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Mechanisms and Impact of Antimicrobial Resistance in *Clostridioides difficile*

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Abstract

The evolution of antimicrobial resistance in *Clostridioides difficile* has markedly shaped its epidemiology and detrimentally impacted patient care. *C. difficile* exhibits resistance to multiple classes of antimicrobials, due to accumulation of horizontally acquired resistance genes and *de novo* mutations to drug targets. Particularly worrying is that declines in clinical success of firstline CDI antimicrobials coincide with the spread of strains that are more resistant to these drugs. Yet, there is still much to learn regarding the prevalence of genetic elements in clinical isolates, their molecular mechanisms, and the extent to which this information can be translated to develop molecular diagnostics that improve antimicrobial prescribing and antimicrobial stewardship approaches for CDI. Thus, this perspective discusses current understanding and knowledge gaps of antimicrobial resistance mechanisms in *C. difficile*, emphasizing on CDI therapies.

Keywords

Antibiotic treatment failure; susceptibility testing; molecular diagnostics

THE IMPACT OF ANTIMICROBIAL RESISTANCE IN CDI

Clostridioides difficile infection (CDI) is a leading cause of antimicrobial-associated diarrhea in hospitalized elderly patients. For over 40 years, metronidazole and vancomycin have been the firstline therapies, while fidaxomicin, approved in 2011, has been largely used to treat recurrent disease (rCDI). Today, the antimicrobial therapeutic model for CDI has changed, where the 2021 IDSA/SHEA and ESCMID guidelines recommend fidaxomicin as the drug of choice, vancomycin as an alternative, and metronidazole only if the other two options are unavailable [1,2]. Prior to the 2021 antimicrobial therapy

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DECLARATIONS OF INTEREST

None.

guidelines, approximately 20% or more patients experience rCDI and 45–65% of these patients experience successive recurrent episodes [3]. Nonetheless, it is generally unclear how resistance to CDI antimicrobials influences treatment outcomes and the onset of rCDI (Figure 1). This is astonishing, since in other infections resistance is a common reason why antimicrobials fail. However, CDI treatment outcomes are not normally explained in the context of antimicrobial resistance (AMR), since anaerobic susceptibility testing of patient isolates is not routinely performed as part of the diagnostic work-up for CDI. Increasing reports of resistance to traditional and new CDI antibiotics warrant reevaluation of this view. *C. difficile* has also evolved resistance to fidaxomicin. In our view, the diagnostic work-up for CDI could be revolutionized by integrating rapid molecular diagnostics to identify AMR mechanisms in *C. difficile*. However, there is a fundamental need to delineate resistance mechanisms in *C. difficile*, in terms of their prevalence among clinical strains, impact on treatment responses, and effects on *C. difficile* pathophysiology. In Figure 2 and Table 1, we summarize the current knowledge of AMR mechanisms in *C. difficile*, with an emphasis on antimicrobial options for CDI. In this opinion article, we address the critical knowledge gaps in current understanding of genetically encoded resistance to antimicrobials in *C. difficile*, with the view of evaluating whether this information can be harnessed for molecular diagnostics and epidemiological surveillance, which could benefit prescribing practices and antimicrobial stewardship policies.

ACQUIRED RESISTANCE TO NON-CDI ANTIMICROBIALS

Almost all major classes of antimicrobials can induce CDI, but ampicillin, amoxicillin, cephalosporins, clindamycin and fluoroquinolones pose higher risk [4]. Although spores are naturally refractory to most antimicrobials, *C. difficile* has acquired resistance determinants to several classes of CDI causing antimicrobials [5,6]. This makes *C. difficile* adept at taking advantage of selection pressures imposed by antimicrobials commonly used in hospital and community settings, including in agriculture. In hospitals, this is best exemplified by the antimicrobials lincomycin, clindamycin, cephalosporins and fluoroquinolones. In the late 1980s and early 1990s, clindamycin's use in U.S. hospitals was associated with outbreaks of clindamycin-resistant strains of the REA (Restriction Endonuclease Analysis) group J that can include Ribotype 001 [7]. Clindamycin resistance in clinical strains of *C. difficile* is primarily due to the erythromycin resistance methylase (ErmB), which methylates the 23S rRNA and prevents binding of clindamycin and related members of the macrolide-lincosamide-streptogramin (MLS) class of antimicrobials. The *ermB* gene is encoded on transposons *Tn5398*, *Tn6194*, *Tn6215* and *Tn6218* [5]. Possession of these mobile elements acts as a reservoir for acquisition of other resistance genes. Indeed, *Tn6218*-like mobile elements were also shown to encode determinants that conferred resistance to multiple unrelated antimicrobials e.g., *cfr*-like 23S rRNA methyltransferases, *matE* (multidrug and toxic compound extrusion) and *aacA-aphD* aminoglycoside resistance determinants) [5]. These observations signify that horizontal gene transfer is integral to both the evolution of multidrug resistance in *C. difficile*, permitting it to respond to various antimicrobial selection pressures, and the maintenance of a reservoir for the intra and inter-species spread of AMR genes in the intestinal ecosystem.

C. difficile is also intrinsically resistant to some β-lactam antimicrobials, such as cephalosporins [8,9], which are commonly prescribed drugs. Recent studies implicate an endogenous class D β-lactamase, BlaCDD (encoded by *CD630_04580*), in intrinsic resistance to some β-lactam antimicrobials since the enzyme hydrolyzes the β-lactam ring of different sub-types of β-lactam antimicrobials (including penicillins, cephalosporins and monobactams) [8,9]. However, *C. difficile* shows varying levels of susceptibility to β-lactams, e.g., MICs for ampicillin, imipenem, ceftriaxone and aztreonam are 4, 4, 64, 2048 μg/mL, respectively) and deletion of *CD630_04580* either had no effect on MICs or reduced MICs by 2–4-fold [8,9]. This suggests that a combination of BlaCDD and other β-lactamases, and/or differences in the affinities of β-lactams to *C. difficile* penicillin-binding proteins (PBPs), might account for variations in susceptibilities. Furthermore, it is debatable whether *blaCDD* is expressed at reduced levels [8] or highly expressed [9], and thus research is needed to define how *C. difficile* regulates its β-lactamase(s) and their involvement in β-lactam resistance and cell physiology. Since carbapenems effectively bind to multiple PBPs, particularly PBPs 1, 2 and 3, and are more resistant to β-lactamases, including BlaCDD, they are used to treat severe infections, including Gram-negative infections. *C. difficile* is still mostly susceptible to carbapenems, but resistance has emerged among various ribotypes, particularly ribotype 017 that is the main *C. difficile* lineage strain in Asia [10]. Imipenem resistance in isolates of RT017 (MIC >32 μg/mL) was associated with mutations near the transpeptidase domains of PBP1 (Ala555Thr) and PBP3 (Tyr721Ser), suggesting they decrease β-lactam affinity [10]. In other organisms, such as *Streptococcus pneumoniae*, mutations within PBPs are a leading cause of resistance to β-lactams [11]. RT017 strains also carry a fifth PBP (PBP5) on a mobile element, which is suggested to facilitate *de novo* mutations causing high-level imipenem resistance [10]. Further research is needed to understand the impact of PBP mutations on *C. difficile* pathophysiology, including the cellular function of PBP5 in facilitating resistance development. Interestingly, cephamycins (analogs of cephalosporins) inhibit sporulation in *C. difficile* by binding to the PBP SpoVD, a homolog of PBP4; cephamycins preferably inhibit PBP4 in bacteria [12]. However, the clinical utility of cephamycins for CDI is debatable [13]. Nonetheless, structural insights into *C. difficile* PBPs, such as SpoVD, could lead to novel therapeutics for CDI.

It has been established that widespread use of fluoroquinolones enabled the global spread fluoroquinolone-resistant epidemic ribotype 027 [14,15]. A Thr82Ile mutation in DNA Gyrase A, a Type II topoisomerase for DNA supercoiling, is the most common substitution in these fluoroquinolone-resistant strains [14,15]. This mutation does not appear to impose a fitness cost and paradoxically may enhance fitness [16,17], implying that fluoroquinolone resistance is unlikely to disadvantage *C. difficile* in the absence of selection pressure. Hence, observed reductions in CDI rates following fluoroquinolone restriction policies [18,19] may not be driven by fitness costs.

RESISTANCE TO CDI ANTIMICROBIALS

Metronidazole

Metronidazole is a nitro-group-containing drug that is bioreductively activated within cells, producing free radicals that damage cellular components, including DNA and metalloclusters of proteins, and deplete cellular low-molecular weight thiols [20,21]. Metronidazole resistance was first reported in the early 2000s, but only recently have resistance mechanisms been elucidated. This is partly due to the inaccurate depiction of metronidazole resistance as an unstable or heterogenous phenotype [22–24]. Rather, recent work has reported that the biological cofactor heme was required to reproducibly detect metronidazole-resistant *C. difficile* [25,26]. Indeed, when susceptibility testing agars lacked heme, or when it was photo-decomposed in agars, then metronidazole-resistant strains appeared to be susceptible [25,26]. After this discovery it was shown that *C. difficile* strains with metronidazole MICs of ≥1 μg/mL were more prone to cause the failure of metronidazole therapy in adult patients diagnosed with CDI [27]. Resistance breakpoints for metronidazole are: >2 μg/mL per the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and ≥32 μg/mL per the Clinical and Laboratory Standards Institute (CLSI). In our opinion since the physiological concentration of metronidazole is on average 9.3 ± 7.5 μg/g wet weight of watery stools, then the CLSI breakpoint may not properly reflect the concentrations of metronidazole in the colon of CDI patients.

Over the last decade genetic mechanisms of metronidazole resistance have been described. Recently, plasmid mediated resistance was reported, involving a high copy number plasmid (pCD-METRO) isolated in a ribotype 020 strain from a patient who failed metronidazole therapy. pCD-Metro was found in ~3.8% of ~585 strains studied and occurred in ribotypes 027, 010, and 020 from different European countries [28]. So far, it is unknown which gene on pCD-METRO confers resistance. Also, resistance mediated by pCD-METRO is not dependent on heme [26], indicating that other mechanisms are likely to be responsible for heme-dependent resistance in most clinical strains. To understand chromosomally mediated resistance, a mutator non-toxigenic strain was constructed [20]. Evolutionary experiments with this mutator identified that mutations to pyruvate:ferredoxin oxidoreductase (PFOR), iron sulfur cluster regulator (IscR) and xanthine dehydrogenase (XDH) conferred resistance in cells deficient in cellular iron, following inactivation of the ferrous iron transporter (FeoB1) [20]. These genetic changes were predicted to impair the reduction of metronidazole to reactive species within cells [20]. Metronidazole-resistant clinical strains (ribotype 027) have also been reported with mutations to catalytic domains of PFOR [20], but complementation of these strains with the wild type gene did not completely restore susceptibility to metronidazole in the presence of heme (Table 1). This implied that other mechanisms contribute to metronidazole resistance in *C. difficile*. A genome-wide association study revealed that non-synonymous mutations (Tyr130Ser, Tyr130Cys) in *C. difficile* NimB (CD1459) and a SNP within the gene's promoter region were associated with strains exhibiting reduced susceptibility to metronidazole; 1501 isolates from the MODIFY I and II clinical trials of bezlotoxumab were examined in this study [29]. Nim proteins are thought to inactivate metronidazole to an amino derivative, bypassing the formation of the antimicrobial's reactive species [30]. Although NimB is common among *C.*

difficile genomes, experimental evidence is needed to establish if it can confer resistance to metronidazole in *C. difficile*.

Vancomycin

The glycopeptide vancomycin binds with high affinity to D-Ala-D-Ala of lipid-II at the C-terminal pentapeptide, thus inhibiting peptidoglycan synthesis and assembly, which weakens the cell wall and causes cells to eventually undergo autolysis. Like metronidazole, the clinical success of vancomycin also declined [31–34]. Interestingly, *C. difficile* isolates from 1984 to 2003 were associated with a vancomycin MIC_{90} of 1 μg/ml, compared to an MIC_{90} of 4 μg/ml for isolates from 2011 to 2012 [35]. This suggests that there has been an increase in strains that are less susceptible to vancomycin. Yet there is no direct correlation between poorer therapeutic outcomes and vancomycin-resistant strains, with MICs of 4–16 μg/mL (i.e., EUCAST breakpoint of >2 μg/mL). It is widely thought that low-level vancomycin-resistant strains (MICs=4–16 μg/mL), are less likely to cause treatment failure since the fecal concentrations of vancomycin (i.e., ~100–1000-fold MIC_{90} [1 μg/ml]), should inhibit the growth of resistant strains (MIC=4–16 μg/ml) [27,36]. However, three aspects complicate the interpretation of MIC data of individual strains in relation to vancomycin treatment failure: *a*) drug concentration at site of infection; *b*) antimicrobial tolerance mechanisms (Eagle effect and resistance to autolysis); and *c*) the microbiome. The actual concentrations of vancomycin along the length of the colon are unknown, as well as whether drug deposition is affected by fulminant disease [37]. In our view, MICs alone do not always predict survivability, especially in a complex dynamic environment such as the gastrointestinal tract. Additionally, less studied phenotypes such as reduced autolytic responses [38] and ‘Eagle effect resistance’ [39] (a paradoxical phenomenon where bacteria grow above bactericidal concentrations), might enable *C. difficile* to survive in physiological concentrations of vancomycin (Table 1 and Figure 3). It has been suggested that higher therapeutic doses of vancomycin should be used (i.e., 500 mg instead of 125 mg, four times/day) [37]. However, it is possible that this could enhance collateral damage to the gut microbiota and promote overgrowth of vancomycin-resistant *Enterococci* (VRE) that is associated with more severe CDI [40]. In our view, these factors suggest that evolution of low-level resistance in *C. difficile* should not be negated, as either a potential driver for increasing recurrence or as a step toward higher level resistance.

Vancomycin resistance mechanisms in enterococci are well-documented, involving modification of the terminal D-Ala with either D-Lac or D-Ser [41]. High-level resistance conferred by D-Ala-D-Lac is encoded by *vanA* and *vanB* gene clusters, whereas low-level resistance is caused by D-Ala-D-Ser encoded by *vanC, vanE and vanG* gene clusters. *C. difficile* isolates encode a *vanG-type* gene cluster ($vanG_{Cd}$) that is cryptic in vancomycin-sensitive strains [38,42]. $vanG_{Cd}$ does not cause resistance in susceptible strains, possibly due to cells favoring use of peptidoglycan precursors ending in the dipeptide D-Ala-D-Ala [43]. Recently, it was described that constitutive expression of $vanG_{Cd}$ occurred in vancomycin-resistant clinical strains (MICs=4–8 μg/mL) and laboratory generated mutants (MICs=8–16 μg/mL), which carried mutations in the VanSR two-component system that regulates $vanG_{Cd}$ [38]. A Thr115Ala mutation in VanR was common in clinical strains from different geographic regions and molecular modelling suggested it locked phosphorylated

VanR into its DNA binding conformation, making it more prone to induce *vanG*$_{Cd}$ transcription. Mutations in the VanS sensor occurred in a conserved region that affected the phosphatase function of VanS and presumably increased cellular levels of phosphorylated of VanR. Interestingly, lab mutants containing a stop codon in *trkA,* a potassium transporter (i.e., Gln26STOP in TrkA; Table 1), were less responsive to autolysis and survived physiological concentrations of vancomycin (i.e., MBCs of ≥1024 μg/mL). It is likely that the loss of TrkA enabled the accumulation of organic osmoprotectants that protect against cell lysis. Although TrkA mutations have not been reported in clinical strains to date, the idea that autolysis resistant strains exhibit enhanced survival in physiological concentrations of vancomycin points to a mechanism for drug tolerance and recurrence by strains with vancomycin susceptible MICs or low-level resistance to vancomycin. Indeed, vancomycin-intermediate *Staphylococcus aureus* showing reduced autolysis can survive in vancomycin concentrations of ≥32-fold higher their MICs [44]. Based on these findings, Figure 3B describes a model whereby low-level vancomycin-resistant *C. difficile* survive physiological concentrations of the drug, by being less responsive to autolysis conditions. Other *in vitro* vancomycin-resistant mutants (MIC=16 μg/mL) were reported [45]. One such lab-generated mutant had a mutation of Asp244Tyr in RNA polymerase β' subunit, while the other mutant carried mutations of ΔAla295 in L-serine deaminase (SdaB), Glu327Stop in cyclic-di-AMP phosphodiesterase (CD630_36590; predicted to be GdpP with 59% homology to *S. aureus* GdpP) and Pro108Leu in peptidoglycan glycosyltransferase (MurG). In unpublished observations, the authors identified a clinical isolate with mutations of ΔAla296 in SdaB, ΔLeu443 in GdpP, and Thr115Ala in VanR (Table 1). This might suggest that mutations to SdaB and GdpP are clinically relevant resistance mechanisms, but this will require experimental validation. We speculate that inactivation of SdaB in *C. difficile* increases the cellular pool of L-serine, enabling the VanT serine racemase of *vanG*$_{Cd}$ to make D-Serine for Lipid-II-D-Ala-D-Ser biosynthesis. In bacteria, GdpP affects several processes including osmotic regulation, cell wall homeostasis, and biofilm development [46]. Since its deletion confers tolerance to cell wall acting antimicrobials [47], it is possible that loss of GdpP in vancomycin-resistant *C. difficile* also promotes tolerance to vancomycin, but this will need to be confirmed experimentally.

Plasmid-mediated reduction of vancomycin susceptibility has recently been reported in isolates (MIC=2 μg/mL) from patients failing to respond to vancomycin therapy [48]. The plasmid, pX18–498, is a large broad host range plasmid with 51 *ORFs*, including a gene encoding a putative N-acetylmuramoyl-L-alanine-amidase, a peptidoglycan remodeling enzyme. Conjugation of pX18–498 into a vancomycin-susceptible strain conferred decreased susceptibility to vancomycin. Possible clinical relevance of pX18–498 was also demonstrated, as mice infected with *C. difficile*-pX18–498 and treated with vancomycin had >1 log greater bioburdens than counterpart mice infected with an isogenic strain lacking the plasmid. This study [48] certainly raises the question of whether there are niche-specific concentrations of vancomycin that favor colonization and survival with low-level resistant mutants and whether there are interactions between determinants on pX18–498 and the core genome.

Biofilm mediated resistance to vancomycin and metronidazole.

Laboratory studies suggest that biofilms might play a role in vancomycin and metronidazole resistance in *C. difficile*. Subinhibitory concentrations of vancomycin and metronidazole were shown to enhance biofilm formation [49,50]. In general, within biofilms, cells have reduced metabolism and are more tolerant to antimicrobials; *C. difficile* biofilms tolerate high concentrations of metronidazole (10–100 μg/mL) [51] and vancomycin (20 μg/mL) [50] that are bactericidal to planktonic cells.

Fidaxomicin

The narrow-spectrum antimicrobial fidaxomicin binds to the RNA polymerase (RNAP) clamp, inhibiting the initiation step of transcription of DNA into RNA [52]. Since its approval for treatment of CDI by the U.S. Food and Drug Administration in 2011, fidaxomicin has been mainly used for rCDI. Fidaxomicin-resistant *C. difficile* (MIC=16 μg/mL) was isolated from a patient with rCDI, following therapy with fidaxomicin [53]. Fidaxomicin resistance arises from mutations in RNAP, in RpoB (Gln1074Lys, Val1143Asp, Gly, Phe) and RpoC (Gln781Arg and Asp1127Glu, Asp237Tyr) [54,55]. Val1143Asp (MIC>64 μg/mL) and Val1143Gly (MIC=16 μg/mL) have also been reported in fidaxomicin-resistant clinical isolates [56,57] (Table 1). Mutations at position 1143 in RpoB affect the fitness and virulence of *C. difficile,* as was shown using recombinant isogenic strains [56]. The clinically occurring mutations of Val1143Asp and Val1143Gly showed reductions in overall growth, competitive fitness, and production of toxins A/B, when compared to their parental strain R20291. Virulence was also reduced in the hamster model of CDI. However, it is unknown whether fidaxomicin-resistant strains, with mutations in RNAP, will survive in physiological fidaxomicin (the fecal concentration of fidaxomicin is reported to be 1396 ± 1019 μg/g) [58]. Because fidaxomicin shows a narrow-spectrum of activity [59], it is possible that the rebounding microbiota could help mitigate the effect of mutants if they emerge during therapy, particularly those with fitness costs. However, if lessons can be learnt from other bacterial pathogens, it is that antimicrobials inhibiting a single drug target are prone to more rapid evolution of resistance. Furthermore, second site compensatory mutations could evolve that reinstate fitness and allow resistance alleles to become fixed. It is interesting that mutations to RNAP are linked to development of vancomycin resistance in *S. aureus* [60,61], by enhancing transcription of cell wall synthesis genes. This certainly raises the question of whether increased use of vancomycin and fidaxomicin might drive co-resistance to these drugs. Therefore, proactive surveillance of fidaxomicin resistance is warranted. Lab-evolved fidaxomicin-resistant mutants (MIC=16 μg/ml) were also found to carry a frameshift mutation in CD2212, a homolog of MarR (multiple antibiotic resistance regulator), but confirmation of the mutation's role in fidaxomicin resistance requires molecular genetic validation [45].

Rifaximin and Tetracyclines

Alternate therapies for CDI include the rifamycin rifaximin and the tetracycline tigecycline [1,2]. Rifaximin is recommended as a follow-up therapy after initial treatment with vancomycin for rCDI. However, *C. difficile* has a mutation frequency of ~10^8 to rifaximin, in which high-level resistant mutants (e.g., MICs >1024 μg/mL) can arise without significant

effects on *in vitro* or *in vivo* fitness [62]. Thus, it has been reported that rifaximin resistance can arise during CDI therapy, resulting in clinical failure of rifaximin (Table 1). Furthermore, rifaximin-resistant *C. difficile* is common in hospitals (i.e., rates of 29.1–48.9%), which increases the risk for therapeutic failure [63]. In contrast to rifaximin, tigecycline has a lower rate of *de novo* resistance. Nonetheless, a recent meta-analysis showed that 20% of *C. difficile* human isolates are tetracycline-resistant [64]. Tetracycline-resistant *C. difficile* strains mainly encode the ribosomal protection protein *tet(M)* on conjugative Tn*916*-like elements. Because tigecycline has a higher affinity for the ribosome than older tetracyclines, it is active against strains bearing Tet(M). Experiments in *E. coli* show that mutations to Tet(M) can engender low-level tigecycline resistance [65]. High-level resistance to tigecycline is encoded by tetracycline destructases, i.e., Tet(X), that enzymatically inactivate tetracyclines. Recent discoveries of *tet*(X) orthologs on mobile elements in commensal, livestock and human isolates also affords a path for tigecycline resistance in *C. difficile* [66]. Because rifaximin and tigecycline are not firstline drugs, it is conceivable that susceptibility testing, and genome sequencing could be applied to improve selection of these therapies for rCDI.

CONCLUDING REMARKS

Over the last decade, knowledge of various mechanisms of resistance to firstline drugs by *C. difficile* now make it possible to explore the effect of resistance on treatment outcomes and epidemiology. For example, because fidaxomicin resistance typically maps to RNAP, mutations can readily be identified by PCR methods or genome sequencing. Similarly, PCR methods can be developed to identify vancomycin resistance mechanisms involving pX18–498 and VanSR mutations. However, further experimental validation and/or determining correlations with clinical failure is required for vancomycin and fidaxomicin resistance mechanisms. Regarding vancomycin, whether the ‘Eagle effect’ or reduced autolysis also contributes to *C. difficile* survival in high doses of the drug will also require further experimentation. It will also be crucial to learn lessons from declining efficacies of metronidazole and vancomycin to better employ these and other anti-*C. difficile* therapeutics. In this regard, whole-genome sequencing-based typing and comparative genomics on patient isolates alongside susceptibility testing results could be essential. There is also an important role to be played by evolutionary genomics (e.g., genome wide-association studies) on global strains to understand the epidemiology of resistance mechanisms and to discover evolving mechanisms that are associated with AMR phenotypes. This information can be harnessed to develop molecular diagnostics that improve therapeutic selection for CDI, including the use of future anti-*C. difficile* therapeutics, which are at various stages of pre-clinical and clinical development.

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HIGHLIGHTS

- Plasmid mediated resistances to metronidazole (pCD-METRO) and vancomycin (pX18–498) may be associated with reduced therapeutic efficacies.
- Heme-dependent resistance to metronidazole found in most metronidazole-resistant *C. difficile* may be associated with clinical failure.
- Resistance to autolysis and the ‘Eagle effect’ could mediate survival in physiological vancomycin.
- Resistance testing should be a part of the diagnostic work-up for CDI.
- Genomic surveillances could track *C. difficile* evolution to improve antimicrobial stewardship policies.

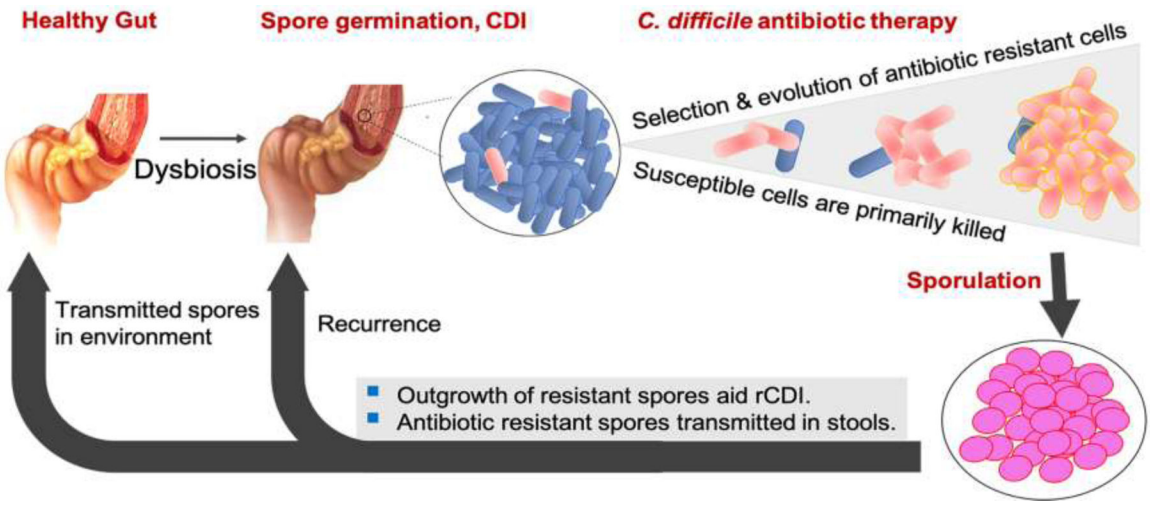

Figure 1.

A Conceptual model demonstrating how genetic resistance to firstline antimicrobials could influence CDI recurrence. Resistant cells, including low-level resistant mutants, formed by spontaneous mutations could be selectively advantaged, even in drugs that achieve high luminal concentrations (e.g., fidaxomicin, vancomycin and rifaximin); low-level resistant mutants might be fitter than their wild type counterparts in niches with sub-physiological concentrations of drug. Sporulation by surviving low-level resistant cells increases the risk for recurrent disease. Drug-resistant spores transmitted in patient stools have an even higher likelihood of causing recurrence or acquiring further mutations that cause higher-level resistance and/or compensate for fitness costs. Strains with high-level resistance are better able to survive physiological concentrations of drug.

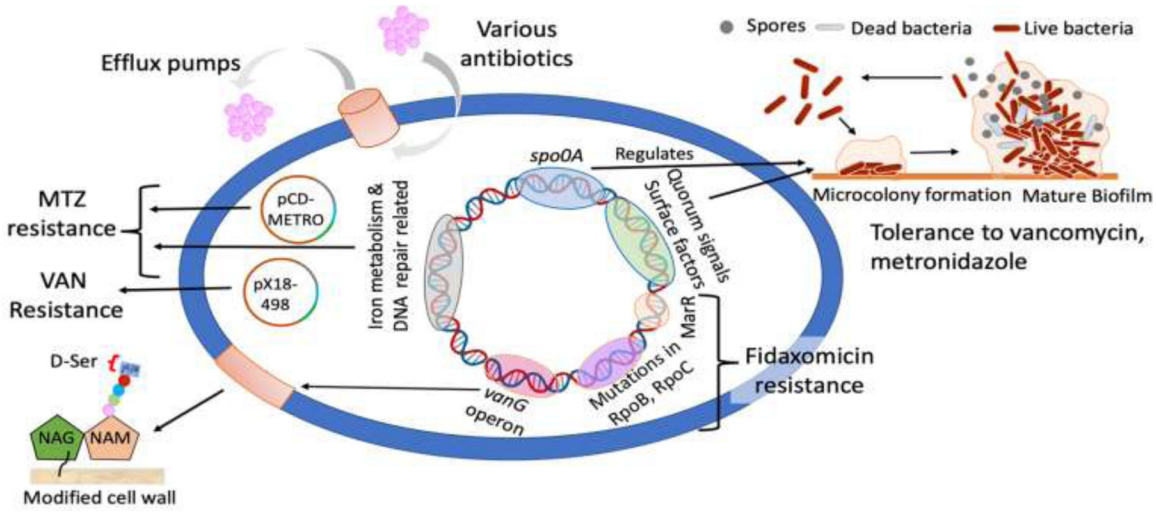

Figure 2. Laboratory and clinically associated mechanisms of resistance to firstline anti-*C. difficile* antimicrobials.

Clinical or laboratory studied mechanisms are indicated below. Metronidazole resistance mechanisms can involve the plasmid pCD-METRO (*clinical*) and endogenous genes (e.g., iron and redox metabolism [*laboratory*]). Vancomycin resistance is either encoded by the plasmid px18–498 (*clinical*) or the $vanG_{Cd}$ operon (*clinical and laboratory*) that modifies the peptidoglycan by replacing the terminal D-Ala with D-Ser. Mutations in *rpoB* and *rpoC* are responsible for the reduced susceptibility to fidaxomicin (*clinical and laboratory*), but a mutation to MarR homolog CD2212 is another putative factor (*laboratory*). Biofilms (*laboratory*) that are metabolically inactive may also provide resistance to metronidazole and vancomycin. Biofilm formation can be promoted by selection pressure from antimicrobials, from quorum sensing signals or controlled by *spo0A* regulatory mechanisms [69]. Efflux pumps (*laboratory*) are also active in *C. difficile* e.g., deletion of the ATP-binding cassette transporter CD2068 in *C. difficile 630Δerm* causes a modest (1.4-fold) reduction in the activity of metronidazole [70].

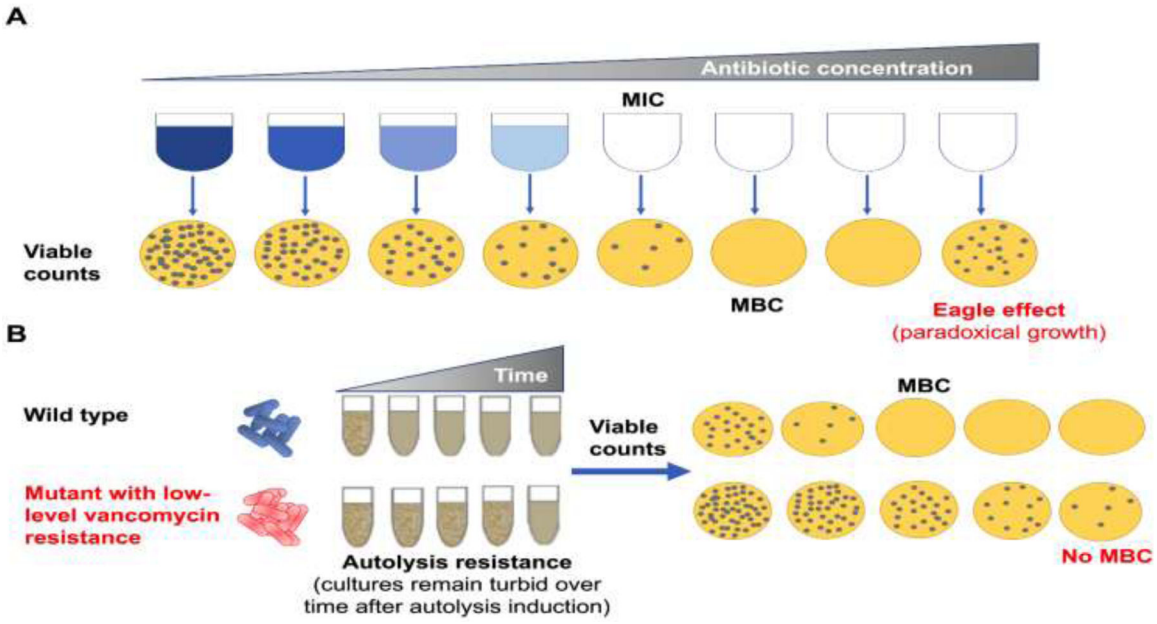

Figure 3. Conceptual models of strains that survive physiological vancomycin by adopting the Eagle resistance or reduced response to autolysis.

Their survival in physiological concentrations of drug could be related to: (**A**) a paradoxical phenomenon known as the Eagle effect, whereby strains that are susceptible, as based on MICs, grow above the MBC; or (**B**) diminished cell lysis under autolysis inducing conditions (autolysis is measured as a loss of optical density over time in Triton-X 100 buffer). Such approaches can be used to characterize strains that survive physiological concentrations of vancomycin.

Table 1.

Mechanisms of resistance to antimicrobials recommended to treat CDI.[a]

Antimicrobial	Mechanism(s) of resistance in clinical or laboratory strains	Reported impact on therapy and phenotype
Vancomycin	**Clinical isolates**	
Inhibits cell wall synthesis by binding Lipid II motif of D-Ala-D-Ala	Putative N-acetylmuramoyl-L-alanine amidase encoded on broad host range plasmid pX18–*498* [48]; predicted to preserve cell wall integrity	Associated with reduced therapeutic response in patients; strains with pX18–498 had MICs=2 μg/ml versus 0.5 μg/ml without the plasmid
	Mutations to VanSR cause constitutive transcription of $vanG_{Cd}$ and production of modified Lipid II; the D-Ala-D-Ser modified Lipid II has ~7-fold educed binding of vancomycin [38]. Mutations in clinical isolates are Ser313Phe and Thr349Ile in VanS and Thr115Ala in VanR.	Unknown clinical impact; strains of MICs 4–8 μg/ml, MBCs ~64 μg/ml; strains were responsive to autolysis inducing conditions
	[b]Clinical isolate with mutations in VanR (Thr115Ala), SdaB (His18Ala) and GdpP (Phe43frameshift)	MIC=8 μg/ml; response to autolysis inducing conditions is unknown
	Laboratory generated mutants	
	Mutations in VanS (Arg314Leu or Gly319Asp) and TrkA (Gln26STOP) [38]	MIC=8–16 μg/ml, MBCs ≥1024 μg/ml; strains were much less responsive to autolysis inducing conditions
	Mutations in MurG (Pro108Leu), GdpP (Glu327Stop) and SdaB (ΔAla295) [45]	MIC=16 μg/ml
	Mutation in RpoC (Asp244Tyr) [45]	MIC=8 μg/ml
Metronidazole	**Identified in clinical isolates**	
Free radicals from nitro group reduction damage DNA and proteins and deplete cellular thiols	Transferable plasmid pCD-METRO (exact resistance gene on the plasmid is unknown) [28]	Clinical isolate from patient with recurrent CDI, treated with metronidazole; MICs ≥ 8 μg/ml
	Ala1018Val mutation in PFOR, predicted to be adjacent to binding domain for 4Fe-4S and thiamine pyrophosphate [20]	MICs 8–16 μg/ml; complementation with wild type PFOR increased susceptibility (MIC=4 μg/ml), but full susceptibility was not restored with complementation suggesting other mechanisms of resistance occurred in the strain
	Laboratory generated mutants	
	Evolved mutants showing mutations to FeoB1 (Glu38frameshift or Lys40frameshift), PFOR (Pro32Leu or Gln803Arg), XDH (synonymous change GA**G**-2070-GA**A**) and IscR (Lys51frameshift or Val76Ala) [20]	Experimental validation showed Δ*feoB1* deletion caused 4-fold decrease in susceptibility (MIC=1 μg/ml in Δ*feoB1* mutant); higher-level resistance (MICs=8 μg/ml) occurred by silencing of genes for PFOR, XDH or IscR in the Δ*feoB1* deletion mutant
	An evolved mutant with mutations in HemN (Tyr214frameshift), ThiH (Ser328Phe), PFOR (Gly423Glu) and GlyC (Ala229Thr) [22]	MIC=8–16 μg/ml; involvement of PFOR in the strain was experimentally validated; complementation with wild type PFOR did not restore full susceptibility [20]
Fidaxomicin	**Identified in clinical isolates**	
Inhibits transcription by binding to RNA polymerase clamp domain	Mutations to RNAP β subunit (Val1143Gly and Val1143Asp) [56,57]	Clinical impact unknown, but strain with Val1143Gly mutation was isolated from a patient with recurrent CDI after fidaxomicin therapy; strains with Val1143Gly and Val1143Asp had MICs of 16 μg/ml and >64 μg/ml, respectively

Antimicrobial	Mechanism(s) of resistance in clinical or laboratory strains	Reported impact on therapy and phenotype
	Laboratory generated mutants	
	Evolved and engineered mutations to RNAP β subunit (Val1143Gly, Gln1073Arg, Val1143Asp, and Val1143Phe) [54,55]	MICs=2 to >64 μg/ml
	An evolved mutant with Phe117frameshift in homolog of transcriptional regulator MarR [45]	MIC=1 μg/ml (4-fold increase over wild type progenitor)
Rifaximin	**Identified in clinical isolates**	
Inhibits transcription by binding to RNA polymerase β subunit (RpoB)	Spectrum of mutations in the rifamycin resistance determining region of RNAP β subunit (e.g., Ser488Tyr, Asp492Tyr, His502Asn/Tyr, Arg505Lys, Ser550Phe/Tyr) [62]	Resistance arose within 32 hours of rifaximin therapy for recurrent CDI; the isolates had mutations of His502Tyr or His502Tyr/Pro496Ser and rifampin MICs ≥32 μg/ml [67] (rifaximin MICs are typically >1024 μg/ml against rifamycin-resistant mutants)
Tetracyclines (tigecycline)	**Identified in clinical isolates**	
Inhibits protein synthesis by binding to 30S ribosomal subunit	Ribosomal protection proteins e.g., Tet(M), Tet(W) found on various mobile genetic elements. TetM is the most common resistance mechanism; these mechanisms do not confer resistance to tigecycline	Clinical outbreaks and evolution of tetracycline resistance in agriculture-associated ribotype ribotype 078 occur in response to tetracycline use; MIC ≥16 μg/ml [68]

[a] Proteins abbreviated above

VanSR (CD630_1625, CD630_1624), VanSR two component system upstream of *vanG*$_{Cd}$ operon; TrkA (CD630_0697), Trk system potassium uptake protein; SdaB (CD630_3222), L-serine deaminase; GdpP (CD630_3659), cyclic-di-AMP phosphodiesterase; MurG (CD630_2725), peptidoglycan glycosyltransferase; PFOR (CD630_2682), pyruvate: ferredoxin oxidoreductase; FeoB1 (CD630_1479), ferrous iron transporter; XDH (CD630_3177), xanthine dehydrogenase; IscR (CD630_1278), iron sulfate cluster regulator; HemN (CD630_2464), oxygen independent coproporphyrinogen III oxidase; ThiH (CD630_1705), thiamine biosynthesis enzyme; GlyC (CD630_2630), glycerol-3-phosphate dehydrogenase; MarR (CD630_2212), multiple antibiotic resistance regulator homolog; RNAP (RNA Polymerase); RpoB (CD630_0066), RNA polymerase subunit beta; RpoC (CD630_0067), RNA polymerase subunit beta'; Tet(M), Tetracycline resistance protein TetM; Tet(W), Tetracycline resistance protein TetW.

[b] Unpublished data by the authors.