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Mechanisms of antibiotic resistance of *Clostridioides difficile*

Ishani Wickramage [1], Patrizia Spigaglia[2] and Xingmin Sun[1]*

[1]*Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, 12901 Bruce B. Down Blvd, Tampa, FL 33612, USA;* [2]*Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy*

*Corresponding author. E-mail: sun5@usf.edu

Clostridioides difficile (CD) is one of the top five urgent antibiotic resistance threats in USA. There is a worldwide increase in MDR of CD, with emergence of novel strains which are often more virulent and MDR. Antibiotic resistance in CD is constantly evolving with acquisition of novel resistance mechanisms, which can be transferred between different species of bacteria and among different CD strains present in the clinical setting, community, and environment. Therefore, understanding the antibiotic resistance mechanisms of CD is important to guide optimal antibiotic stewardship policies and to identify novel therapeutic targets to combat CD as well as other bacteria. Epidemiology of CD is driven by the evolution of antibiotic resistance. Prevalence of different CD strains and their characteristic resistomes show distinct global geographical patterns. Understanding epidemiologically driven and strain-specific characteristics of antibiotic resistance is important for effective epidemiological surveillance of antibiotic resistance and to curb the inter-strain and -species spread of the CD resistome. CD has developed resistance to antibiotics with diverse mechanisms such as drug alteration, modification of the antibiotic target site and extrusion of drugs via efflux pumps. In this review, we summarized the most recent advancements in the understanding of mechanisms of antibiotic resistance in CD and analysed the antibiotic resistance factors present in genomes of a few representative well known, epidemic and MDR CD strains found predominantly in different regions of the world.

Introduction

Antibiotics are no longer the 'miracle cure' they once were in saving lives from bacterial infections, with resistance being reported in all countries of the world.[1] In what can unfortunately be called a 'post-antibiotic era' today,[1] antibiotic resistance is one of the biggest threats to global health, food security and development.[2] Since the beginning of use of antibiotics in humans and animals in the 20th century, a constant trend was seen globally that the release of each new antibiotic was followed a few years or decades later by the emergence of bacterial strains resistant to that antibiotic.[1] *Clostridioides difficile* (CD), the bacterium which causes the most common healthcare-associated infection in the USA, has been recognized by the CDC as one of the top five urgent antibiotic resistance threats in USA.[1] Understanding the mechanisms of antibiotic resistance in CD is of paramount importance in designing therapeutic strategies to circumvent the resistance, guide clinical antibiotic therapy and develop novel effective antibiotics.

CD is a Gram-positive, spore-forming and toxin-producing anaerobic bacterium. CD infection (CDI) manifests as a wide range of clinical presentations, from mild diarrhoea to life-threatening pseudomembranous colitis, toxic megacolon, sepsis and death.[3,4] In 2017, CDI caused 223 900 estimated hospitalizations, 12 800 deaths and $1 billion attributable healthcare costs.[1,5] CDI is a leading cause of nosocomial antibiotic-associated diarrhoea and poses a grave threat especially to immunocompromised patients and older people, with a high incidence of recurrence even after successful treatment.[4]

The relationship between antibiotic use and CDI is complex. Use of antibiotics has been identified as the most significant risk factor for the development of CDI.[4] On one hand, use of broad-spectrum antibiotics such as cephalosporins, clindamycin and fluoroquinolones disrupts the endogenous intestinal microbiota, which facilitates the colonization of the gastrointestinal tract by CD and establishment of CDI;[3] on the other hand, antibiotics are the main therapeutic option available for CDI. Currently, only a few antibiotics are found to be effective in treating CDI. Vancomycin and fidaxomicin are recommended as first-line therapy to treat an initial episode of CDI and for recurrences.[6] While no longer recommended as first-line therapy, metronidazole is used to treat non-severe CDI in adults and children.[6] Since recently, rifamycins such as rifaximin are also being explored as adjunctive therapy against CDI.[6] Unfortunately, resistance or decreased susceptibility to all these antibiotics have recently been reported,[6–12] presenting a grave challenge to patients and clinicians with the lack of therapeutic options currently available to treat CDI.

Since the early 21st century, there has been an alarming increase in the incidence, severity and recurrence in cases of CDI globally, predominantly with the emergence of epidemic strains such as PCR ribotype (RT) 027 (BI/NAPI/027).[13] These epidemic strains are also resistant to multiple antibiotics. Antibiotic resistance

appears to drive outbreaks of CDI with high morbidity and mortality, as widespread usage of a particular antibiotic is often followed up by the emergence of resistant and epidemic strains. For example, a period of high usage of fluoroquinolones in North America was followed by the emergence and spread of fluoroquinolone-resistant RT027 strains, and this led to the global emergence of CDI in the early 2000s.[14,15] Moreover, restricting the prescription of fluoroquinolones has been associated with a decrease in infections caused by fluoroquinolone-resistant CD isolates, and has been suggested to explain the decline of CDI in UK.[16] While the epidemic ribotypes RT027 and RT078 are predominantly found in Europe and North America, the strain DH/NAP11/106 has now surpassed others as the most common cause of CDI in adults in USA.[17] The epidemiology of CD shows distinct geographical distributions. RT017, which is suggested to have originated in Asia, is the most common ribotype found in this continent.[18,19]

CD has developed resistance to antibiotics with a wide variety of mechanisms such as alteration of the antibiotic, modification of the antibiotic target site and extrusion of the drugs via efflux pumps. Additionally, innate properties of CD such as biofilm and dormant spore formation improve its survival in environments containing antibiotics and increase its tolerance to antibiotics. Numerous genes and mutations of these genes encode mediators of these resistance mechanisms. CD has a highly mobile genome with a high number of mobile genetic elements such as conjugative and mobilizable transposons, prophages and IStrons.[20] These mobile elements, especially transposons, contain several known and putative mediators of antibiotic resistance.[20] Therefore, horizontal gene transfer may play an important role in dissemination of antibiotic resistance between CD and other species of bacteria, as well as among different CD strains. Hence, understanding the resistance mechanisms in CD is important for devising methods to overcome antibiotic resistance. Additionally, CD is known to have low genome conservation with high genome variability between different strains.[21,22] Therefore, study of strain specific factors that drive antibiotic resistance is necessary to gain a comprehensive understanding of the resistance mechanisms of CD.

In this work, we have reviewed the most recent known information on the mechanisms of antibiotic resistance in CD (Table 1, Figures 1 and 2). We have also analysed the antibiotic resistance factors present in the genomes of a few representative epidemic CD strains found throughout the globe (Table 2).

Antibiotics used for the treatment of CDI

Vancomycin

Vancomycin is now recommended as first-line therapy for initial, recurrent and fulminant CD infections.[6] This glycopeptide antibiotic was released for usage in 1958 and has usually been reserved as a last resort drug for the treatment of severe infections caused by select organisms.[6,23] While it was initially considered a wonder drug, which was immune to antimicrobial resistance, vancomycin-resistant *Enterococcus* species were first reported in 1988, followed by *Staphylococcus aureus* in 2002.[6,23] While vancomycin has been successful in treating CDI for many years, until recently being reserved for severe or recurrent cases, CD strains with resistance or reduced susceptibility to vancomycin have emerged in recent years, posing a grave concern.[6–9]

Vancomycin elicits its bactericidal activity by binding with high affinity to peptidoglycan precursors and inhibiting the bacterial cell wall synthesis. It forms a network of hydrogen bonds with the D-Ala-D-Ala C-terminus of uracil diphosphate-*N*-acetylmuramyl-pentapeptide and prevents the transglycosylation reaction which adds late precursors to the nascent peptidoglycan chain. It thereby inhibits the subsequent transpeptidation-based cross-linking, which is necessary for the formation of a mature peptidoglycan layer.[23,24] Resistance to vancomycin has been reported to occur in enterococci through the presence of operons of enzymes known as Van operons.[24] The enzymes encoded by these operons act by either synthesizing low-affinity peptidoglycan precursors where the D-Ala C-terminus has been replaced by D-Lac or D-Ser, or by cleaving the high affinity precursors thus eliminating the vancomycin binding site.[24] *vanG* is an inducible chromosomal operon that has been described to induce vancomycin resistance in enterococci and consists of two sets of genes called a sensor operon and a resistance operon that work together to produce the altered peptidoglycan precursor D-Ala-D-Ser. The sensor operon constitutes a two-component regulatory system with a membrane-bound sensor histidine kinase (VanS) and a response regulator (VanR) transcriptional activator, which in response to vancomycin stimulates the expression of downstream resistance genes. The resistance operon consists of VanT, a serine racemase that converts L-Ser to D-Ser; VanG, a D-Ala-D-Ser ligase; and VanY, a D, D-carboxypeptidase that removes D-Ala residues from the C-terminus of peptidoglycan precursors.[24] A *vanG* operon-like gene cluster, named $vanG_{CD}$, has been detected in about 85% of CD clinical isolates.[25] However, the presence of this functional gene cluster was not shown to mediate vancomycin resistance in CD.[26,27] But recently, mutations in genes of this cluster were found to be associated with vancomycin resistance in some novel CD strains reported in Israel[7] and USA,[7] (I. Wickramage and X. Sun, unpublished data) which had genomic sequences and antibiotic resistance patterns that have not been previously observed. Two RT027 clinical isolates from Texas that were resistant to vancomycin revealed a different mutation each in $VanS_{CD}$, Ser313Phe and Thr349Ile.[7] Other clinical isolates that showed resistance to vancomycin, also of RT027, from Texas ($n = 7$) and Israel ($n = 2$) showed the substitution Thr115Ala in $VanR_{CD}$.[7] Our study also detected this mutation, encoded by the SNP A343G in the receptor domain of $vanR_{CD}$, in two novel RT027 CD strains isolated in Florida from CDI diagnosed patients (I. Wickramage and X. Sun, unpublished data). The vancomycin-resistant clinical isolates that carried these mutations in $VanS_{CD}$ and $VanR_{CD}$ showed constitutive expression of the VanG operon resistance genes,[7] and the increased vancomycin MICs for these strains could be reversed by gene silencing of $vanG_{CD}$.[7] These mutated strains also showed reduced binding of vancomycin to the maturing cell wall. Through homology modelling-based analysis of the Thr115Ala substitution in $VanR_{CD}$, it is proposed that the mutated position 115 of this protein, through interaction with a conserved flexible loop in the effector domain, stabilizes the dimeric, DNA-binding conformation of $VanR_{CD}$, thereby enhancing its ability to transcriptionally activate the resistance genes of the operon. The change from polar threonine to non-polar alanine may energetically facilitate the hydrophobic interactions with lipophilic loop residues (Figure 1).[7]

Other mechanisms and mutations have also been suggested to explain vancomycin resistance in CD. Genetic changes were

Table 1. Summary of mechanisms of antibiotic resistance of *C. difficile*

Antibiotic	Mechanism of antimicrobial action	Proposed mechanism/s of resistance
Vancomycin	Inhibits bacterial cell wall synthesis by binding to the D-Ala-D-Ala C-terminus of uracil diphosphate-*N*-acetylmuramyl-pentapeptide late peptidoglycan precursor[23,24]	Alteration of vancomycin binding site in peptidoglycan precursors. Mediated by mutations in $vanG_{CD}$ operon enzymes: Ser313Phe and Thr349Ile in $VanS_{CD}$ and Thr115Ala in $VanR_{CD}$[7] (clinical isolates)
Metronidazole	Bacterial DNA breakage and cytotoxicity[31]	Inhibiting reductive activation of metronidazole by impairing oxidoreductive metabolic pathways • Genetic changes which may impair electron transport chain: glycerol-3-phosphate dehydrogenase *glyC* (Ala229Thr) (presumptive mechanism) and PFOR *nifJ* (Gly423Glu) detected in a clinical CD strain (CD26A54_R) maintained metronidazole resistance by *in vitro* passages under subinhibitory drug concentrations.[33,34] Importance of PFOR in CD metronidazole resistance was confirmed by gene complementation[32] • Changes that cause reduction of intracellular iron levels, shifting the cells toward flavodoxin-mediated oxidoreductase reactions: ferric uptake regulator fur (Glu41Lys) detected CD26A54_R strain (presumptive mechanism), ferrous iron transporter *feoB1* gene may be implicated[32] (presumptive mechanism in a laboratory-generated mutant) • Other genetic changes detected in the CD26A54_R strain: oxygen-independent coproporphyrinogen III oxidase *hemN* (frameshift mutation Tyr214fs), thiamine biosynthesis protein peptidase *thiH* (Ser328Phe): associated with nutrient limitation and growth rate reduction[34] (unknown and presumptive mechanisms) • pCD-METRO plasmid—exact mechanism unknown[11] (clinical isolates) • CD2068 ABC transporter efflux pump[42] (a clinical strain; mechanism molecularly confirmed)
Fidaxomicin	Inhibits bacterial transcription by inhibiting bacterial RNA polymerase[43]	Induced mutations in RNA polymerase subunit β: Gln1073Arg, Val1143Asp, Val1143Gly and Val1143Phe mediated by A3221G, T3428A, T3428G and G3427C of *rpoB*, respectively, and putative transcriptional regulator MarR: frameshift after amino acid 117 encoded by ΔT349[28,44,109] (laboratory-generated mutants)
Rifamycins	Inhibits bacterial transcription by binding to the β subunit of RNA polymerase, RpoB[12]	Potential impairment of drug binding by mutations in the RRDR of RpoB: Arg505Lys (commonest), His502Asn, His502Tyr, Ser488Tyr, Ser550Phe, Ser550Tyr, Asp492Tyr, Ser507Leu, Gln489Leu, Gly510Arg and Leu584Phe[45] (clinical isolates)
Clindamycin/ MLS_B family	Inhibits bacterial protein synthesis. Clindamycin inhibits peptide bond formation between the A- and P-site tRNAs during translation[47]	• Alteration of drug binding site by methylation of the ribosome at a specific site of 23S rRNA via *erm*(B)-encoded erythromycin ribosomal methylase[48] (clinical strains) • 23S rRNA methyltransferase activity mediated by genes *cfrB, cfrC* and *cfrE*[53] (clinical isolates) • Potential efflux pump activity of *cme* gene-encoded MFS secondary multidrug transporter[54] (laboratory-based heterologous expression)
Cephalosporins	Inhibits bacterial cell wall synthesis by acylating the PBP transpeptidases in bacterial cell wall, thereby inhibiting the transpeptidase-mediated cross-linking reaction of peptidoglycan synthesis[55]	• Drug inactivation by β-lactamase enzymes: e.g. class D β-lactamases and putative β-lactamase encoded by genes such as *CD630_04580*[20,56,57] (clinical strains) • CD2068 ABC transporter efflux pump[42] (a clinical strain; mechanism molecularly confirmed)
Fluoroquinolones	Inhibits bacterial DNA replication and transcription by inhibiting bacterial DNA gyrase, thus negative supercoiling[58]	• Alteration of drug target sites by mutations in the QRDR of *gyrA* and/or *gyrB* genes leading to amino acid substitutions such as Thr82Ile in GyrA[59,61,62,75] (clinical isolates and laboratory-generated mutants) • CD2068 ABC transporter efflux pump[42] (a clinical strain; mechanism molecularly confirmed)

Continued

Table 1. *Continued*

Antibiotic	Mechanism of antimicrobial action	Proposed mechanism/s of resistance
		• Potential efflux pump activity of *cdeA* gene-encoded sodium-dependent efflux pump of the MATE subfamily of secondary multidrug transporters[66] (laboratory-based heterologous overexpression)
Tetracycline	Inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit and blocking the association of aminoacyl-tRNA at the A-site[58,67]	Prevention of binding of drug to the ribosome by production of ribosomal protectant proteins Tet(M), Tet(W) and Tet(44), usually located on mobile or conjugative elements, such as the conjugative elements of the Tn*916* family (e.g. Tn*6190*) and Tn*6164*[68,69] (clinical strains)
Chloramphenicol	Inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit at A2451 and A2452 residues and preventing the binding of tRNA to the P-site of the larger ribosomal subunit thus the elongation of polypeptide chain[58]	Enzyme-mediated antibiotic modification and inactivation: relocation of an acetyl group from acetyl CoA to the primary hydroxyl group of chloramphenicol by *catD*-encoded chloramphenicol acetyltransferase enzyme, at the mobile regions Tn*4453a* and Tn*4453b* transposons[71,72] (clinical isolates)
Linezolid	Inhibits bacterial protein synthesis by binding to bacterial 23S rRNA of the 50S subunit and preventing the formation of the 70S ribosomal unit[73]	Target methylation and subsequent disruption of drug-target interaction: methylation of 8-methyladenosine at A2503 position in 23S rRNA of the large ribosomal subunit by *cfr*-encoded rRNA methyltransferase Cfr[53] (clinical isolates)

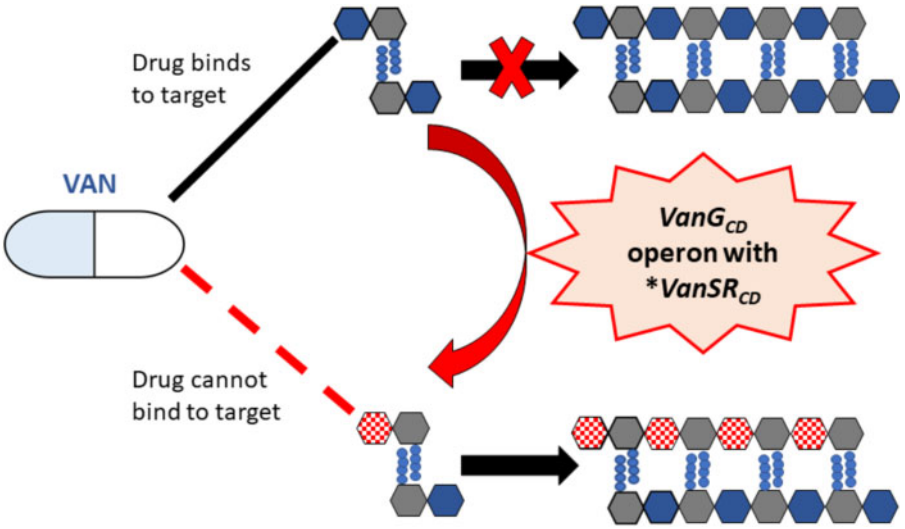

Figure 1. Mechanisms of vancomycin resistance. Vancomycin (VAN) acts by binding with high affinity to the D-Ala-D-Ala C-terminus of uracil diphosphate-*N*-acetylmuramyl-pentapeptide and prevents the transglycosylation reaction which adds late precursors to the nascent peptidoglycan chain, thus inhibiting bacterial cell wall synthesis.[23,24] Vancomycin resistance in *C. difficile* is associated with mutations in VanS$_{CD}$ sensor histidine kinase and VanR$_{CD}$ response regulator of the *vanG* operon-like gene cluster, *vanG$_{CD}$*, which alter peptidoglycan precursors and thereby the vancomycin binding site[7] (I. Wickramage and X. Sun, unpublished data). *, mutated. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

identified in a CD strain and clinical isolates serially passaged *in vitro* under increasing concentrations of vancomycin and were detected to have reduced susceptibility to this antibiotic after multiple passages.[28] In the previously described CD strain thus passaged, a novel mutation Pro108Leu was detected in MurG N-acetylglucosaminyltransferase, which catalyses the conversion of peptidoglycan precursor lipid I to lipid II, an important step in bacterial cell wall synthesis. The same strain also revealed two other mutations—Glu327stop substitution in the putative RNA/single-stranded DNA exonuclease CD3659 and the deletion of a single amino acid in a stretch of alanines between positions 292 and 295 in the L-Ser deaminase encoded by the *sdaB* gene.[28] One of the clinical isolates that developed low susceptibility to vancomycin after exposure to increasing drug concentrations displayed the G733T SNP in the *rpoC* gene, which encodes the Asp244Tyr substitution in the β′ subunit of RNA polymerase.[28] This genetic change possibly mediates resistance by affecting multiple gene expression pathways.[28] However, the causality of these mutations

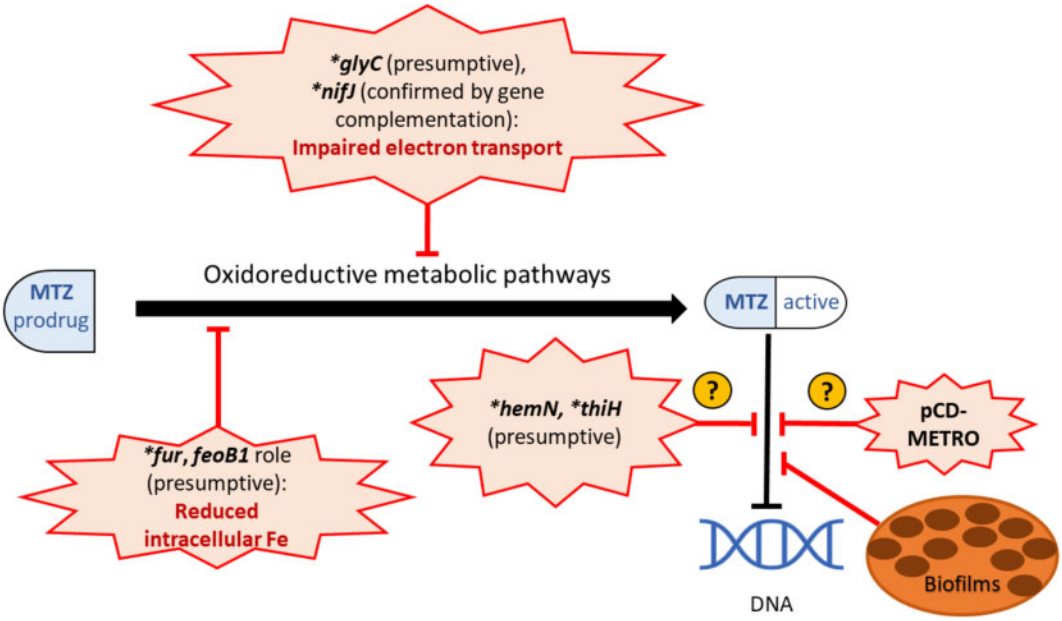

Figure 2. Mechanisms of metronidazole resistance. Metronidazole (MTZ) acts by inducing DNA strand breakage and cytotoxicity, causing bacterial cell death.[31] It is administered as an inactive prodrug that is activated under reductive conditions inside the cell.[31] *C. difficile* (CD) resistance to MTZ may be achieved by factors that prevent the generation of the active form of the drug, which are possibly mediated by multigenetic mechanisms involved in oxidoreductive and iron-dependent metabolic pathways.[32–34] While the high copy number plasmid pCD-METRO is associated with CD MTZ resistance, its mechanism is unknown.[11] CD growing in biofilms have shown increased tolerance to MTZ.[40] *, mutated. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

on vancomycin resistance in CD has not been verified by testing in naive hosts, and the MurG Pro108Leu substitution is also detected in the phenotypically vancomycin-susceptible CD630 reference strain.

CD may also use intrinsic barriers to survive in adverse environments containing antibiotics, such as formation of biofilms and spores. CD cells growing *in vitro* in biofilms have been shown to have higher percentages of survival under exposure to high concentrations of vancomycin than those grown planktonically.[29] Also, biofilm formation was demonstrated to be induced in the presence of subinhibitory and inhibitory concentrations of vancomycin *in vitro*.[29] Spores can survive antibiotic therapy and when treatment is completed or the antibiotic concentration in the body falls below an inhibitory threshold, they may germinate and cause a relapse of CDI. While vancomycin has successful bactericidal activity against vegetative forms of CD, it has no effect on spores.[30] Therefore, sporulation has been found to play a role in the tolerance of some CD strains to vancomycin.

Metronidazole

Although for about 30 years metronidazole was recommended as first-line therapy for CDI, recent evidence has shown that it has inferior clinical benefits compared with vancomycin.[6] Therefore, currently, metronidazole is reserved for use for an initial episode of non-severe CDI in settings where access to vancomycin or fidaxomicin is limited, and in paediatric patients.[6] Low levels of resistance of CD to metronidazole have been reported in many countries.[11]

Metronidazole is a bactericidal nitroimidazole class of antibiotic, which is administered as a prodrug.[31] Inside the cell, it is activated by the reduction of its nitro group in anaerobic enzymatic reactions with low redox potentials. This leads to generation of free radicals, leading to cytotoxicity and cell death in anerobic bacteria.[31] The process of reductive activation itself may be cytotoxic, as metronidazole acts as an alternative electron acceptor and inhibits the proton motive force and ATP production.[31]

Metronidazole resistance in CD may involve multigenetic mechanisms that are possibly involved in oxidoreductive and iron-dependent metabolic pathways.[32] Proteins that are involved in electron transfer reactions play important roles in the reduction of metronidazole to generate the active form of the drug.[33] Genomic and proteomic analyses of the CD clinical isolate CD26A54_R, which maintained metronidazole resistance by serial passages under sublethal concentrations of metronidazole, identified mutations in genes involved in electron transport such as the glycerol-3-phosphate dehydrogenase-encoding gene *glyC* (Ala229Thr) and the pyruvate-flavodoxin oxidoreductase (PFOR)-encoding gene *nifJ* (Gly423Glu).[33,34] Another *in vitro* study supported the importance of PFOR in CD metronidazole resistance, where laboratory-generated mutations in the catalytic domains of PFOR gave rise to metronidazole resistance in CD that was reversed with gene complementation.[32]

Impairment of intracellular iron content has been implicated in CD resistance to metronidazole. In a laboratory-generated CD mutant, the truncation of *feoB1* gene, which encodes a ferrous iron transporter, led to reduced intracellular iron content and a low level of resistance to metronidazole.[32] The authors reasoned that a decrease in intracellular iron shifts cells toward flavodoxin-mediated oxidoreductase reactions, thus impairing the

Table 2. Antibiotic resistance genes detected in some epidemic strains of *C. difficile* through genomic analysis via the Comprehensive Antibiotic Resistance Database (CARD)

C. difficile strain and GenBank accession no.	RT	RGI criteria for gene search	ARO term; SNP	Detection criteria	AMR gene family	Drug class	Resistance mechanism	Identity of matching region (%)	Length of reference sequence (%)
CD630 (CP010905.2)	012	perfect	*cdeA*	protein homologue model	MATE transporter	fluoroquinolone, acridine dye	antibiotic efflux	100.0	100.00
		strict	*vanRG*	protein homologue model	glycopeptide resistance gene cluster, *vanR*	glycopeptide	antibiotic target alteration	77.87	99.15
		strict	*vanXYG*	protein homologue model	glycopeptide resistance gene cluster, *vanXY*	glycopeptide	antibiotic target alteration	58.82	105.51
		strict	*ermB* (2 hits)	protein homologue model	Erm 23S ribosomal RNA methyltransferase	streptogramin, lincosamide, macrolide	antibiotic target alteration	97.55	98.79
R20291 (FN545816.1)	027	strict	*cdeA*	protein homologue model	MATE transporter	fluoroquinolone, acridine dye	antibiotic efflux	99.09	100.00
		strict	*vanRG*	protein homologue model	glycopeptide resistance gene cluster, *vanR*	glycopeptide	antibiotic target alteration	77.87	99.15
		strict	*vanXYG*	protein homologue model	glycopeptide resistance gene cluster, *vanXY*	glycopeptide	antibiotic target alteration	58.82	105.51
CD196 (NC_013315.1)	027	strict	*vanRG*	protein homologue model	glycopeptide resistance gene cluster, *vanR*	glycopeptide	antibiotic target alteration	77.87	99.15
		strict	*vanXYG*	protein homologue model	glycopeptide resistance gene cluster, *vanXY*	glycopeptide	antibiotic target alteration	58.82	105.51
		strict	*Clostridioides difficile* 23S rRNA with mutation conferring resistance to erythromycin and clindamycin; C656T	rRNA gene variant model	23S rRNA with mutation conferring resistance to macrolide antibiotics	macrolide, lincosamide	antibiotic target alteration	99.07	99.93
M120 (NC_017174.1)	078	perfect	*ant(6)-Ia*[110]	protein homologue model	ANT(6)	aminoglycoside	antibiotic inactivation	100.0	100.00
		perfect	*ant(9)-Ia*	protein homologue model	ANT(9)	aminoglycoside	antibiotic inactivation	100.0	111.16
		perfect	*ant(6)-Ib*	protein homologue model	ANT(6)	aminoglycoside	antibiotic inactivation	100.0	100.00
		strict	*cdeA*	protein homologue model	MATE transporter	fluoroquinolone, acridine dye	antibiotic efflux	97.51	100.00
		strict	*tet(W/N/W)*	protein homologue model	tetracycline-resistant ribosomal protection protein	tetracycline	antibiotic target protection	68.5	100.00
		strict	*tet(W/N/W)*	protein homologue model	tetracycline-resistant ribosomal protection protein	tetracycline	antibiotic target protection	68.45	100.16

DH/NAP11/106 (NZ_CP022524)	106	strict	*vanRG*	protein homologue model	glycopeptide resistance gene cluster, *vanR*	glycopeptide	antibiotic target alteration	77.87	99.15
		strict	*vanXYG*	protein homologue model	glycopeptide resistance gene cluster, *vanXY*	glycopeptide	antibiotic target alteration	58.82	105.51
		strict	*Clostridioides difficile* 23S rRNA with mutation conferring resistance to erythromycin and clindamycin; C656T	rRNA gene variant model	23S rRNA with mutation conferring resistance to macrolide antibiotics	macrolide, lincosamide	antibiotic target alteration	99.97	99.97
CF5 (NC_017173)	017	strict	*vanRG*	protein homologue model	glycopeptide resistance gene cluster, *vanR*	glycopeptide	antibiotic target alteration	77.87	99.15
		strict	*vanXYG*	protein homologue model	glycopeptide resistance gene cluster, *vanXY*	glycopeptide	antibiotic target alteration	59.22	105.51
		strict	*Clostridioides difficile* 23S rRNA with mutation conferring resistance to erythromycin and clindamycin; C656T	rRNA gene variant model	23S rRNA with mutation conferring resistance to macrolide antibiotics	macrolide, lincosamide	antibiotic target alteration	99.03	−99.93
M68 (NC_017175.1)	017	perfect	*tetM*	protein homologue model	tetracycline-resistant ribosomal protection protein	tetracycline	antibiotic target protection	100.0	100.00
		perfect	*aac(6')-Ie-aph(2'')-Ia*	protein homologue model	APH(2''), AAC(6')	aminoglycoside	antibiotic inactivation	100.0	100.00
		perfect	*catI*	protein homologue model	CAT	phenicol	antibiotic inactivation	100.0	100.00
		strict	*ermB*	protein homologue model	Erm 23S ribosomal RNA methyltransferase	macrolide, lincosamide, streptogramin	antibiotic target alteration	98.78	98.79
		strict	*Clostridioides difficile* 23S rRNA with mutation conferring resistance to erythromycin and clindamycin; C656T	rRNA gene variant model	23S rRNA with mutation conferring resistance to macrolide antibiotics	macrolide, lincosamide	antibiotic target alteration	98.93	99.93

ARO; antibiotic resistance ontology, AMR; antimicrobial resistance; CAT, chloramphenicol acetyltransferase.

cellular action of metronidazole.[32] Proteomic analysis of the metronidazole-resistant CD26A54_R isolate, generated as described above, showed a significant increase in expression the ferrous iron transport B (FeoB) protein in the absence of metronidazole, suggesting that metronidazole-resistant strains may be defective in iron uptake and/or regulation.[33] In *Helicobacter pylori*, laboratory-generated mutations in the ferric uptake regulator Fur protein, a regulatory protein that controls the transcription of numerous genes in response to iron availability and oxidative stress, have been implicated in metronidazole resistance.[35] Mutational disruption of the *fur* gene alters binding of Fur to superoxide dismutase and reduces cellular oxidative stress and subsequent metronidazole activation.[34,35] Genomic analysis of the previously described serially passaged metronidazole-resistant CD26A54_R strain revealed a point mutation in the *fur* gene (Glu41Lys), which was absent in the metronidazole-susceptible variant of this strain CD26A54_S.[34] However, the exact role of this mutation in metronidazole resistance in CD is not understood.

Several other presumptive mechanisms of CD resistance to metronidazole have been suggested based on *in vitro* studies. However, these proposed mechanisms have not been validated in naive hosts. Mutations found only in the resistant variant of the serially passaged CD strain CD26A54 (CD26A54_R)—such as the frameshift mutation Tyr214fs in the *hemN* gene encoding oxygen-independent coproporphyrinogen III oxidase, which produces a product involved in haem biosynthesis, and Ser328Phe in the *thiH* gene, which encodes a thiamine biosynthesis protein peptidase—have been proposed to contribute to nutrient limitation, which may lead to the aberrant growth seen in the culture of this strain.[34] But the role of altered growth in CD metronidazole resistance is unclear.

CD has been observed to be heteroresistant to metronidazole, where the culture of a CD isolate consists of subpopulations with variable susceptibility to this antibiotic.[36,37] *In vitro* studies showed that subjecting initially metronidazole-resistant CD clinical isolates to a freeze-thaw cycle rendered them metronidazole susceptible,[33,36] while maintaining slow growing subpopulations.[36] Although in other anaerobic bacteria such as *Bacteroides* species heteroresistance has been associated with the presence of *nim* genes, which encode nitroimidazole reductases that render the drug inactive,[38] the presence of *nim* genes has not been implicated in CD metronidazole resistance.[36] Interestingly, induction of metronidazole resistance is seen with prolonged *in vitro* exposure to subinhibitory concentrations of this drug.[33,36] However, the clinical significance of inducible heteroresistance of CD to metronidazole remains unclear.

Even though several plasmids of various sizes had been described in CD, until recently they were considered to not encode for any virulence or antibiotic resistance factors.[39] Recently, a novel 7 kb high copy number plasmid, named pCD-METRO, was identified in strains belonging to diverse ribotypes in several countries, and was correlated to metronidazole resistance in CD.[11] However, the exact gene(s) in this plasmid that confer the resistance are currently unknown (Figure 2). While the authors propose that this plasmid may have been acquired via horizontal gene transfer, they failed to identify a potential donor organism from the NCBI sequence data.[11]

Some virulence factors of CD such as formation of biofilms have been found to be associated with increased tolerance to metronidazole.[40] CD cells growing in biofilms displayed survival under a 100-fold higher concentration of metronidazole than the cells growing in a liquid media culture.[40] Moreover, exposure to subinhibitory concentrations of metronidazole was found to significantly increase the *in vitro* biofilm formation in some CD strains.[41] The efflux pump CD2068 of the ATP-binding cassette (ABC) transporter class was also shown to reduce susceptibility of CD to metronidazole, together with other antibiotics.[42]

Fidaxomicin

Fidaxomicin was approved by the US FDA for the treatment of CDI in 2011 and is recommended for treating initial episodes and recurrences of CDI.[6,43] Some studies suggest superior clinical benefits by fidaxomicin compared with vancomycin.[6]

Fidaxomicin is a macrolide antibiotic that elicits its bactericidal action by inhibiting bacterial RNA polymerase, and thereby transcription and subsequent protein synthesis.[43] It has a narrow spectrum of activity with higher potency displayed for inhibiting RNA polymerase of clostridial species rather than other bacteria.[43] Therefore, it poses a lower risk for disruption of the gut microflora, an important characteristic for a successful antibiotic that is used to treat CDI when taking the pathogenesis of CDI into consideration. It also achieves a high concentration in the intestine with minimal systemic absorption and a prolonged post-antibiotic effect.[10,43] Resistance of CD to fidaxomicin is not widely known, although a single CD strain isolated from a patient experiencing a recurrence of CDI showed reduced susceptibility.[10] Induced mutations in RNA polymerase subunit β—namely A3221G of *rpoB* leading to Gln1073Arg substitution of RpoB[28] and genetically engineered mutations T3428A, T3428G and G3427C of *rpoB* resulting in Val1143Asp, Val1143Gly and Val1143Phe, respectively[44]—were found to be associated with CD resistance to fidaxomicin in two separate studies, and the latter three mutations were found in conjunction with reduced *in vitro* fitness and *in vivo* virulence.[44] These alterations in the RNA polymerase likely impair its interaction with fidaxomicin. Moreover, a thymine deletion at the 349th position of *marR*, a gene encoding a homologue to the MDR-associated transcriptional regulator MarR, resulting in a frameshift of the resulting protein after amino acid 117, was found in a CD strain rendered fidaxomicin resistant via serial *in vitro* passages.[28] The role of these alterations in clinical resistance to fidaxomicin in CD is yet to be verified.

Rifamycins

Rifamycins such as rifaximin and rifampicin are being tried as adjunct therapy for CDI.[6] They inhibit bacterial RNA synthesis by binding to the β subunit of RNA polymerase, RpoB, at a site and step of RNA synthesis distinct from those of fidaxomicin.[12,43] Although CD rifamycin resistance has been reported in several countries,[12] there have been no reports of overlapping resistance between fidaxomicin and rifamycins.

Mutations in the rifamycin resistance-determining region (RRDR) of RpoB found in clinical isolates of CD have been found to be associated with rifamycin resistance,[45] possibly leading to impairment of drug binding. The most common mutation

Arg505Lys, as well as other mutations such as His502Asn, His502Tyr, Ser488Tyr, Ser550Phe, Ser550Tyr, Asp492Tyr, Ser507Leu, Gln489Leu, Gly510Arg and Leu584Phe, have been described in numerous strains resistant to rifamycins.[45] However, most of these mutations did not impose fitness cost to the bacteria *in vitro*,[45] suggesting that other unknown mechanisms may also contribute to rifamycin resistance in CD.

Antibiotics highly associated with pathogenesis of CDI

Clindamycin and other members of the macrolide-lincosamide-streptogramin B (MLS_B) family of antibiotics

Clindamycin is a lincosamide antibiotic with broad spectrum activity and belongs to the MLS_B family of antibiotics.[46] The drugs in this family act by disrupting bacterial protein synthesis, which is achieved with clindamycin by inhibiting peptide bond formation between the A- and P-site tRNAs during translation.[47] Orally administered clindamycin is excreted in bile and gets highly concentrated in stools, disrupting the bacterial species diversity of the intestinal microbiota.[46] Clindamycin administration is considered a highly important risk factor for the development of CDI.[46]

Erythromycin ribosomal methylase genes such as *erm*(B) are considered to mediate resistance of CD to antibiotics of the MLS_B family such as clindamycin and erythromycin, despite the *in vitro* fitness cost.[48,49] The protein encoded by this gene, ErmB, methylates the ribosome at a specific site of 23S rRNA and prevents the binding of the antibiotics.[48] This gene is usually located on mobilizable genetic elements, such as Tn*5398* or E4 elements related to the conjugative transposon Tn*6194*, thus is capable of interspecies horizontal transfer.[48,49] Tn*5398* has been reported to be transferred between CD strains and between CD and *Bacillus subtilis* by conjugation and homologous recombination.[48,50–52] The CD reference strain CD630, which is a well-known erythromycin-resistant strain, contains two copies of *erm*(B) in its genome (Table 2). Other genes such as *cfrB, cfrC* and *cfrE* encoding a 23S rRNA methyltransferase have been implicated in resistance of CD to MLS_B antibiotics.[53] Efflux pumps may also play a role in CD resistance to the MLS_B family of antibiotics. The expression of the CD *cme* gene, which encodes a secondary multidrug transporter of the major facilitator superfamily (MFS), was shown to confer erythromycin resistance in *Enterococcus faecalis*.[54]

Cephalosporins

Cephalosporins are bactericidal, β-lactam type antibiotics, which act by acylating the penicillin-binding protein (PBP) transpeptidases in bacterial cell wall, thereby inhibiting the transpeptidase-mediated cross-linking reaction of peptidoglycan synthesis, consequently resulting in the lysis of bacterial cells.[55] They are also considered to contribute a very high risk for the development of CDI.[56] While the mechanism of the widespread CD resistance to cephalosporins is not fully understood, some CD strains are known to encode β-lactamase enzymes, such as class D β-lactamases and putative β-lactamase encoded by genes such as *CD630_04580*, which destroy the β-lactam ring rendering the drugs inactive.[20,56,57] Efflux pumps may also play a role in CD resistance to cephalosporins. The second-generation cephalosporin cefoxitin was found to be potentially extruded from CD cells via the ABC transporter CD2068, thus showing reduced susceptibility together with other antibiotics.[42]

Fluoroquinolones

Fluoroquinolones mediate their bactericidal action by inhibiting the bacterial DNA gyrase, topoisomerase II, thereby preventing the action of this enzyme of formation of a negative supercoil in the DNA, which is necessary for replication or transcription.[58] Widespread use of fluoroquinolones and the subsequent development of fluoroquinolone resistance are associated with the emergence of epidemic RT027 strains.[14,15]

Resistance in CD was detected to be higher for the second-generation fluoroquinolone ciprofloxacin than for the fourth-generation fluoroquinolones moxifloxacin and gatifloxacin.[59,60] Therefore, the newer generations of fluoroquinolones may provide a therapeutic alternative for treating CDI.[59] Mutations in the quinolone resistance-determining regions (QRDR) of *gyrA* and/or *gyrB* genes result in several amino acid substitutions, which render CD resistant to fluoroquinolones.[59,61,62] The commonest of these mutations is the amino acid substitution Thr82Ile in GyrA.[59–61] A recent study showed that the Thr82Ile substitution in GyrA resulted in no detectable fitness cost in CD.[63] This suggests that even in the absence of antibiotic selective pressure this substitution can be maintained. The propensity of fluoroquinolone-susceptible CD strains to acquire mutations and develop reduced susceptibility was studied using five clinical parent strains of CD that were susceptible to moxifloxacin (MIC = 1 mg/L) and levofloxacin (MIC = 2 mg/L).[60] CLSI's tentative susceptibility breakpoint for moxifloxacin in anerobic organisms is given as $\leq$2 mg/L , [64] while the EUCAST epidemiological cut-off value for moxifloxacin in CD is 4 mg/L.[65] These values have not been enumerated for levofloxacin. The fluoroquinolone-susceptible parent CD strains were grown under 2 mg/L of moxifloxacin or 4 mg/L of levofloxacin.[60] Subsequently, colonies were selected, sequenced, their MICs determined, and were further passaged under double the concentration of moxifloxacin and levofloxacin compared with the MIC levels. Isolates thus selected in the presence of increasing concentrations of moxifloxacin and levofloxacin showed higher MICs for moxifloxacin (MIC = 8–128 mg/L) and levofloxacin (MIC = 8–32 mg/L) and also exhibited substitutions in GyrA and/or GyrB, which were previously not detected in the parent strains.[60] This work suggests the potential of suboptimal concentrations of fluoroquinolones to select for GyrA and/or GyrB mutant fluoroquinolone-resistant CD isolates. While *in vivo* studies are needed to support these findings, this stresses the need for optimal dosing of antibiotics to prevent the development of antibiotic resistance.

Different types of efflux pumps have also been implicated in CD resistance to fluoroquinolones. The previously described ABC transporter CD2068 appeared to mediate MDR to ciprofloxacin and levofloxacin.[42] Additionally, overexpression of the CD *cdeA* gene, which encodes the sodium-dependent efflux pump of the multidrug and toxic compound extrusion (MATE) subfamily of secondary multidrug transporters, was observed to induce fluoroquinolone resistance in *Escherichia coli*.[66]

Other antibiotics

Tetracycline

Tetracyclines inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit and blocking the association of aminoacyl-tRNA at the A-site.[58,67] The tetracycline-resistant CD strains produce ribosomal protectant proteins such as Tet(M), Tet(W) and Tet(44), which prevent the binding of the antibiotics to the ribosome.[68,69] The majority of these tetracycline resistance genes encoding ribosomal protectant proteins in CD are located on mobile or conjugative elements, such as *tet*(M)-containing conjugative elements of the Tn*916* family (e.g. Tn*6190*) and *tet*(44)-containing Tn*6164*.[68,69] Tetracycline resistance in CD is usually mediated by its most widespread gene class *tet*(M).[69] To date, these resistance mechanisms have not been shown to mediate resistance in CD to newer tetracyclines such as tigecycline and omadacycline. The tigecycline-resistance genes *tet*(X3) and *tet*(X4) recently detected in Gram-negative bacteria and the acquired mutations in Tet proteins that reduce susceptibility to tigecycline have not been reported in CD.[70]

Chloramphenicol

Chloramphenicol elicits its bacteriostatic activity by binding to the 50S ribosomal subunit at A2451 and A2452 residues and inhibiting bacterial protein synthesis by preventing the binding of tRNA to the P-site of the larger ribosomal subunit, thus the elongation of polypeptide chain.[58] CD resistance to chloramphenicol is achieved via enzyme-mediated antibiotic modification. CD has two copies of the *catD* gene, which encodes the chloramphenicol acetyltransferase enzyme, at the mobile regions Tn*4453a* and Tn*4453b* transposons.[71] This enzyme relocates an acetyl group from acetyl CoA to the primary hydroxyl group of chloramphenicol, rendering the drug inactive and unable to bind to the ribosome to elicit its antibiotic action.[72]

Linezolid

Linezolid, a bacteriostatic oxazolidinone type of antibiotic recently introduced to the market, inhibits bacterial protein synthesis by binding to the 23S rRNA of the 50S ribosomal subunit, thus preventing the formation of the 70S ribosomal unit.[73] Linezolid is currently not recommended for the treatment of CDI.[6] However, a retrospective study over a period of 4 years on a cohort of patients who developed ventilator-associated pneumonia following major heart surgery suggested a potential protective role of linezolid against the development of CDI.[74]

Genes that encode the rRNA methyltransferase Cfr—*cfrB*, *cfrC* and *cfrE*—have been detected among clinical isolates of CD and these isolates exhibited higher MICs of linezolid.[53] The Cfr protein catalyses the methylation of 8-methyladenosine at A2503 position in 23S rRNA of the large ribosomal subunit. This disrupts the interaction between the target and the antibiotic.[53]

Genomic analysis of antibiotic resistance in *C. difficile*: new insights into CDI evolution

The recent advancements in rapid and affordable WGS technologies and the availability of bioinformatics tools and online accessible databases have provided important information on current and emerging antibiotic resistance trends in CD and new insights on CDI dynamics of evolution.

The number of CD strains resistant to several classes of antibiotics is increasing worldwide. About 60% of clinical CD strains have been reported as MDR in Europe,[75] representing one of the major threats to vulnerable patient populations, particularly in the intensive care units and the long-term care settings.

Besides the well-known RT027, several other CD RTs causing severe infections and outbreaks are reported as MDR, including RT012, RT017 and recent emerging types such as RT106, RT018, RT356 and RT078.[18,76–81]

Some CD types, such as RT017, display a higher prevalence of antibiotic resistance than other RTs. Genomic analysis of RT017 CD strains of recent isolation demonstrates that these strains have acquired new mechanisms of antibiotic resistance compared with the reference strain M68, isolated in 2003.[22,82,83]

Almost all (93%–100%) RT017 CD strains from China, Korea and Europe are resistant to MLS_B antibiotics.[84–87] In strain M68, this resistance has been associated with an *erm*(B) gene located on Tn*6194*, while a novel *erm*(G) gene, located on a mobile genetic element, capable of interspecies horizontal transfer, has recently been reported in RT017 CD strains.[88] Interestingly, 8%–12% of recent RT017 strains have also developed high MIC values of imipenem, rarely observed in the CD population.[85,89] In these strains, resistance to imipenem has been associated with two missense mutations (Ala555Thr and Tyr721Ser) near the active site of the *pbp1* and *pbp3* genes.[90] Similarly, resistance to linezolid, very uncommon in CD (1%–6%),[91,92] has been described in RT017 strains that have acquired mobile genetic elements carrying a *cfr* methyltransferase gene.[93] Furthermore, more than 80% of RT017 strains have been reported resistant to fluroquinolones for alteration of the GyrB[62,82,94] and a similar percentage have acquired a Tn*916*-like element, resulting in resistance to tetracycline.[82,95] Finally, RT017 CD has been reported to have a higher prevalence of resistance (about 32%) to rifaximin than other RTs[84,87] due to missense mutations in the *rpoB* gene.[96] The high propensity of RT017 CD for acquiring antibiotic resistance and the high prevalence of MDR strains, particularly in Asia, where antibiotics are poorly regulated, raises several concerns about a further expansion of this ribotype not only in that region but also in other countries with a consequent increase of associated hospital outbreaks.

Surveillance data indicate that the incidence of CDI in the community (CA-CDI) has globally increased and now CA-CDIs accounts for 41% of all CDI cases in the USA,[97] 30% in Australia[98] and 14% in Europe.[99]

Differently from the healthcare-associated RT027, diffusion of RT078, the most common cause of CA-CDI, is probably via other routes/sources outside the hospitals.[100] CD reservoirs have been identified in animals (particularly farm animals), the natural environment (soil, water) and food (animal food and vegetables).[101] Genomic analysis has demonstrated that CD RTs common to humans and farm animals share a recent evolutionary history and support CDI as a zoonotic disease with a consequent spillover of CD into the environment and food. In particular, genomic analysis has evidenced a potential bidirectional spread of RT078 CD strains between pigs and farmers.[102] Tetracycline, widely used in animal husbandry, has a key role in driving RT078 CD diffusion. In fact, phylogenetic analysis performed on hundreds of international

RT078 genomes has demonstrated a global spread of this RT, with multiple independent clonal expansions associated with the acquisition of tetracycline resistance.[103] In particular, phenotypic and genotypic analysis of 185 RT078 strains has demonstrated that 48% of them contain one or more tetracycline resistance genes [*tet*(M), *tet*-40, *tet*(O) and *tet*-44], often associated with mobile genetic elements [Tn*6190* for *tet*(M) and Tn*6164* for *tet*-44].[80] Among strains analysed, 36.1% were also resistant to MLS_B but only 13% of them showed an *erm*(B) gene, prevalently associated with Tn*6194*; the remaining strains were negative for the other *erm* classes and for ribosomal proteins (L4/L22) and 23S rRNA gene mutations, suggesting the presence of an alternative mechanism. Among the other resistance loci identified, one or more aminoglycoside/streptothricin resistance genes were observed in 45% of the strains, while the *aph3-III–sat4A–ant6-Ia* cassette was reported in 40% of strains. Furthermore, all strains were positive for the β-lactamase-inducing PBP gene *blaR* and the efflux resistance gene *cme*.

Recent genomic investigations indicate that, besides toxigenic CD strains, non-toxigenic (NT) CD strains could also represent a source of antibiotic resistance determinants. Resistance and MDR have been reported in NT strains of human and animal origin and, in particular, several NT RT010 strains were found to be MDR and resistant to metronidazole.[104,105] Interestingly, this resistance in RT010 has been correlated with the presence of the plasmid pCD-METRO.[11] Furthermore, Tn*6215*-like elements have been reported in 33% of human and environmental RT010 strains.[106] The Tn*6215* is a peculiar mobilizable transposon, conferring resistance to MLS_B and able to transfer between CD strains using different mechanisms (conjugation-like mechanism, phage ΦC2 transduction and a transformation-like mechanism).[52,107] Since NT CD strains can colonize animals and humans and are widely diffused in the natural environment, they could have an important role in the spreading of antibiotic resistance among the CD population.

All data indicate that antibiotic resistance in CD is constantly evolving, with consequent important impacts on epidemiology of CDI. Recent studies strongly suggest that there is a potential circulation of CD strains between reservoirs where, depending on the selective forces present, antibiotic resistance determinants may be acquired, reinforcing the concept that CD resistome is shared between the environment and clinic. In alignment with the One Health surveillance framework of CDI, genomic analysis and the application of online tools for real-time detection and tracking of antibiotic resistance determinants may represent a useful weapon to expand monitoring across CD reservoirs, besides phenotypic surveillance, in order to guide the development of optimal antibiotic stewardship policies to prevent and limit mobilization of the genetic reservoir of resistance among the CD population.

Mechanisms of antibiotic resistance in representative strains of the prevalent epidemic ribotypes

We identified the antibiotic resistance factors found in the genomes of a few selected representative CD RT strains, using the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) software, with perfect or strict criteria for resistome prediction based on homology and SNP models[108] to study CD strain-specific resistance factors (Table 2). Strains were selected based on their epidemic nature and to represent the most common ribotypes found in different areas of the world. While the presence of a resistance gene or a mutation itself is not indicative of phenotypic resistance, it is important to be aware of the potential resistance-mediating genes present in epidemiologically prevalent strains, to be vigilant for future emergence of resistance via occurrence of mutations or changes in gene expression. Moreover, predicting potential resistance patterns will be useful in guiding antibiotic stewardship policies and for identification of novel therapeutic targets to combat CDI.

Conclusions

Antibiotic resistance of CD is an urgent problem faced all over the world today. There is a global increase in MDR of CD, with emergence of novel strains that are often more virulent and MDR. Antibiotic resistance in CD is perpetually evolving with acquisition of novel resistance-determining mechanisms. These resistance-mediating factors can be transferred between different species of bacteria and among different strains of CD present in the clinical setting, community and in the environment, including animal reservoirs, food sources, soil and water. Community-acquired CDI is now increasing worldwide, and environmental sources of CD are considered to be important for this phenomenon, especially with zoonotic spillover and bidirectional transfer. Non-toxigenic CD strains are also now emerging as an important source of antibiotic resistance and MDR of CD, as these strains are widely diffused in the natural environment and can colonize both humans and animals, thus can vastly contribute to spreading CD antibiotic resistance.

Prevalence of different strains of CD and their characteristic antibiotic resistance patterns show distinct geographical patterns in different areas of the world. Epidemiological characteristics of CDI are largely determined by antibiotic resistance, driven by variable stringencies of regulation of antibiotic use in different regions of the world. Understanding epidemiologically driven and strain-specific characteristics of antibiotic resistance is important for effective surveillance of antibiotic resistance and for limiting spread of resistance-determining factors, not only between different strains of CD but also among various bacterial species.

Understanding the mechanisms of antibiotic resistance in CD and vigilant monitoring of CD for new genotypic and phenotypic characteristics and evolution of antibiotic resistance are also important for identifying potential therapeutic targets. Using targets thus identified, new and improved therapies need to be developed to prevent and curb antibiotic resistance of CD. Supplementing phenotypic methods of antibiotic resistance detection with genome analysis, bioinformatic tools and use of online databases is vital for efficient detection and monitoring of CD antibiotic resistance and evolution. Effective antibiotic stewardship, with prescription and usage of optimal doses of antibiotics for the optimal duration of time and avoiding unnecessary use of antibiotics, is essential to prevent and control the development of CD antibiotic resistance.

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