30], and seems more common in non-toxicogenic strains, with 7–ninefold increased MIC values in non-toxicogenic RT010 compared to toxicogenic RT027, RT078 and RT001 [30]. One non-toxicogenic clinical strain from China was reported with an MIC as high as 256 mg/L [4]. Saha et al. found a statistically significant decrease in resistance between pre-2012 to post-2012 studies, but these results were strongly impacted by testing methodology [5]. *C. difficile* RTs 027, 106, 001/072, 206, 010 and 356 strains had increased mean metronidazole MICs compared to other strains [6, 27, 28]. *C. difficile* RTs 176, 001, 126, 078 and 198 were also associated with reduced susceptibility to metronidazole [6, 26, 27]. Overall, mechanisms of metronidazole resistance remain unclear but increasingly appear to be multigenic with a role for iron metabolism.

Fidaxomicin

Fidaxomicin, a tiacumicin antimicrobial, inhibits RNA synthesis by interfering with the formation of the DNA–RNA polymerase complex before transcription can be initiated [39]. For *C. difficile*, it is a bactericidal and narrow spectrum, with minimal side effects, similar rates of initial cure as vancomycin and a significant reduction in recurrence [40]. However, Sears et al. found a mean faecal concentration of ~1396 mg/kg, and at such high concentrations, its impact on the gut microbiome may be broader than expected [41]. Similar to vancomycin, this high concentration is far above known MICs, and so future increases in MIC values may not result in reduced susceptibility in clinical settings [27].

Both fidaxomicin and rifamycins inhibit bacterial transcription, binding at adjacent but not overlapping target sites [4, 6]. Mutations in *rpoB* are responsible for resistance in both, but fortunately, they require separate mutations and are not cross-protective [6]. To examine the effect of *rpoB* mutations on fidaxomicin MICs, Kuehne et al. generated isogenic mutants, each with a single SNP in *rpoB*, resulting in the following changes: Val1143Asp, Val1143Gly and Val1143Phe [40]. These mutants had reduced susceptibility to fidaxomicin and reduced virulence and fitness (sporulation, growth and toxin production) in vitro [40]. This indicates that fidaxomicin-resistant mutations may be rarer due to negative effects on fitness, a finding supported by in vitro work demonstrating reduced susceptibility developed easily but was paired with severe fitness defects [42]. This study detected three resistance mutations in *rpoB* but also identified one isolate that showed a dramatically increased MIC of 64 mg/L with less severe reductions in toxin production and sporulation, and without the expected reduction

in replication rate [42]. Mutation in a *marR* (repressor for a multiple AMR operon) homologue (CD22120) or *rpoC* (Asp273Tyr change) may also increase fidaxomicin MICs [11, 23].

Despite approval for fidaxomicin use for CDI in 2010, uptake has been limited [21], possibly due to its higher cost [43], and there are currently no breakpoints available [8]. Unlike rifamycins, fidaxomicin resistance is very rare [5, 8, 27, 28, 44].

Rifamycins

Rifamycins inhibit bacterial DNA-dependent RNA polymerase. The bacterial RNA polymerase RpoB, especially the β -subunit [6], is the primary site for resistance-conferring mutations [4]. These mutations may disrupt the direct interaction between target and antimicrobial or modify the rifamycin-binding pocket [11]. Figure 2 depicts the location of resistance-conferring amino acid substitutions in *rpoB*. Mutations in *rpoB* resulting in rifamycin resistance in *C. difficile* do not seem to confer a fitness cost, in contrast to species such as *Neisseria meningitidis* and *M. tuberculosis* [40]. Resistant strains may have MICs 1000-fold higher than susceptible strains [45], and there is a high agreement between phenotypic and genotypic resistance in rifaximin [46]. However, a US study found that epidemic *C. difficile* RT027 strains had unusual intermediate MICs to rifamycin, suggesting the potential for alternate resistance mechanisms [25]. The susceptibility of rifampin correlates with that of rifaximin, and thus, both are discussed in the following section.

While effective at reducing recurrence, the rapid development of resistance makes rifamycin use risky. In one case, resistance developed in a *C. difficile* RT056 strain within 3 days of rifaximin therapy, with the MIC increasing from 0.002 to > 32 mg/L [47]. Rifamycins in long-term regimes are commonly used in therapy for tuberculosis, a leading cause of infectious disease-related deaths globally [44, 48]. This combination of long-term, frequent use and the single mutation required leaves rifamycins particularly vulnerable to the development of resistance. In a study of several Asia–Pacific countries, Lew et al. noted that rifaximin resistance was not found in *C. difficile* strains from countries with low tuberculosis rates (Singapore, Australia and Japan) [44].

A 2020 meta-analysis and a 2018 European study found 42.4% and ~15% rifampin resistance, respectively [6, 8]. A separate European study from 2015 found rifampin resistance in 17/22 countries (breakpoint 16 mg/L) [28]. Resistance was not uniform across a location or RT [6], e.g. rifampicin resistance was present in 62.4% of Italian isolates compared to 13.4% of the whole collection [28], and mutations in *rpoB* were strongly associated with high-risk strains,

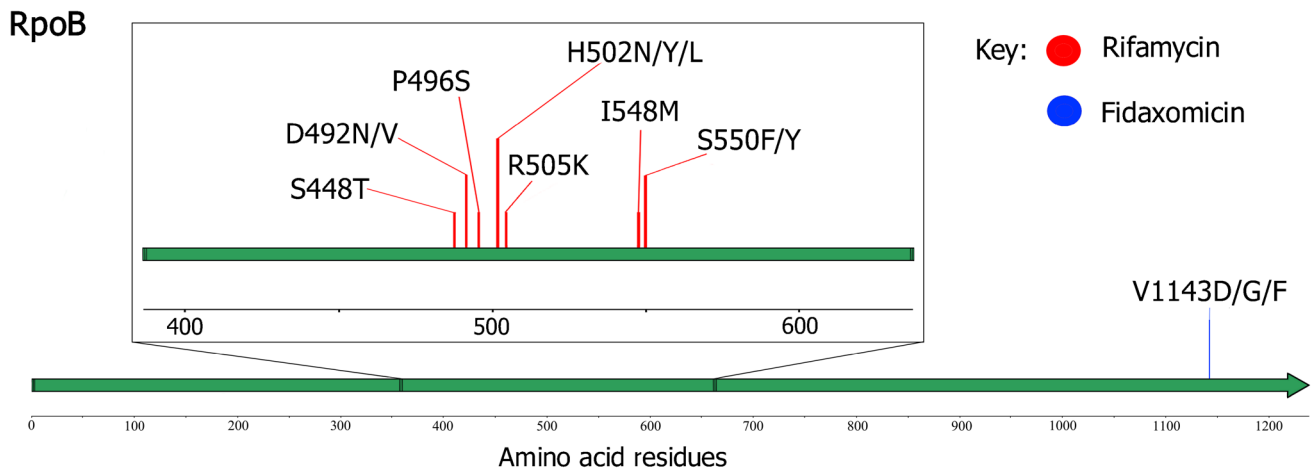


Fig. 2 Resistance-conferring mutations in RpoB. Known amino acid substitutions in RpoB that confer resistance to rifamycins or fidaxomicin. Mutation-rich regions have been enlarged with line breaks indicated by triple black lines. Substitutions are displayed in standard notation (single-letter amino acid codes and amino acid site; S=ser-

ine, T=threonine, D=aspartic acid, N=asparagine, V=valine, P=proline, H=histidine, Y=tyrosine, L=leucine, R=arginine, K=lysine, I=isoleucine, M=methionine, F=phenylalanine) [35, 42, 46, 47, 49, 123–127]

e.g. multi-locus sequence types (ST) 11 (e.g. RT078) and 37 (e.g. RT017) [49]. While both location and strain appear to be linked to AMR, they do not always agree; e.g. RT001/072 from the Czech Republic demonstrated rifampicin resistance, but RT001/072 from Germany, Latvia or Slovakia did not [28].

Ridinilazole

Ridinilazole is a narrow-spectrum, potent (*C. difficile* MIC₉₀ 0.2 mg/L) antimicrobial currently in phase III trials that has a novel but unclear mechanism of action [16]. High-resolution microscopy suggests an effect on cell division resulting in a filamentous cell [50]. Ridinilazole also reduces in vitro production of toxins A and B in *C. difficile* RT027 at both sub- and supra-MIC levels. It is poorly absorbed, and high concentrations can be found in stool. In vitro testing suggested that higher concentrations were required to kill gut microbiota than fidaxomicin, thus reducing gut dysbiosis [50]. In both animal models and human trials, ridinilazole demonstrated improved sustained cure rates over vancomycin without significant adverse effects [16, 51]. Sustained cure rates consider both initial cure and recurrence rates, and the superiority of ridinilazole over vancomycin was principally driven by a significant reduction in recurrences [51]. In a phase II trial, those patients who developed a recurrence all had strains that remained susceptible to ridinilazole, suggesting an alternate reason for treatment failure [50].

Treatments under development

Several potential treatments are currently under development but have not yet completed phase III trials, including ramizol, clofazimine, DS-2969b, CRS3123, ibezapolstat, DNV3837/DNV3681, MGB-BP-3, LFF571 and ramoplanin [16]. Some have similar mechanisms or targets to existing antimicrobials, e.g. DS-2969b which targets GyrB and thus may be vulnerable to amino acid substitutions as with the fluoroquinolones. Others, such as ibezapolstat that inhibits DNA polymerase III ϵ , have novel mechanisms of action. Surotomycin, a potent bactericidal cyclic lipopeptide, completed phase III trials but did not meet its goals of non-inferiority to vancomycin and is no longer in development [16]. Studies determined resistance was likely to be extremely rare, involving proteins with potential roles in membrane structure or biosynthesis [45, 52]. Similarly, cadazolid, a novel oxazolidinone, did not progress after phase III trials [16].

Resistance to other antimicrobials

Fluoroquinolones

Fluoroquinolones are bactericidal antimicrobials that target the DNA replication enzymes DNA gyrase and type IV topoisomerase, inhibiting their ligase functions while continuing to allow them to generate double-stranded DNA breaks in the chromosome [53]. Newer generation fluoroquinolones such as moxifloxacin show increased activity against Gram-positive and anaerobic bacteria [53, 54]. However,

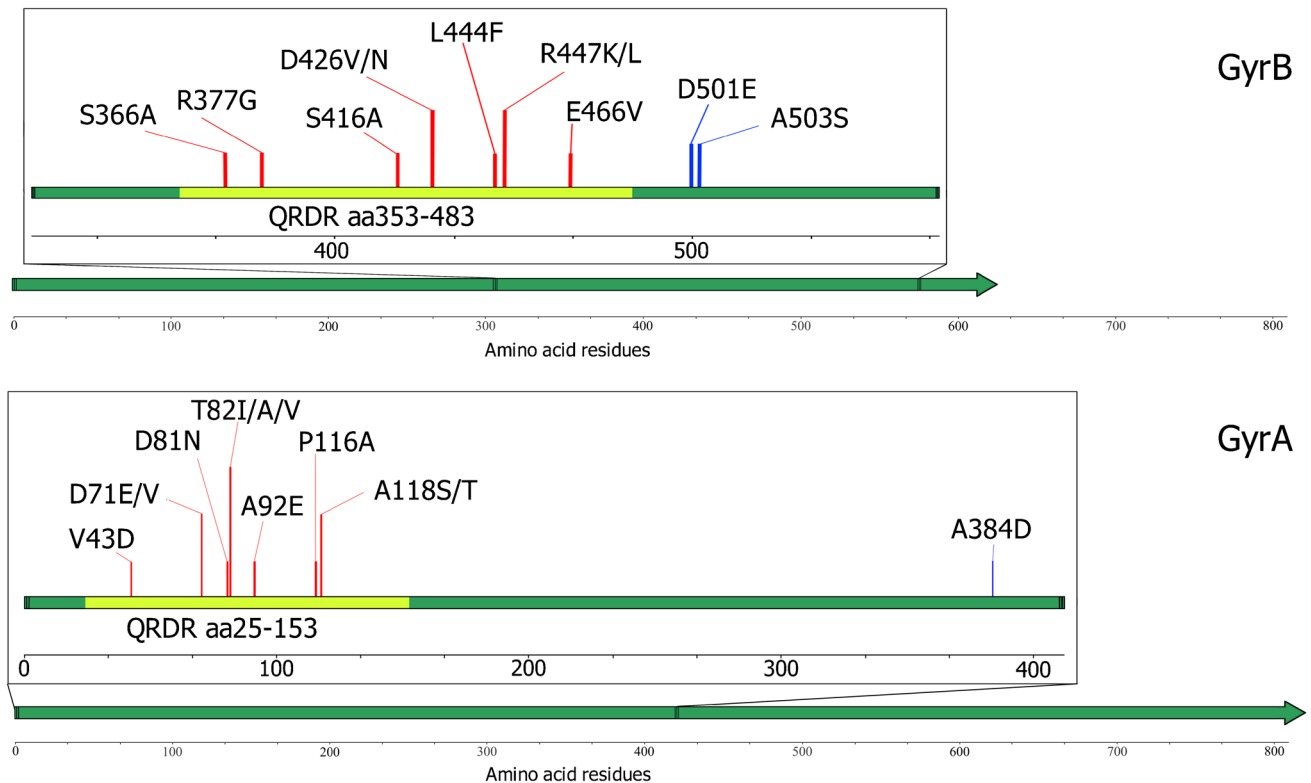


Fig. 3 Fluoroquinolone resistance mutations in GyrA and GyrB. Known amino acid substitutions in GyrA and GyrB conferring resistance to fluoroquinolones. Mutation-rich regions have been enlarged, with line breaks indicated by triple black lines. The quinolone resistance-determining region (QRDR) is identified by a yellow box, with extra-QRDR substitutions noted in blue. Substitutions are displayed

in standard notation (single-letter amino acid codes and amino acid site; S=serine, A=alanine, R=arginine, G=glycine, D=aspartic acid, V=valine, N=asparagine, L=leucine, F=phenylalanine, K=lysine, E=glutamic acid, T=threonine, P=proline) [11, 35, 46, 49, 54, 55, 58, 70, 112–116, 128, 129]

moxifloxacin and levofloxacin may fail to reach inhibitory concentrations in the intestine in early treatment, and resistance develops frequently after exposure [6].

Resistance in *C. difficile* occurs via mutations that reduce binding affinity in the target site, the quinolone resistance-determining region (QRDR) of DNA gyrase subunits A and B [9, 55]. Figure 3 depicts the major GyrA and GyrB substitutions that confer fluoroquinolone resistance. While there are many amino acid substitutions responsible for resistance, Thr82Ile in GyrA is the most common [56]. In one study, 179/186 (96.2%) strains with reduced susceptibility had this alteration [26]. This mutation lacks a detectable fitness cost [57], and in at least one study, resistant strains exhibited increased fitness [26], suggesting it may be maintained in the population regardless of the presence of antimicrobials [6]. The prevalence of this mutation in particular may be explained by higher fitness costs and increased mutation requirements for other amino acid changes such as Thr82Val in GyrA [57]. Alterations in GyrB are less common than those in GyrA [57]. Different mutations provide different levels of resistance, with Thr82Ile associated with high-level resistance while others lead to lower MICs in comparison

[57]. While QRDR mutations are the major mechanism, some resistance-conferring mutations have been found outside the QRDR and may be more common than previously thought [58]. Resistance to newer fluoroquinolones appears to be somewhat cross-protective, with one study finding all strains resistant to moxifloxacin were also resistant to gatifloxacin, levofloxacin and ciprofloxacin [35]. While there is generally high concordance between phenotypic and genotypic (*gyrA* and *gyrB*) resistance typing [46], one study has identified ciprofloxacin-resistant strains with no mutations in any part of the *gyrA/gyrB* regions [58], so other resistance mechanisms may yet be discovered.

Interestingly, antimicrobial adjuncts may improve the efficacy of some antimicrobials for CDI. A recent study by Pellissery et al. found that sub-MIC levels of sodium selenite significantly increased the susceptibility of *C. difficile* to ciprofloxacin in vitro while not affecting beneficial microbiota [59]. Such findings indicate that AMR may not condemn a drug to permanent disuse; instead, more research is needed on potential combinations.

Fluoroquinolone resistance is widely regarded as having played a significant role in the spread of hypervirulent strain

C. difficile RT027 which caused substantial outbreaks across North America and Europe in the 2000s. In the 1990s and 2000s, North America had a heavy reliance on fluoroquinolones [60] such as levofloxacin which was recommended by IDSA/Thoracic Society for therapy for community-acquired pneumonia [61]. In contrast, Australia had strict limitations on fluoroquinolone use, in both humans and livestock, which contributed to the much-delayed importation and limited local spread of RT027 [62–64]. *C. difficile* RT027 was characterised by binary toxin production and fluoroquinolone resistance and responsible for dramatically increased mortality rates [65]. These epidemic strains of *C. difficile* RT027 had increased resistance to fluoroquinolones compared to pre-epidemic strains [6], with the prevalence of moxifloxacin resistance in Europe rising from 6.6% (1991–1997, before the introduction of moxifloxacin) to 39.9% (2011–2012) [54].

The prevalence of *C. difficile* RT027 has decreased significantly over the last decade, from 31% in 2011–2012 to 14% in 2015–2017 in the USA, resulting in a drop in moxifloxacin resistance rates [25]. While *C. difficile* RT027 is the best-known fluoroquinolone-resistant RT, resistance is found in many strains and appears to be becoming more common [6], with *C. difficile* RT001, RT017, ST35, ST3 and ST54 in particular with a high prevalence of up to 99% [8, 54, 66], although clonal outbreaks may significantly impact resistance rates. There were statistically significant differences in resistance rates across continents, with the highest rates found in South America, followed by Asia and North America [8].

Tetracyclines

Tetracyclines are broad-spectrum antimicrobials that target the 30S ribosome, preventing attachment of the aminoacyl-tRNA to inhibit protein synthesis [11]. Resistance in *C. difficile* involves protection of the ribosomes via elongation factor homologs encoded on various *tet* genes (e.g. *tetM*, *tetW*, *tetA(P)* and *tetB(P)*) carried by transposons (e.g. Tn916, Tn5397 and Tn4453) [9, 11, 54, 56, 67–69]. The tetracycline efflux pump gene *tet40* has also been detected in *C. difficile* RT078 [69]. A study of UK, European and North American isolates found that *tetM* was the most common tetracycline resistance determinant [69]. Some strains have multiple *tet* genes, but it is unclear whether or not this affects the MIC [11]. Interestingly, isolates that were *tetM* positive but tetracycline susceptible were discovered in a study of hospital strains in China, although no mechanism was described [70].

Several tetracycline resistance determinants show evidence of movement between species due to their location on MGEs such as plasmids and transposons, and the widespread use of tetracyclines. Selection pressure from extensive tetracycline use in agriculture [8, 69], rather than human

medicine, appears to be driving an expansion of tetracycline-resistant clones, particularly in RT078 [69]. *C. difficile* RT078, commonly found in livestock, has since spread to humans, with increasing numbers of cases (particularly community-acquired cases) first detected in 2005–2008 across Europe and America [69]. It has high rates of tetracycline resistance and frequently possesses tetracycline resistance determinants with high sequence identity to other zoonotic pathogens. Human and animal isolates of RT078 are highly genetically similar [69], and a large-scale phylogenetic study of isolates from 22 countries identified frequent zoonotic transmission of RT078 across geographic borders [71]. Whole-genome sequencing of isolates from humans and pigs has found genetically identical or closely related strains in both [71–74]. A 2019 study found close genetic links (in both sequence similarity and location) between *tetP* genes in *Paenoclostridium sordellii* and *C. difficile*, suggestive of horizontal gene transfer between clostridial species [75].

As with other antimicrobials, strain differences exist; increasing tetracycline resistance rates were found in *C. difficile* RT027 clinical strains [25], while *C. difficile* RT078 isolates contain more of the *tetM* determinant than other strains [69]. Location plays a significant role in resistance distribution even within single strains; higher rates of tetracycline resistance were detected in *C. difficile* RT017 isolates from China (82.4–85.7%) than from European countries (27.8%) [54]. The highest rates of tetracycline resistance were found in Oceania, Asia and Europe [8].

Several tetracyclines have been proposed as potential alternative CDI treatments. Tigecycline has been suggested for the treatment of severe CDI [4], as it is efficacious, inhibits spore formation and decreases toxin production; however, it reduces the healthy microbiota of the gut [16] and thus may contribute to CDI recurrence. A pan-European study of 2698 isolates in 2011–2014 found no resistance to tigecycline [27], but a more recent study characterising over 500 isolates from phase III surtomycin clinical trials found 2.5% were resistant [76]. Omadacycline, another tetracycline that has completed phase III clinical trials for various infections, demonstrated in vitro activity against *C. difficile* (with lower MICs than vancomycin) and does not induce CDI in in vitro models [77]; however, it also has a significant impact on the gut microbiota [16]. Further research in vivo is required, as well as a comprehensive study of the effect tetracycline resistance determinants may have on this drug. Eravacycline also shows activity against *C. difficile*, but once again is broad-spectrum and thus not ideal for treatment [16].

Macrolide-lincosamide-streptogramin B antimicrobials

Macrolide-lincosamide-streptogramin B (MLS_B) antimicrobials (e.g. erythromycin and clindamycin) bind to the 50S

subunit of bacterial ribosomes, preventing elongation of peptides and thus inhibiting protein synthesis. MLS_B resistance in *C. difficile* is principally conferred by ribosomal methylation, but other mechanisms have been implicated and much is still unknown. The most common determinants of resistance are 23S ribosomal RNA methyltransferases encoded by *erm* genes, which are typically carried on transposons. The most prevalent *erm* gene is *ermB*, with two copies carried on a 9.6-kb conjugative transposon Tn5398 [6]. In one study in China, 89.3% of MLS_B resistant isolates carried *ermB* [78]. Alternative genetic contexts for *ermB* have been discovered, including a 12.8-kb plasmid fragment [49], a truncated version of Tn5398 that does not confer resistance (Δ *ermB* isolates) [79] and Tn6215 transduced by phage ϕ C2 [80]. Other *erm* genes including *ermG*, *ermQ*, *ermFS* and *ermC* have also been detected on transposons including Tn6398, Tn6194, Tn6149 and Tn6215, some of which are transmissible across species boundaries [4, 9, 46, 55, 68, 81].

An as-yet unexplained but common phenomenon is the existence of *C. difficile* strains with MLS_B resistant phenotype but lacking *erm* genes of any type, indicating the presence of other resistance mechanisms [6, 9, 11, 46, 67, 78]. Alterations to ribosomal proteins and rDNA are present in non-resistant isolates, and pump inhibitors such as reserpine had no effect despite suggestions of a role for the *cme* efflux pump [11], providing evidence against target alteration or efflux pump mechanisms [35, 55]. Several other putative mechanisms and genes of interest have been identified; erythromycin-resistant but clindamycin-susceptible strains have been identified with the *msrA* gene [49] which acts as a protein-oxidation repair enzyme in *Escherichia coli*, and one strain with high-level clindamycin and erythromycin resistance but lacking any known resistance genes encoded an rRNA methyltransferase enzyme identical to one found in *Erysipelotrichaceae* (accession AYM48329.1) [46].

A cluster of MLS_B resistance-associated genes was found in MDR *C. difficile* isolates, encoding *ermG*, *mefA* and *msrD* (macrolide efflux) and *vat* (streptogramin A acetyltransferase) [81]. This cluster appeared to be on a putative mobile genetic element, exhibiting a mosaic structure and including a type I restriction-modification (RM) system, an intact prophage and an IS66 family transposase as well as the AMR determinants. The element was later detected in several other *C. difficile* genomes in GenBank [81].

The *cfr* and *cfr*-like 23S rRNA methyltransferases confer resistance to a large group of antimicrobials (including phenicol, lincosamides, oxazolidinones, pleuromutilins, streptogramin A and large macrolides) in several bacterial species [82, 83]. While several *cfr* genes have now been detected in *C. difficile*, often carried on MGEs [84], their contribution to resistance requires further research. Some variants of Tn6218 contain *cfrB*, suggested to be a

mechanism of clindamycin and erythromycin resistance in *C. difficile* [6, 49]. The *cfrC* gene has also been found in clindamycin-resistant strains [49], while Stojkovic et al. identified a new *cfr*-like gene named *cfrE* in a clinical isolate from South America [84]. In vitro testing showed that both *cfrC* and *cfrE* had methylation activity in vitro in *E. coli*, but poor activity in *C. difficile* [84]. A study of an MDR (erythromycin, chloramphenicol, clindamycin and florfenicol, linezolid and streptogramin A) *C. difficile* isolate discovered a constitutively expressed *cfrC* gene which was later identified in search of several other *C. difficile* genomes [83]. The genomic context surrounding the gene indicated that *C. difficile* acquired *cfrC* some time ago and may be a reservoir for other bacteria [83]. Putative plasmids containing *cfr* genes have been detected in *C. difficile*; a putative plasmid containing *cfrC* was detected in silico (which has not yet been experimentally verified) [30], and a 6961 bp-sized plasmid named pCd13-Lar was identified carrying both the *aphA3* aminoglycoside resistance gene and a *cfrC* gene 99% similar to one found in the pTx40 plasmid from *Campylobacter coli* [49]. All *cfr* genes were uncommon in *C. difficile*, with a ~4% (*cfrC*), 1.3% (*cfrB*) and 0.09% (*cfrE*) detection rate according to examination of protein sequences from > 2000 genomes [84].

Clindamycin use poses a high risk for inducing CDI [6], and it was the first antimicrobial to be recognised as such [54]. Indeed, the disease was initially called clindamycin-associated colitis. Resistance is most prevalent in Asia and South America [8] and very common in human isolates in Australia (84%) [85]. A meta-analysis found no significant [79] difference in resistance rates for clindamycin between historic and modern strains [8]; however, a study in Eastern China found a statistically significant increase in MICs for erythromycin and clindamycin between 2015 and 2020 [78], which were the most common resistances found in China along with ciprofloxacin [66].

Oxazolidinones

Oxazolidinones such as linezolid are broad-spectrum antimicrobials active against Gram-positive bacteria. They inhibit protein synthesis by binding to the 50S ribosomal subunit and preventing the formation of the initiation complex [83, 86]. Linezolid inhibits protein synthesis by targeting the 23S rRNA and inhibited *C. difficile* cytotoxin production in in vitro models [11]; however, it is not recommended for CDI treatment [84]. Linezolid causes gut dysbiosis due to its broad spectrum of activity [87], but this appears short-lived [88]. Reports have emerged of linezolid-induced *C. difficile* colitis, and there is a suggestion that faecal concentrations may not be sufficient due to renal metabolism [89].

The *cfpC* gene carried via a transposon and a mutation in the *rplC* (ribosomal protein 3) gene has been associated with resistance to linezolid [11, 54]. While *C. difficile* is generally susceptible to linezolid, a small number of resistant strains have been reported in Europe, with estimates of prevalence ranging from 1–5.7% [54].

Chloramphenicol

Chloramphenicol inhibits protein synthesis by binding to the 50S subunit of the ribosome and blocking the action of peptidyl transferase and thus peptide bond formation. It is active against a wide range of bacteria including Gram-positives and anaerobes. Resistance mechanisms focus on protecting the ribosomes via methylation or inactivating the antimicrobial via enzymes such as the chloramphenicol acetyltransferase encoded by the *catD* gene [55]. In *C. difficile*, *catD* has been found on transposons Tn4453a and Tn4453b [6]. Problems with toxicity to human cells limit the use of chloramphenicol for *C. difficile*, so research is rare. Resistance is uncommon [55] with <5% of strains from prevalent RTs resistant in in vitro tests according to the ClosER study of 22 European countries [28].

Aminoglycosides

Aminoglycosides target the 16S rRNA aminoacyl-tRNA recognition site and inhibit protein synthesis. Intrinsic resistance in anaerobes means that the presence of aminoglycoside genes is likely a poor indicator of phenotype [81]. Despite intrinsic resistance, some strains of *C. difficile* also contain acquired aminoglycoside resistance genes, indicating transfer from other bacteria [46]. Resistance mechanisms mainly involve aminoglycoside-inactivating enzymes [90]. Multiple resistance genes have been identified such as *npmA* (a 16S rRNA methyltransferase), *aph(3')-III* (an aminoglycoside phosphotransferase) and *ant(6)-Ia* (an aminoglycoside nucleotidyltransferase) [91], as well as a range of putative resistance determinants commonly found in other species, such as *aadE* (an aminoglycoside 6-adenylyltransferase) and *aac(6')-Ie-aph(2'')-Ia* [81]. CLSI breakpoints are not defined for aminoglycosides in *C. difficile* [91].

Newer aminoglycosides such as amikacin, arbekacin and plazomicin are less vulnerable to some resistance mechanisms than the earlier gentamicin and tobramycin but are still affected by some mechanisms such as 16S-rRNA methyltransferases and certain *aac* and *aph* genes [90]. In *C. difficile*, resistance to apramycin, a newer aminoglycoside under development, is rare and associated with the *npmA* gene [90].

β-Lactams

β-Lactam antimicrobials, including penicillins, carbapenems and cephalosporins, feature a β-lactam ring and inactivate cell wall synthesis enzymes. *C. difficile* is intrinsically resistant to many β-lactams due to the presence of chromosomal class D β-lactamase genes (*blaCDDs*) which confer broad-spectrum resistance to β-lactam antimicrobials including penicillins, monobactams and all generations of cephalosporins [92, 93]. Unlike many β-lactamases, the *blaCDD* genes appear not to be mobile elements, as they are not surrounded by known MGE genes and are only distantly related to other β-lactamases [46] while maintaining high amino acid sequence identity with *blaCDDs* from other *C. difficile* strains [92]. They remain associated with the cell surface and are only active under reducing conditions [93]. The regulation of *blaCDDs* is debated, with Toth et al. finding *blaCDD* enzymes were poorly expressed [92], while Sandhu et al. found a high expression that was inducible and both dosage- and antimicrobial-dependent [93]. Regulation is controlled by *blaR* (sensor) and *blaI* (repressor), with disruption of *blaI* resulting in improved growth in ampicillin [93]. A co-transcribed lipoprotein, *blaX*, enhances *blaCDD* activity but is not essential [93].

β-Lactamase inhibitors in combination with antimicrobials (e.g. amoxicillin-clavulanate) were effective against *C. difficile* in at least one study, although these agents are not often included in AMR surveillance [54]. Carbapenems possess a unique structure that confers resistance to most β-lactamases [94]; they bind to PBPs 1, 2 and 3 [94]. *C. difficile* has generally been considered susceptible to carbapenems, but resistance has begun to be reported [54] primarily in RT017 [95], which have been found with imipenem-resistance mutations in the active site of penicillin-binding protein genes *pbp1* and *pbp3* [54, 81]. A fifth *pbp5* on a mobile element was also found in resistant strains, but its effects are unclear [95].

Resistance to cephalosporins is high in *C. difficile*, and their widespread use is recognised as a high risk for inducing CDI [6], while penicillins and carbapenems have significantly lower MICs [92]. According to Toth et al., testing of several β-lactam antimicrobials (ampicillin, penicillin G, oxacillin, cephalothin, cefotaxime, ceftriaxone, ceftazidime, cefepime, aztreonam, imipenem and meropenem) revealed that the highest MICs were to aztreonam, followed by ceftazidime, cefotaxime and ceftazidime, with the lowest MICs to meropenem, followed by ampicillin, penicillin G and imipenem [92].

Efflux mechanisms

Efflux pumps are classified into ATP-binding cassette (ABC) transporters or secondary multidrug transporters, which are further divided into resistance-nodulation-cell division (RND), small multidrug resistance, major facilitator superfamily (MFS) and multidrug and toxic compound extrusion (MATE) groups [23, 96]. Their role in *C. difficile* AMR has not been clearly described; however, *C. difficile* does possess efflux pump genes known to confer resistance when tested in other species; the *cdeA* gene encodes an MATE efflux mechanism that confers resistance to norfloxacin and ciprofloxacin in *E. coli*, while the *cme* MFS mechanism confers erythromycin resistance in *E. faecalis* [23, 97]. The *cdeA* transporter conferred resistance only under the control of the *Plac* promoter, not its natural promoter [98].

ABC transporters conferring AMR have been found in other *Clostridium* spp. as well, including *cmpA* in *C. hathewayi* and CPE1506 in *C. perfringens* [99]. A putative ABC transporter is also present in *C. difficile*; *cd2068* expression is induced by antimicrobials (cloxacillin, ampicillin, ceftiofur, ciprofloxacin, gentamicin and levofloxacin) and confers increased resistance to these antimicrobials [99]. Interestingly, *C. difficile* has an efflux pump complex homologous to the TolC pump found in *E. coli*, in which it locates in the outer membrane and thus has not otherwise been found in Gram-positive species [96]. Mutants lacking this complex had their growth inhibited by tetracyclines and penicillins as well as acidic pH, fungicides and some inorganic and organic compounds not used against *C. difficile* [96].

Multidrug resistance

MDR in *C. difficile*, defined as resistance to three or more antimicrobial agents, is common and usually involves clindamycin, erythromycin, fluoroquinolones and cephalosporins [6, 44]. Like AMR rates, MDR rates vary by location and strain type. The majority of epidemic and emerging strains such as *C. difficile* RTs 176 and 356/607 are MDR [6]. In Europe, MDR estimates varied between 28 and 77%, with RTs 027, 017 and 198 resistant to five or more antimicrobials and some strains even resistant to nine antimicrobials [6, 27, 35]. Oddly, rifampicin resistance was always associated with moxifloxacin resistance in one study [35]. In the Asia-Pacific region, multidrug resistance was present in 100% of RT369, 92.7% of RT018 and 66.1% of RT017 [44]. Li et al. found MDR varied from 5.8% in Southwest China to 43.75% in South China and suggested increasing wealth correlated to increasing MDR [66], possibly due to the availability of more drug classes. MDR also remained

common in US RT027 strains, with a significant increase in co-resistance to clindamycin, moxifloxacin, rifamycins and tetracyclines over time [25].

Difficulties in determining AMR in *C. difficile*

Characterising AMR in a strain of *C. difficile* can be surprisingly difficult. Heteroresistance (small numbers of resistant cells within a susceptible population) and reduced susceptibility can prevent accurate determination by standard susceptibility tests [4]. Treatment failure unrelated to known resistance mechanisms also occurs. While known mutations in *gyrA*, *gyrB* and *rpoB* were highly concordant with their respective resistance phenotypes [46], the same cannot be said for other resistance mechanisms, highlighting a key issue with modern methods of AMR determination *in silico*: While *in silico* methods can provide rapid, high-volume detection of many antimicrobial resistance determinants at once, genotype does not always correlate with phenotype. In a recent study of Australian *C. difficile* ST11 strains, *in silico* AMR screening matched well (100%) with agar dilution MIC results for fluoroquinolones and tetracycline, but only poorly for MLS_{Bs} (36%, screened using *ermB*), further indicating alternate mechanisms exist [100].

In silico methods also depend on comprehensive knowledge of genetic features to search for, and in many cases, this information is not yet known, or there is a delay between discovery and implementation in databases. Figure 4 provides an overview of potential resistance-conferring loci. While large AMR gene databases and search tools exist, there are several, each with differences, and they require a separate set of skills from *in vitro* testing. *In vitro* testing is not immune to these challenges either, with a variety of methods and standards in use making comparisons difficult. Antimicrobial susceptibility tests include the agar dilution assay (gold standard), E-tests (most commonly used [6, 8]) and broth microdilution [4]. The E-test is less laborious than agar dilution but can have discrepancies in MICs which are generally lower than those determined by agar dilution [4, 67]. Many methods are based on set ‘checkpoints’ of concentration or dilution, thus limiting exact measurements, and chemical properties, e.g. solubility, may limit testing of concentrations outside a set range [11]. Even only using the E-test, one study found clindamycin MICs changed around the resistance breakpoint depending on how MIC endpoints were read, either via the product insert or using methods described in the literature [25]. When MICs cluster around breakpoints (as is common with cephalosporins), small changes (e.g. changes in media or testing method) can have large consequences for perceived resistance rates [101].

Similar strains may also compromise testing; in one study, a newly discovered clindamycin-resistant strain

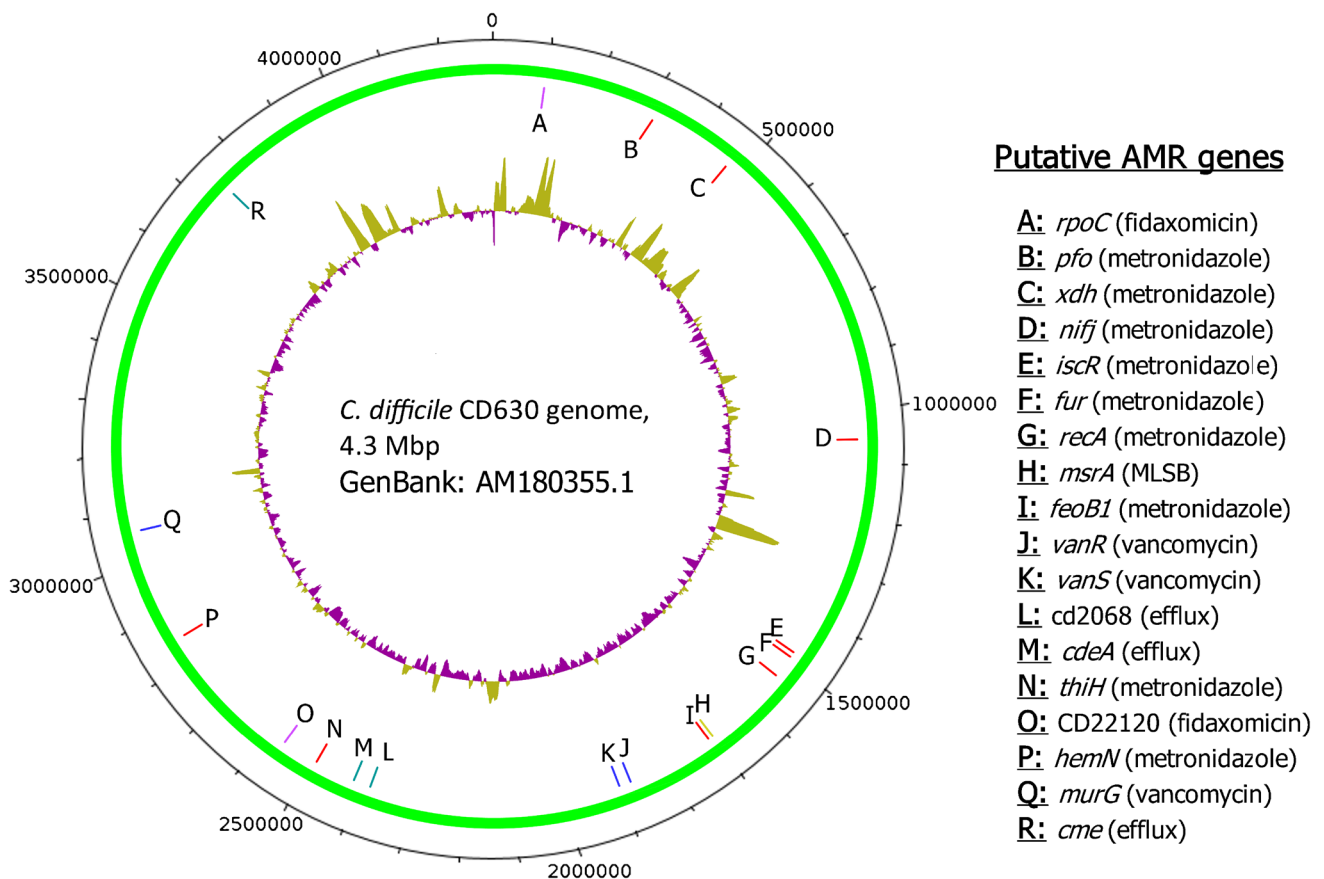


Fig. 4 Genomic loci suggested to play a role in AMR in *C. difficile*. Graphical representation of the *C. difficile* genome with loci that have been implicated as potentially playing a role in AMR indicated by coloured lines (red=metronidazole, blue=vancomycin,

purple=fidaxomicin, teal=efflux, yellow=MLSB). These genes are listed beside with gene names and the suggested antimicrobial affected. GC content is indicated on the inner circle, with above average in yellow and below average in purple

RT591 was misidentified via Xpert *C. difficile* PCR (Cepheid) as RT027 due to similar genetic composition of the PaLoc and presence of *cdtB* [102]. *C. difficile* RT244 in an Australian outbreak was also misidentified as RT027 by the same test [103].

One of the most common issues for in vitro testing is the lack of a single standard, making comparisons between studies difficult. Various measures are reported such as weighted pooled resistance (WPR), MIC₅₀ and MIC₉₀, S/I/R classifications, cumulative mean resistance, high- or low-level resistance, or resistance prevalence. Clinical breakpoints characterise microbes as susceptible, intermediate or resistant depending on whether a level of antimicrobial activity is associated with a likelihood of treatment success or failure [104]. Breakpoints from CLSI, EUCAST and prior literature are used, with some taken from their use in other bacteria as breakpoints are not available [5]. Epidemiological cut-off values may also be used, characterising microbes as wild type (absence of acquired or mutational resistance mechanism) or non-wild type (presence of acquired or mutational resistance mechanism) [104].

EUCAST and CLSI guidelines are the most commonly used in determining the classification of bacteria as susceptible/intermediate/resistant [67]; however, the breakpoints of each are not equivalent. For metronidazole, for example, the CLSI breakpoint is ≥ 32 mg/L [105], while the EUCAST breakpoint is ≥ 2 mg/L [106]. One study comparing AMR in several Gram-negative bacteria found that resistance rates were dramatically different with different breakpoints, as much as 52.3% (EUCAST) vs 19.9% (CLSI) for amoxicillin-clavulanic acid [107]. Also, the CLSI breakpoint is based on serum rather than intestinal concentration, but ESCMID guidelines are based on intestinal activity [67]. Due to differences in classification schemes and disagreement between methods, some groups have suggested that the use of direct MIC data would better enable comparisons between studies [5].

On a broader scale, it can be difficult to definitively state levels of resistance of certain *C. difficile* populations. Saha et al. analysed 60 studies between 1982 and 2017, including data from 27 countries and over 8000 isolates [5], and found the majority of reports were from Europe or North America.

Unfortunately, this is a consistent issue with limited publications from many regions, e.g. Africa and many parts of Asia. Antimicrobial susceptibility testing is not routinely performed for *C. difficile* in most places [30], and diagnostic assays for CDI differ between locations, affecting the recruitment of cases/strains for study [44]. The quality of studies also has a statistically significant impact on AMR, with vancomycin resistance threefold higher in studies rated low quality compared to those rated high quality [8].

Adding to these challenges is the sheer diversity in this species. *C. difficile* has one of the lowest gene conservation rates of all bacterial species, with only an estimated 16–32% of genes conserved [108, 109] out of a large, open pangenome of over 10,000 genes [100, 108, 109]. Moreover, *C. difficile* is taxonomically incoherent, displaying genomic characteristics representative of distinct species divided along with the major evolutionary clades [110]. It also has a highly mobile genome with a demonstrated capacity to exchange genes with other species [46, 108], enhanced by the ability of *C. difficile* to persist as spores, and its niche within environments considered hotspots for genetic exchange (such as the human gut, soil and sewage) [49, 62]. In Southeast Asia, more than half the non-toxicogenic strains studied carried mobile elements featuring AMR genes, many of which were similar to those found in other bacterial species of the porcine gut, such as *E. rhusiopathiae* and *S. suis* [46]. AMR genes included *tetM*, *ermB*, *tetO*, *tetB*, *catP*, the *ant(6)-iae aph(30)-III sat4A* cluster and *aac(60)-Ie-aph(200)-Ia* [46]. While non-toxicogenic strains have been suggested as an option for reducing CDI due to the protective effect they have against toxicogenic strains [111], their ability to gain or pass on resistance genes to other bacteria remains a risk.

Epidemic strains are typically more resistant than non-epidemic counterparts [62, 67], with a lower diversity of RTs also correlated with increased AMR [28]. *C. difficile* RT017 (common in Asia) was reported to have greater resistance to several antimicrobials [44] as well as higher rates of MDR. Analysis of isolates from the Asia–Pacific region found that RT014/020 and RT002 (both common in Australia) to have lower rates of resistance [44, 54]. *C. difficile* RTs 027, 001/072, 017, 018 and 356 were associated with resistance in Europe [28]. Surviving antimicrobial treatment may not be the only benefit to resistant strains, with Baines and Wilcox suggesting strains with reduced susceptibility hold a competitive advantage as they would be able to recolonise the gut sooner after antimicrobial treatment than other strains [11].

Several analyses showed that rates of AMR varied significantly not just with strain but with location (country and continent), wealth and patient age (with AMR increasing to a peak in the 65–85 year age group) [8, 44, 66, 67]. As the evolution of resistance is largely driven by selective

pressure, differences in population dynamics and antimicrobial use policies explain much of this finding [9, 28]. An excellent example of this is the rapid expansion of *C. difficile* RT027 across North America where fluoroquinolone usage was high [9], compared to the scarcity and much-delayed development of the same strain in Australia, where fluoroquinolones were strictly limited [62], although the lack of a local reservoir likely contributed to the failure of RT027 to establish in Australia.

Economic factors may also play a part. The association with the gross domestic product (inversely correlated to combined AMR) [44] may be due to a host of factors such as less stringent antimicrobial stewardship, poorer health-care infrastructure and increased rates of other pathogens requiring antimicrobial treatment. Many Asian countries have some of the world's highest antimicrobial consumption rates, with availability often not requiring prescriptions nor appropriate diagnosis [44]. Australia, in contrast, with higher wealth and strict antimicrobial stewardship policies, had the lowest mean cumulative resistance score (2.58) of 12 Asia–Pacific countries [44].

A solution to these issues is unlikely to be easily found. In silico testing appears promising with its ability to avoid much of the methodological variance of in vitro testing and the increasing use of genome sequencing. Further research to identify currently unknown genetic determinants, as well as a greater scope of gene search databases to include potential AMR acquisition from other species, would greatly improve the predictive value of in silico testing.

Barring the development of a quick, easy, cheap and effective testing method suitable for both clinical use and the research lab, there are simply too many factors at play to easily resolve the variance in physical testing around the world. Perhaps a more achievable solution would be to improve data sharing and understanding of these factors so that they may be taken into account when determining resistance. Large databases are already widely used for a variety of purposes, such as the ENA, GenBank or CARD, and enable large-scale analyses by pooling research from around the globe. Integration of susceptibility testing data, along with methods used, into a centralised database may enable a greater understanding of the factors influencing the determination of resistance. Such a database would ideally allow for filtering and comparison of methodology factors (such as test used, media, storage and breakpoints) to account for their effects. This would enable comparisons of resistance between datasets using different methods without requiring global standardisation of laboratories with different needs and resources.

Concluding remarks

AMR in *C. difficile* remains a serious threat and burden to healthcare systems, with many mechanisms of resistance still poorly understood and MDR widespread. Treatment options are narrowing, with both metronidazole and vancomycin seeing increasing treatment failures with no clear answer, while rifaximin is especially vulnerable to the rapid development of resistance. Evidence is increasingly pointing towards a diverse array of environmental and animal reservoirs of *C. difficile* with the transfer of AMR genes to and from other species. Resistance-conferring plasmids have now been identified, while previously, they were assumed to play no role in *C. difficile* AMR.

With high diversity, a mobile resistome and new resistance mechanisms being discovered, ongoing genomic-focused public health surveillance of *C. difficile* is critical for understanding and combating CDI. Yet, determining AMR in *C. difficile* remains challenging for a variety of genetic, epidemiological and practical reasons. Continuing AMR research with a One Health perspective, as well as improvements to collaborative data collection, distribution and use, will be key to combating this pathogen in the future.

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References

- O'Neill J (2016) Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance. Web citation: <https://amr-review.org/>. Accessed 20 Apr 2021
- Centers for Disease Control and Prevention (2013) CDC antibiotic resistance threats in the United States, 2013. Web citation: <http://www.cdc.gov/drugresistance/threat-report-2013/>. Accessed 20 Apr 2021
- Centers for Disease Control and Prevention (2019) CDC antibiotic resistance threats in the United States, 2019. Web citation: <https://www.cdc.gov/drugresistance/biggest-threats.html>. Accessed 20 Apr 2021
- Peng Z, Jin D, Kim HB, Stratton CW, Wu B, Tang YW, Sun X (2017) Update on antimicrobial resistance in *Clostridium difficile*: resistance mechanisms and antimicrobial susceptibility testing. *J Clin Microbiol* 55(7):1998–2008
- Saha S, Kapoor S, Tariq R, Schuetz AN, Tosh PK, Pardi DS, S K, (2019) Increasing antibiotic resistance in *Clostridioides difficile*: a systematic review and meta-analysis. *Anaerobe* 58:35–46
- Spigaglia P, Mastrantonio P, Barbanti F (2018) Antibiotic resistances of *Clostridium difficile*. Springer International Publishing, Cham, pp 137–159
- Slimings C, Riley TV (2014) Antibiotics and hospital-acquired *Clostridium difficile* infection: update of systematic review and meta-analysis. *J Antimicrob Chemother* 69(4):881–891
- Sholeh M, Krutova M, Forouzesh M, Mironov S, Sadeghifard N, Molaeipour L, Maleki A, Kouhsari E (2020) Antimicrobial resistance in *Clostridioides (Clostridium) difficile* derived from humans: a systematic review and meta-analysis. *Antimicrob Resist Infect Control* 9(1):158
- Hong S, Knight DR, Riley TV (2019) The impact of antimicrobial resistance on induction, transmission and treatment of *Clostridium difficile* infection. *Microbiol Aust* 40(2):77–81
- Deshpande A, Pasupuleti V, Thota P, Pant C, Rolston DDK, Sfera TJ, Hernandez AV, Donskey CJ (2013) Community-associated *Clostridium difficile* infection and antibiotics: a meta-analysis. *J Antimicrob Chemother* 68(9):1951–1961
- Baines SD, Wilcox MH (2015) Antimicrobial resistance and reduced susceptibility in *Clostridium difficile*: potential consequences for induction, treatment, and recurrence of *C. difficile* infection. *Antibiotics (Basel)* 4(3):267–298
- Rao K, Malani PN (2020) Diagnosis and treatment of *Clostridioides (Clostridium) difficile* infection in adults in 2020. *J Am Med Assoc* 323(14):1403–1404
- McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH (2018) Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* 66(7):e1–e48
- Trubiano JA, Cheng AC, Korman TM, Roder C, Campbell A, May MLA, Blyth CC, Ferguson JK, Blackmore TK, Riley TV, Athan E (2016) Australasian Society of Infectious Diseases updated guidelines for the management of *Clostridium difficile* infection in adults and children in Australia and New Zealand. *J Intern Med* 46(4):479–493
- Debast SB, Bauer MP, Kuijper EJ (2014) European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection. *Clin Microbiol Infect* 20:1–26
- Carlson TJ, Gonzales-Luna AJ (2020) Antibiotic treatment pipeline for *Clostridioides difficile* infection (CDI): a wide array of narrow-spectrum agents. *Curr Infect Dis Rep* 22(8):20
- Pu M, Cho JM, Cunningham SA, Behera G, Becker S, Amjad T, Greenwood-Quaintance KE, Mendes-Soares H, Jones-Hall Y, Jeraldo PR, Chen J, Dunny G, Patel R, Kashyap PC (2020) Plasmid acquisition alters vancomycin susceptibility in *Clostridioides difficile*. *Gastroenterology* 160(3):941–945
- The European Committee on Antimicrobial Susceptibility Testing (2021) Breakpoint tables for interpretation of MICs and

- zone diameters. Web citation: https://eucast.org/clinical_break_points/?debug=1. Accessed 02 Mar 2021
19. Woods E, Wetzl D, Mukerjee M, McBride S (2018) Examination of the *Clostridioides (Clostridium) difficile* VanZ ortholog, CD1240. *Anaerobe* 53:108–115
 20. Ammam F, Marvaud J, Lambert T (2012) Distribution of the vanG-like gene cluster in *Clostridium difficile* clinical isolates. *Can J Microbiol* 58:547–551
 21. Shen WJ, Deshpande A, Hevener KE, Endres BT, Garey KW, Palmer KL, Hurdle JG (2020) Constitutive expression of the cryptic vanGCd operon promotes vancomycin resistance in *Clostridioides difficile* clinical isolates. *J Antimicrob Chemother* 75(4):859–867
 22. Knight DR, Androga GO, Ballard SA, Howden BP, Riley TV (2016) A phenotypically silent vanB2 operon carried on a Tn1549-like element in *Clostridium difficile*. *mSphere* 1(4):e00177-00116
 23. Harnvoravongchai P, Pipatthana M, Chankhamhaengdech S, Janvilisri T (2017) Insights into drug resistance mechanisms in *Clostridium difficile*. *Essays Biochem* 61(1):81–88
 24. Jarrad AM, Blaskovich MAT, Prasetyoputri A, Karoli T, Hansford KA, Cooper MA (2018) Detection and investigation of eagle effect resistance to vancomycin in *Clostridium difficile* with an ATP-bioluminescence assay. *Front Microbiol* 9:1420
 25. Tickler IA, Obradovich AE, Goering RV, Fang FC, Tenover FC, Consortium TH, Consortium HAI (2019) Changes in molecular epidemiology and antimicrobial resistance profiles of *Clostridioides (Clostridium) difficile* strains in the United States between 2011 and 2017. *Anaerobe* 60:102050
 26. Krutova M, Capek V, Nycova E, Vojackova S, Balejova M, Geigerova L, Tejkalova R, Havlina L, Vagnerova I, Cermak P, Ryskova L, Jezek P, Zamazalova D, Vesela D, Kucharova A, Nemcova D, Curdova M, Nyc O, Drevinek P (2020) The association of a reduced susceptibility to moxifloxacin in causative *Clostridium (Clostridioides) difficile* strain with the clinical outcome of patients. *Antimicrob Resist Infect Control* 9(1):98
 27. Freeman J, Vernon J, Pilling S, Morris K, Nicholson S, Shearman S, Longshaw C, Wilcox MH (2018) The ClosER study: results from a three-year pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes, 2011–2014. *Clin Microbiol Infect* 24(7):724–731
 28. Freeman J, Vernon J, Morris K, Nicholson S, Todhunter S, Longshaw C, Wilcox MH (2015) Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. *Clin Microbiol Infect* 21(3):248.e249–248.e216
 29. Dingsdag SA, Hunter N (2017) Metronidazole: an update on metabolism, structure–cytotoxicity and resistance mechanisms. *J Antimicrob Chemother* 73(2):265–279
 30. Boekhoud IM, Hornung BVH, Sevilla E, Harmanus C, Bos-Sanders I, Terveer EM, Bolea R, Corver J, Kuijper EJ, Smits WK (2020) Plasmid-mediated metronidazole resistance in *Clostridioides difficile*. *Nat Commun* 11(1):598
 31. Moura I, Monot M, Tani C, Spigaglia P, Barbanti F, Norais N, Dupuy B, Bouza E, Mastrantonio P (2014) Multidisciplinary analysis of a nontoxigenic *Clostridium difficile* strain with stable resistance to metronidazole. *Antimicrob Agents Chemother* 58(8):4957–4960
 32. Chong PM, Lynch T, McCorrister S, Kibsey P, Miller M, Gravel D, Westmacott GR, Mulvey MR, the Canadian Nosocomial Infection Surveillance P (2014) Proteomic analysis of a NAP1 *Clostridium difficile* clinical isolate resistant to metronidazole. *Plos One* 9(1):e82622
 33. Deshpande A, Wu X, Huo W, Palmer KL, Hurdle JG (2020) Chromosomal resistance to metronidazole in *Clostridioides difficile* can be mediated by epistasis between iron homeostasis and oxidoreductases. *Antimicrob Agents Chemother* 64(8):e00415-00420
 34. Boekhoud IM, Sidorov I, Nooij S, Harmanus C, Bos-Sanders IMJG, Viprey V, Spittal W, Clark E, Davies K, Freeman J, Kuijper EJ, Smits WK, Consortium C-C (2021) Haem is crucial for medium-dependent metronidazole resistance in clinical isolates of *Clostridioides difficile*. *J Antimicrob Chemother*:dkab09776(7):1731–1740. <https://doi.org/10.1093/jac/dkab097>
 35. Spigaglia P, Barbanti F, Mastrantonio P, on behalf of the European Study Group on *Clostridium difficile*, Ackermann G, Balmelli C, Barbut F, Bouza E, Brazier J, Delmée M, Drudy D, Kuijper E, Ladas H, Mastrantonio P, Nagy E, Pituch H, Poxton I, Rupnik M, Wullt M, Yücesoy M (2011) Multidrug resistance in European *Clostridium difficile* clinical isolates. *J Antimicrob Chemother* 66(10):2227–2234
 36. Lynch T, Chong P, Zhang J, Hizon R, Du T, Graham MR, Beniac DR, Booth TF, Kibsey P, Miller M, Gravel D, Mulvey MR, Canadian Nosocomial Infection Surveillance Program (2013) Characterization of a stable, metronidazole-resistant *Clostridium difficile* clinical isolate. *PLoS One* 8(1):e53757
 37. Peláez T, Cercenado E, Alcalá L, Marín M, Martín-López A, Martínez-Alarcón J, Catalán P, Sánchez-Somolinos M, Bouza E (2008) Metronidazole resistance in *Clostridium difficile* is heterogeneous. *J Clin Microbiol* 46(9):3028–3032
 38. Krutova A, Kinross P, Barbut F, Hajdu A, Wilcox MH, Kuijper EJ (2017) How to: surveillance of *Clostridium difficile* infections. *Clin Microbiol Infect* 24:469–475
 39. Artsimovitch I, Seddon J, Sears P (2012) Fidaxomicin is an inhibitor of the initiation of bacterial RNA synthesis. *Clin Infect Dis* 55(Suppl 2):S127–S131
 40. Kuehne SA, Dempster AW, Collery MM, Joshi N, Jowett J, Kelly ML, Cave R, Longshaw CM, Minton NP (2018) Characterization of the impact of rpoB mutations on the in vitro and in vivo competitive fitness of *Clostridium difficile* and susceptibility to fidaxomicin. *J Antimicrob Chemother* 73(4):973–980
 41. Sears P, Crook DW, Louie TJ, Miller MA, Weiss K (2012) Fidaxomicin attains high fecal concentrations with minimal plasma concentrations following oral administration in patients with *Clostridium difficile* infection. *Clin Infect Dis* 55:S116–S120
 42. Schwanbeck J, Riedel T, Laukien F, Schober I, Oehmig I, Zimmermann O, Overmann J, Groß U, Zautner AE, Bohne W (2019) Characterization of a clinical *Clostridioides difficile* isolate with markedly reduced fidaxomicin susceptibility and a V1143D mutation in *rpoB*. *J Antimicrob Chemother* 74(1):6–10
 43. Nelson RL, Suda KJ, Evans CT (2017) Antibiotic treatment for *Clostridium difficile*-associated diarrhoea in adults. *Cochrane Database Syst Rev* 3(3):CD004610
 44. Lew T, Putsathit P, Sohn KM, Wu Y, Ouchi K, Ishii Y, Tateda K, Riley TV, Collins DA (2020) Antimicrobial susceptibilities of *Clostridium difficile* isolates from 12 Asia-Pacific countries in 2014 and 2015. *Antimicrob Agents Chemother* 64(7):e00296-e220
 45. Mascio CT, Chesnel L, Thorne G, Silverman JA (2014) Surotomycin demonstrates low in vitro frequency of resistance and rapid bactericidal activity in *Clostridium difficile*, *Enterococcus faecalis*, and *Enterococcus faecium*. *Antimicrob Agents Chemother* 58(7):3976–3982
 46. Imwattana K, Kiratisin P, Riley TV, Knight DR (2020) Genomic basis of antimicrobial resistance in non-toxigenic *Clostridium difficile* in Southeast Asia. *Anaerobe* 66:102290
 47. Carman RJ, Boone JH, Grover H, Wickham KN, Chen L (2012) In vivo selection of rifamycin-resistant *Clostridium difficile* during rifaximin therapy. *Antimicrob Agents Chemother* 56(11):6019–6020

48. World Health Organisation (2020) Global Tuberculosis Report 2020. Web citation: <https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2020>. Accessed 10 May 2021
49. Chatedaki C, Voulgaridi I, Kachrimanidou M, Hrabak J, Papagiannitsis CC, Petinaki E (2019) Antimicrobial susceptibility and mechanisms of resistance of Greek *Clostridium difficile* clinical isolates. *J Glob Antimicrob Resist* 16:53–58
50. Carlson TJ, Endres BT, Bassères E, Gonzales-Luna AJ, Garey KW (2019) Ridinilazole for the treatment of *Clostridioides difficile* infection. *Expert Opin Investig Drugs* 28(4):303–310
51. Vickers RJ, Tillotson GS, Nathan R, Hazan S, Pullman J, Lucasti C, Deck K, Yacyshyn B, Maliakkal B, Pesant Y, Tejura B, Roblin D, Gerding DN, Wilcox MH, Dsg Co (2017) Efficacy and safety of ridinilazole compared with vancomycin for the treatment of *Clostridium difficile* infection: a phase 2, randomised, double-blind, active-controlled, non-inferiority study. *Lancet Infect Dis* 17(7):735–744
52. Adams HM, Li X, Mascio C, Chesnel L, Palmer KL (2015) Mutations associated with reduced surotomycin susceptibility in *Clostridium difficile* and *Enterococcus* species. *Antimicrob Agents Chemother* 59(7):4139–4147
53. Oliphant CM, Green GM (2002) Quinolones: a comprehensive review. *Am Fam Physician* 65(3):455–464
54. Imwattana K, Knight DR, Kullin B, Collins DA, Putsathit P, Kiratisin P, Riley TV (2020) Antimicrobial resistance in *Clostridium difficile* ribotype 017. *Expert Rev Anti Infect Ther* 18(1):17–25
55. Spigaglia P (2016) Recent advances in the understanding of antibiotic resistance in *Clostridium difficile* infection. *Ther Adv Infect Dis* 3(1):23–42
56. Mackin KE, Elliott B, Kotsanas D, Howden BP, Carter GP, Korman TM, Riley TV, Rood JI, Jenkin GA, Lyras D (2015) Molecular characterization and antimicrobial susceptibilities of *Clostridium difficile* clinical isolates from Victoria, Australia. *Anaerobe* 34:80–83
57. Wasels F, Kuehne S, Cartman S, Spigaglia P, Barbanti F, Minton N, Mastrantonio P (2014) Fluoroquinolone resistance does not impose a cost on the fitness of *Clostridium difficile* in vitro. *Antimicrob Agents Chemother* 59(3):1794–1796
58. Mac Aogáin M, Kilkenny S, Walsh C, Lindsay S, Moloney G, Morris T, Jones S, Rogers TR (2015) Identification of a novel mutation at the primary dimer interface of GyrA conferring fluoroquinolone resistance in *Clostridium difficile*. *J Glob Antimicrob Resist* 3(4):295–299
59. Pellissery AJ, Vinayamohan PG, Yin H-B, Mooyottu S, Venkitanarayanan K (2019) In vitro efficacy of sodium selenite in reducing toxin production, spore outgrowth and antibiotic resistance in hypervirulent *Clostridium difficile*. *J Med Microbiol* 68(7):1118–1128
60. Linder JA, Huang ES, Steinman MA, Gonzales R, Stafford RS (2005) Fluoroquinolone prescribing in the United States: 1995 to 2002. *Am J Med* 118(3):259–268
61. Niederman MS, Mandell LA, Anzueto A, Bass JB, Broughton WA, Campbell GD, Dean N, File T, Fine MJ, Gross PA, Martinez F, Marrie TJ, Plouffe JF, Ramirez J, Sarosi GA, Torres A, Wilson R, Yu VL (2001) Guidelines for the management of adults with community-acquired pneumonia. *Am J Respir Crit Care Med* 163(7):1730–1754
62. Knight DR, Riley TV (2016) *Clostridium difficile* clade 5 in Australia: antimicrobial susceptibility profiling of PCR ribotypes of human and animal origin. *J Antimicrob Chemother* 71(8):2213–2217
63. Knight DR, Giglio S, Huntington PG, Korman TM, Kotsanas D, Moore CV, Paterson DL, Prendergast L, Huber CA, Robson J, Waring L, Wehrhahn MC, Weldhagen GF, Wilson RM, Riley TV (2015) Surveillance for antimicrobial resistance in Australian isolates of *Clostridium difficile*, 2013–14. *J Antimicrob Chemother* 70(11):2992–2999
64. Australian Commission on Safety and Quality in Health Care (2018) *Clostridium difficile* infection. Monitoring the national burden of *Clostridium difficile*. Sydney. Web citation: <https://www.safetyandquality.gov.au/publications-and-resources/resource-library/monitoring-national-burden-clostridium-difficile-infection>. Accessed 21 Oct 2021
65. Alyousef AA (2018) *Clostridium difficile*: epidemiology, pathogenicity, and an update on the limitations of and challenges in its diagnosis. *J AOAC Int* 101(4):1119–1126
66. Li H, Li W-G, Zhang W-Z, Yu S-B, Liu Z-J, Zhang X, Wu Y, Lu J-X (2019) Antibiotic resistance of clinical isolates of *Clostridioides difficile* in China and its association with geographical regions and patient age. *Anaerobe* 60:102094
67. Banawas SS (2018) *Clostridium difficile* infections: a global overview of drug sensitivity and resistance mechanisms. *Biomed Res Int* 2018:8414257
68. Schmidt C, Löffler B, Ackermann G (2007) Antimicrobial phenotypes and molecular basis in clinical strains of *Clostridium difficile*. *Diagn Microbiol Infect Dis* 59(1):1–5
69. Dingle KE, Didelot X, Quan TP, Eyre DW, Stoesser N, Marwick CA, Coia J, Brown D, Buchanan S, Ijaz UZ, Goswami C, Douce G, Fawley WN, Wilcox MH, Peto TEA, Walker AS, Crook DW (2019) A role for tetracycline selection in recent evolution of agriculture-associated *Clostridium difficile* PCR ribotype 078. *mBio* 10(2):e02790-02718
70. Dong D, Zhang L, Chen X, Jiang C, Yu B, Wang X, Peng Y (2013) Antimicrobial susceptibility and resistance mechanisms of clinical *Clostridium difficile* from a Chinese tertiary hospital. *Int J Antimicrob Agents* 41(1):80–84
71. Knetsch CW, Kumar N, Forster SC, Connor TR, Browne HP, Harmanus C, Sanders IM, Harris SR, Turner L, Morris T, Perry M, Miyajima F, Roberts P, Pirmohamed M, Songer JG, Weese JS, Indra A, Corver J, Rupnik M, Wren BW, Riley TV, Kuijper EJ, Lawley TD (2018) Zoonotic transfer of *Clostridium difficile* harboring antimicrobial resistance between farm animals and humans. *J Clin Micro* 56(3):e01384-e1317
72. Knetsch CW, Connor TR, Mutreja A, van Dorp SM, Sanders IM, Browne HP, Harris D, Lipman L, Keessen EC, Corver J, Kuijper EJ, Lawley TD (2014) Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011. *Euro Surveill* 19(45):20954
73. Werner A, Mölling P, Fagerström A, Dyrkell F, Arnellos D, Johansson K, Sundqvist M, Norén T (2020) Whole genome sequencing of *Clostridioides difficile* PCR ribotype 046 suggests transmission between pigs and humans. *PLoS One* 15(12):e0244227
74. Knight DR, Squire MM, Collins DA, Riley TV (2017) Genome analysis of *Clostridium difficile* PCR ribotype 014 lineage in Australian pigs and humans reveals a diverse genetic repertoire and signatures of long-range interspecies transmission. *Front Microbiol* 7:2138
75. Vidor CJ, Bulach D, Awad M, D L, (2019) *Paeniclostridium sordellii* and *Clostridioides difficile* encode similar and clinically relevant tetracycline resistance loci in diverse genomic locations. *BMC Microbiol* 19(1):53
76. Cheknis A, Devaris D, Chesnel L, Dale SE, Nary J, Sambol SP, Citron DM, Goering RV, Johnson S (2020) Characterization of *Clostridioides difficile* isolates recovered from two phase 3 surotomycin treatment trials by restriction endonuclease

- analysis, PCR ribotyping and antimicrobial susceptibilities. *J Antimicrob Chemother* 75(11):3120–3125
77. Begum K, Bassères E, Miranda J, Lancaster C, Gonzales-Luna AJ, Carlson TJ, Rashid T, Eyre DW, Wilcox MH, Alam MJ, Garey KW (2020) In vitro activity of omadacycline, a new tetracycline analog, and comparators against *Clostridioides difficile*. *Antimicrob Agents Chemother* 64(8):e00522–e520
 78. Zhao L, Luo Y, Bian Q, Wang L, Ye J, Song X, Jiang J, Tang Y-W, Wang X, Jin D (2020) High-level resistance of toxigenic *Clostridioides difficile* genotype to macrolide-lincosamide-streptogramin B in community acquired patients in Eastern China. *Infect Drug Resist* 13:171–181
 79. Ramírez-Hernández V, Ramírez-Vargas G (2020) A putative *erm* gene is present in Δ ermB *Clostridioides difficile* isolates showing high levels of resistance to clindamycin. *bioRxiv*:2020.2011.2020.391128. <https://doi.org/10.1101/2020.11.20.391128>
 80. Goh S, Hussain H, Chang BJ, Emmett W, Riley TV, Mullaney P (2013) Phage ϕ C2 mediates transduction of Tn6215, encoding erythromycin resistance, between strains. *mBio* 4(6):e00840-00813
 81. Isidro J, Menezes J, Serrano M, Borges V, Paixão P, Mimoso M, Martins F, Toscano C, Santos A, Henriques AO, Oleastro M (2018) Genomic study of a *Clostridium difficile* multidrug resistant outbreak-related clone reveals novel determinants of resistance. *Front Microbiol* 9(2994):2994
 82. Vester B (2018) The *cfr* and *cfr*-like multiple resistance genes. *Res Microbiol* 169(2):61–66
 83. Candela T, Marvaud JC, Nguyen TK, Lambert T (2017) A *cfr*-like gene *cfr*(C) conferring linezolid resistance is common in *Clostridium difficile*. *Int J Antimicrob Agents* 50(3):496–500
 84. Stojković V, Ulate MF, Hidalgo-Villeda F, Aguilar E, Monge-Cascante C, Pizarro-Guajardo M, Tsai K, Tzoc E, Camorlinga M, Paredes-Sabja D, Quesada-Gómez C, Fujimori DG, Rodríguez C (2019) *cfr*(B), *cfr*(C), and a new *cfr*-like gene, *cfr*(E), in *Clostridium difficile* strains recovered across Latin America. *Antimicrob Agents Chemother* 64(1):e01074–e1019
 85. Lim S-C, Androga GO, Knight DR, Moon P, Foster NF, Riley TV (2018) Antimicrobial susceptibility of *Clostridium difficile* isolated from food and environmental sources in Western Australia. *Int J Antimicrob Agents* 52(3):411–415
 86. Bozdogan B, Appelbaum PC (2004) Oxazolidinones: activity, mode of action, and mechanism of resistance. *Int J Antimicrob Agents* 23(2):113–119
 87. von der Hartmut Lode N, Höh SZ, KB, Carl Erik Nord, (2001) Ecological effects of linezolid versus amoxicillin/clavulanic acid on the normal intestinal microflora. *Scand J Infect Dis* 33(12):899–903
 88. Baines SD, Noel AR, Huscroft GS, Todhunter SL, O'Connor R, Hobbs JK, Freeman J, Lovering AM, Wilcox MH (2011) Evaluation of linezolid for the treatment of *Clostridium difficile* infection caused by epidemic strains using an in vitro human gut model. *J Antimicrob Chemother* 66(7):1537–1546
 89. Zabel LT, Worm S (2005) Linezolid contributed to *Clostridium difficile* colitis with fatal outcome. *Infection* 33(3):155–157
 90. Plattner M, Gysin M, Haldimann K, Becker K, Hobbie SN (2020) Epidemiologic, phenotypic, and structural characterization of aminoglycoside-resistance gene *aac*(3)-IV. *Int J Mol Sci* 21(17):6133
 91. Marsh JW, Pacey MP, Ezeonwuka C, Ohm SL, Snyder D, Cooper VS, Harrison LH, Doi Y, Mustapha MM (2019) *Clostridioides difficile*: a potential source of *npmA* in the clinical environment. *J Antimicrob Chemother* 74 (2):521–523
 92. Toth M, Stewart NK, Smith C, Vakulenko SB (2018) Intrinsic class D β -lactamases of *Clostridium difficile*. *mBio* 9(6):e01803-01818
 93. Sandhu BK, Edwards AN, Anderson SE, Woods EC, McBride SM (2019) Regulation and anaerobic function of the *Clostridioides difficile* β -lactamase. *Antimicrob Agents Chemother* 64(1):e01496–e1419
 94. Codjoe FS, Donkor ES (2017) Carbapenem resistance: a review. *Med Sci (Basel, Switzerland)* 6(1):1
 95. Isidro J, Santos A, Nunes A, Borges V, Silva C, Vieira L, Mendes AL, Serrano M, Henriques AO, Gomes JP, Oleastro M (2018) Imipenem resistance in *Clostridium difficile* ribotype 017. *Portugal Emerging infectious diseases* 24(4):741–745
 96. Lopez JME (2017) Characterization of an efflux pump system, the CD_AcrA-AcrB-ToIC complex, in *Clostridium difficile*. Kansas State University, Department of Plant Pathology, p 66
 97. Lebel S, Bouttier S, Lambert T (2004) The *cme* gene of *Clostridium difficile* confers multidrug resistance in *Enterococcus faecalis*. *FEMS Microbiol Lett* 238(1):93–100
 98. Dridi L, Tankovic J, Petit JC (2004) CdeA of *Clostridium difficile*, a new multidrug efflux transporter of the MATE family. *Microb Drug Resist* 10(3):191–196
 99. Ngernombat C, Sreesai S, Harnvoravongchai P, Chankhamhaengdecha S, Janvilisri T (2017) CD2068 potentially mediates multidrug efflux in *Clostridium difficile*. *Sci Rep* 7(1):9982–9982
 100. Knight DR, Kullin B, Androga GO, Barbut F, Eckert C, Johnson S, Spigaglia P, Tateda K, Tsai P-J, Riley TV (2019) Evolutionary and genomic insights into *Clostridioides difficile* sequence type 11: a diverse zoonotic and antimicrobial-resistant lineage of global one health importance. *mBio* 10(2):e00446-00419
 101. Brook I, Wexler HM, Goldstein EJC (2013) Antianaerobic antimicrobials: spectrum and susceptibility testing. *Clin Microbiol Rev* 26(3):526–546
 102. Skinner AM, Petrella L, Siddiqui F, Sambol SP, Gulvik CA, Gerding DN, Donskey CJ, Johnson S (2020) Unique clindamycin-resistant *Clostridioides difficile* strain related to fluoroquinolone-resistant epidemic BI/RT027 strain. *Emerg Infect Dis* 26(2):247–254
 103. Lim SK, Stuart RL, Mackin KE, Carter GP, Kotsanas D, Francis MJ, Easton M, Dimovski K, Elliott B, Riley TV, Hogg G, Paul E, Korman TM, Seemann T, Stinear TP, Lyras D, Jenkin GA (2014) Emergence of a ribotype 244 strain of *Clostridium difficile* associated with severe disease and related to the epidemic ribotype 027 strain. *Clin Infect Dis* 58(12):1723–1730
 104. The European Committee on Antimicrobial Susceptibility Testing (2021) EUCAST definitions of clinical breakpoints and epidemiological cut-off values, https://eucast.org/clinical_breakpoints/?debug=1. Accessed 18 Mar 2021
 105. Clinical and Laboratory Standards Institute (CLSI) (2020) Performance standards for antimicrobial susceptibility testing. CLSI supplement M100. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA (30th ed). <https://standards.globalspec.com/std/14371884/CLSI%20M100>
 106. The European Committee on Antimicrobial Susceptibility Testing (2021) Breakpoint tables for interpretation of MICs and zone diameters Version: 11.0. <http://www.eucast.org>
 107. Cusack TP, Ashley EA, Ling CL, Rattanavong S, Roberts T, Turner P, Wangrangsimakul T, Dance DAB (2019) Impact of CLSI and EUCAST breakpoint discrepancies on reporting of antimicrobial susceptibility and AMR surveillance. *Clin Microbiol Infect* 25(7):910–911
 108. Elliott B, Androga GO, Knight DR, Riley TV (2017) *Clostridium difficile* infection: evolution, phylogeny and molecular epidemiology. *Infect Genet Evol* 49:1–11
 109. Ferguson JK, Cheng AC, Gilbert GL, Gottlieb T, Korman T, McGregor A, Richards M, Roberts S, Robson J, Van Gessel H, Riley TV (2011) *Clostridium difficile* laboratory testing in

- Australia and New Zealand: national survey results and Australasian Society for Infectious Diseases recommendations for best practice. *Pathology* 43(5):482–487
110. Knight DR, Imwattana K, Kullin B, Guerrero-Araya E, Paredes-Sabja D, Didelot X, Dingle KE, Eyre DW, Rodríguez C, Riley TV (2020) The *Clostridioides difficile* species problem: global phylogenomic analysis uncovers three ancient, toxigenic, genospecies. *bioRxiv:2020.2009.2021.307223*
 111. Gerding DN, Sambol SP, Johnson S (2018) Non-toxicogenic *Clostridioides* (formerly *Clostridium*) *difficile* for prevention of *C. difficile* infection: from bench to bedside back to bench and back to bedside. *Front Microbiol* 9:1700
 112. Spigaglia P, Barbanti F, Mastrantonio P, Brazier JS, Barbut F, Delmée M, Kuijper E, I. RP, on behalf of the European Study Group on *Clostridium difficile* (2008) Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. *J Med Microbiol* 57 (6):784–789
 113. Kuwata Y, Tanimoto S, Sawabe E, Shima M, Takahashi Y, Ushizawa H, Fujie T, Koike R, Tojo N, Kubota T, Saito R (2015) Molecular epidemiology and antimicrobial susceptibility of *Clostridium difficile* isolated from a university teaching hospital in Japan. *Eur J Clin Microbiol Infect Dis* 34(4):763–772
 114. Liao CH, Ko WC, Lu JJ, Hsueh PR (2012) Characterizations of clinical isolates of *Clostridium difficile* by toxin genotypes and by susceptibility to 12 antimicrobial agents, including fidaxomicin (OPT-80) and rifaximin: a multicenter study in Taiwan. *Antimicrob Agents Chemother* 56(7):3943–3949
 115. Walkty A, Boyd DA, Gravel D, Hutchinson J, McGeer A, Moore D, Simor A, Suh K, Taylor G, Miller M, Mulvey MR (2010) Molecular characterization of moxifloxacin resistance from Canadian *Clostridium difficile* clinical isolates. *Diagn Microbiol Infect Dis* 66(4):419–424
 116. Huang H, Weintraub A, Fang H, Nord CE (2009) Antimicrobial resistance in *Clostridium difficile*. *Int J Antimicrob Agents* 34(6):516–522
 117. He M, Sebahia M, Lawley TD, Stabler RA, Dawson LF, Martin MJ, Holt KE, Seth-Smith HM, Quail MA, Rance R, Brooks K, Churcher C, Harris D, Bentley SD, Burrows C, Clark L, Corton C, Murray V, Rose G, Thurston S, van Tonder A, Walker D, Wren BW, Dougan G, Parkhill J (2010) Evolutionary dynamics of *Clostridium difficile* over short and long time scales. *Proc Natl Acad Sci U S A* 107(16):7527–7532
 118. Dingle KE, Elliott B, Robinson E, Griffiths D, Eyre DW, Stoesser N, Vaughan A, Golubchik T, Fawley WN, Wilcox MH, Peto TE, Walker AS, Riley TV, Crook DW, Didelot X (2014) Evolutionary history of the *Clostridium difficile* pathogenicity locus. *Genome Biol Evol* 6(1):36–52
 119. Wang H, Roberts AP, Lyras D, Rood JI, Wilks M, Mullany P (2000) Characterization of the ends and target sites of the novel conjugative transposon Tn5397 from *Clostridium difficile*: excision and circularization is mediated by the large resolvase. *TnX J Bacteriol* 182(13):3775–3783
 120. Roberts AP, Mullany P (2011) Tn916-like genetic elements: a diverse group of modular mobile elements conferring antibiotic resistance. *FEMS Microbiol Rev* 35(5):856–871
 121. Corver J, Bakker D, Brouwer MSM, Harmanus C, Hensgens MP, Roberts AP, Lipman LJA, Kuijper EJ, van Leeuwen HC (2012) Analysis of a *Clostridium difficile* PCR ribotype 078 100 kilobase island reveals the presence of a novel transposon, Tn6164. *BMC Microbiol* 12:130–130
 122. Lyras D, Storie C, Huggins AS, Crellin PK, Bannam TL, Rood JI (1998) Chloramphenicol resistance in *Clostridium difficile* is encoded on Tn4453 transposons that are closely related to Tn4451 from *Clostridium perfringens*. *Antimicrob Agents Chemother* 42(7):1563–1567
 123. O'Connor JR, Galang MA, Sambol SP, Hecht DW, Vedantam G, Gerding DN, Johnson S (2008) Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*. *Antimicrob Agents Chemother* 52(8):2813–2817
 124. Pecavar V, Blaschitz M, Hufnagl P, Zeinzinger J, Fiedler A, Allerberger F, Maass M, Indra A (2012) High-resolution melting analysis of the single nucleotide polymorphism hot-spot region in the *rpoB* gene as an indicator of reduced susceptibility to rifaximin in *Clostridium difficile*. *J Med Microbiol* 61(Pt 6):780–785
 125. Miller MA, Blanchette R, Spigaglia P, Barbanti F, Mastrantonio P (2011) Divergent rifamycin susceptibilities of *Clostridium difficile* strains in Canada and Italy and predictive accuracy of rifampin Etest for rifamycin resistance. *J Clin Microbiol* 49(12):4319–4321
 126. Carman RJ, Genheimer CW, Rafii F, Park M, Hiltonsmith MF, Lyrerly DM (2009) Diversity of moxifloxacin resistance during a nosocomial outbreak of a predominantly ribotype ARU 027 *Clostridium difficile* diarrhea. *Anaerobe* 15(6):244–248
 127. Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI, Pasculle AW, Harrison LH (2009) High frequency of rifampin resistance identified in an epidemic *Clostridium difficile* clone from a large teaching hospital. *Clin Infect Dis* 48(4):425–429
 128. Ackermann G, Tang YJ, Kueper R, Heisig P, Rodloff AC, Silva J Jr, Cohen SH (2001) Resistance to moxifloxacin in toxigenic *Clostridium difficile* isolates is associated with mutations in *gyrA*. *Antimicrob Agents Chemother* 45(8):2348–2353
 129. Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit JC (2002) *gyrA* and *gyrB* mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. *Antimicrob Agents Chemother* 46(11):3418–3421

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