Recent advances in the understanding of antibiotic resistance in *Clostridium difficile* infection

Ther Adv Infect Dis
(2016) 3(1) 23–42
DOI: 10.1177/2049936115622891

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***Abstract*:** *Clostridium difficile* epidemiology has changed in recent years, with the emergence of highly virulent types associated with severe infections, high rates of recurrences and mortality. Antibiotic resistance plays an important role in driving these epidemiological changes and the emergence of new types. While clindamycin resistance was driving historical endemic types, new types are associated with resistance to fluoroquinolones. Furthermore, resistance to multiple antibiotics is a common feature of the newly emergent strains and, in general, of many epidemic isolates. A reduced susceptibility to antibiotics used for *C. difficile* infection (CDI) treatment, in particular to metronidazole, has recently been described in several studies. Furthermore, an increased number of strains show resistance to rifamycins, used for the treatment of relapsing CDI. Several mechanisms of resistance have been identified in *C. difficile*, including acquisition of genetic elements and alterations of the antibiotic target sites. The *C. difficile* genome contains a plethora of mobile genetic elements, many of them involved in antibiotic resistance. Transfer of genetic elements among *C. difficile* strains or between *C. difficile* and other bacterial species can occur through different mechanisms that facilitate their spread. Investigations of the fitness cost in *C. difficile* indicate that both genetic elements and mutations in the molecular targets of antibiotics can be maintained regardless of the burden imposed on fitness, suggesting that resistances may persist in the *C. difficile* population also in absence of antibiotic selective pressure. The rapid evolution of antibiotic resistance and its composite nature complicate strategies in the treatment and prevention of CDI. The rapid identification of new phenotypic and genotypic traits, the implementation of effective antimicrobial stewardship and infection control programs, and the development of alternative therapies are needed to prevent and contain the spread of resistance and to ensure an efficacious therapy for CDI.

***Keywords*:** antimicrobial susceptibility, clindamycin, *Clostridium difficile*, fluoroquinolones, metronidazole, mobile genetic elements, multidrug resistance, rifamycins

Introduction

Clostridium difficile is recognized as the major cause of healthcare antibiotic-associated diarrhea [To and Napolitano, 2014]. Antibiotics used for treating every kind of infection may potentially promote *C. difficile* infection (CDI). After antibiotic therapy, the protective intestinal microbiota is disrupted allowing ingested or resident *C. difficile* to colonize the gastrointestinal tract and infect the host. Antibiotic resistance enables *C. difficile* to grow in the presence of drugs, so strains resistant to multiple agents may have a selective advantage for their diffusion from the usage of these antibiotics.

An alarming increase in incidence of CDI has been observed across the United States, Canada and Europe over the last decade, with a significant financial burden on the healthcare system [Redelings *et al.* 2007; Burckhardt *et al.* 2008; Bauer *et al.* 2011; Gravel *et al.* 2009; Miller *et al.* 2011a; Dubberke and Olsen, 2012; Lessa *et al.* 2012]. These changes have been associated with the emergence of highly virulent

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(or hypervirulent) strains of *C. difficile*. The most prominent hypervirulent type is recognized as polymerase chain reaction (PCR) ribotype (RT) 027, North American pulsed field gel electrophoresis type I (NAPI) and restriction endonuclease analysis group B1, depending on the typing method used. Strains RT027, responsible for severe infections, characterized by a high rate of recurrences, mortality and refractory to traditional therapy, have spread globally over recent years [Pépin *et al.* 2004, 2005b; McDonald *et al.* 2005; Muto *et al.* 2005; Goorhuis *et al.* 2007; Clements *et al.* 2010]. *C. difficile* epidemiology is rapidly evolving. In recent years, CDI has emerged as a cause of diarrhea in the community, especially in populations previously considered at low risk, such as young individuals, healthy peripartum women, antibiotic-naive patients or those with no recent health care exposure [Kim *et al.* 2008; Pituch, 2009; Freeman *et al.* 2010]. In addition to RT027, a number of emergent highly virulent RTs, correlated to RT027 or not, have recently been identified [Cartman *et al.* 2010; Valiente *et al.* 2012]. Among these types, the hypervirulent RT078 has been recognized as a cause of infections in humans, in hospitals [Rupnik *et al.* 2008; Bauer *et al.* 2011], in the community [Limbago *et al.* 2009], and also in animals [Kee *et al.* 2007; Goorhuis *et al.* 2008].

Antibiotic resistance plays an important role in driving the current epidemic of CDI and the emergence of new types. A glaring example of this role is represented by the emergence and spread of *C. difficile* RT027, that has been correlated with the massive use of fluoroquinolones (FQs) and the acquisition of resistance to these antibiotics, a trait not present in historic strains of the same type [He *et al.* 2013].

C. difficile show a high capability to adapt to the environment through metabolic and genomic changes. Molecular investigations have demonstrated that *C. difficile* has a versatile genome content, with a wide range of mobile elements, many of them encoding for predicted antibiotic resistances [Sebaihia *et al.* 2006; He *et al.* 2010, 2013]. In addition to horizontal gene transfer, other mechanisms may contribute to antibiotic resistance in this pathogen, and recent studies support the multifactorial nature of this phenomenon.

The present paper will review phenotypic and genotypic traits of antibiotic resistance in *C. difficile* taking into consideration the most recent data.

Antibiotics promoting CDI

Certain antibiotics, such as cephalosporins (CFs), clindamycin (CLI) and, more recently, FQs, are known to carry a higher risk for CDI than others [Gerding, 2004; Slimings and Riley, 2014].

In the 1970s, CLI was recognized as the highest-risk agent for CDI [Bartlett *et al.* 1977]. Since then, many CDI outbreaks involving CLI-resistant *C. difficile* strains have been described [Samore *et al.* 1994, 1997; Johnson *et al.* 1999]. Antimicrobial stewardship policies were implemented to control the use of CLI in the US and Europe, therefore the attributable risk of CLI-associated diarrhea and CDI was reduced in the following years [Wiström *et al.* 2001]. CFs had become the antibiotics with the highest relative and attributable risk of CDI, between the 1980s and 1990s for their frequent use in hospitals [Bignardi, 1998]. A decrease in the numbers of patients with *C. difficile* has been observed in the hospitals that have curtailed the use of these antibiotics [de Lalla *et al.* 1989; Khan and Cheesbrough, 2003]. Currently, the risk of hospital-acquired CDI remains high for CFs and CLI, so their importance as promoting agents should not be minimized. The rise in the FQ-associated CDI has been concomitant with the increasing incidence of *C. difficile* RT027 in the early 2000s. Current strains RT027 show high-level resistance to FQs, never observed in historical isolates of the same RT [McDonald *et al.* 2005]. Although infection control procedures and antimicrobial stewardship have led to a significant reduction in the incidence of CDI by *C. difficile* RT027, this RT is still predominant in both Europe and the US [Muto *et al.* 2007; Lessa *et al.* 2015; Freeman *et al.* 2015].

C. difficile *resistance patterns*

Rates of antibiotic resistance vary considerably in the different studies, probably depending on the geographic regions and local or national antibiotic policy. Data extrapolated from 30 studies published between 2012 and 2015 (Table 1) indicate that resistance to CLI and CFs is very common in *C. difficile* clinical isolates (55% and 51%, respectively), as the resistance to erythromycin (ERY) and FQs (47%). Most of the strains tested for susceptibility to second-generation CFs [cefotetan (CTT) and cefoxitin (FOX)] were

Table 1. Antimicrobial susceptibility of *C. difficile* clinical isolates from 30 studies published between 2012 and 2015.

Year of isolation	Country	Number of clinical isolates	Percentage of clinical isolates resistant to											Predominant *C. difficile* types (method different from PCR-ribotyping)	Susceptibility method	References
			CFs						FQs							
			CTT	FOX	CRO	CTX	CLI	ERY	CIP	MXF	GAT	RIF	MTZ	VAN		
2000–2009	Germany	34						76	88	68					001, 027	Etest	Reil *et al.* [2012]
2002–2005	Japan	73			93.2		87.7	87.7	100				0.0	0.0		Etest	Oka *et al.* [2012]
2006–2007	Canada	432			35.4		50.5			45.1			0.0	0.0	NAP1, 2, 4, 6 (PFGE)	AD	Karlowsky *et al.* [2012]
2006–2008	Switzerland	86			30.4		52.3		98.8				0.0	1.2		Etest	Büchler *et al.* [2014]
2008–2009	US and Canada	316					41.5			38		7.9	0.0		002, 017, 027, 053, 078,104, 106	Etest	Tenover *et al.* [2012]
2009	France	224					34.8	19,2		8			0.0	0.0	014/020/077, 078/126, 015, 002, 005, 027	Etest	Eckert *et al.* [2013]
2009	Spain	154					74	49	100	43		24	0.0	0.0		Etest	Rodríguez-Pardo *et al.* [2013]
2000–2009	Korea	120	19				81	80	100	42					001, 018, 017, 014/020	AD	Lee *et al.* [2014]
2008–2010	South Korea	131					67.9			62.6		19.1	0.0	0.0	018, 017, 001	Etest	Kim *et al.* [2012]
2005–2010	Taiwan	403					73.5			17.9			0.0	0.5		AD	Liao *et al.* [2012]
2007–2010	Texas	271		96.3					98.5				13.3	0.0		Etest	Norman *et al.* [2014]
2008–2010	Poland	17					100	100	100	100	100	0.0	0.0	0.0	027, 176	Etest	Obuch-Woszczatynski *et al.* [2014]
2008–2010	Hungary	88								19.7		14.9	0.0	0.0		Etest	Eitel *et al.* [2015]
2008–2010	Hungary	200					29.5	31		21.5		11.5	0.0			Etest	Terhes *et al.* [2014]
2009–2010	Poland	10										80.0	0.0	0.0	046, 017	Etest	Obuch-Woszczatynski *et al.* [2013]
2010	US	46					8.3			78.0			0.0		027,001, 014/020,005	Etest	Zhou *et al.* [2014]
2010–2011	Croatia	23					39	30	96	22	26		0.0	0.0	001, 014	Etest	Novak *et al.* [2014]
2007–2011	Spain	196						18.0		20.0			0.0	0.0	014, 078, 001	Etest	Weber *et al.* [2013]
2008–2011	Slovenia	92	1.1	98.9			42.4	13.0	100	11.9		2.2	0.0	0.0	002, 014/020, 012, 029, 046	BD	Pirš *et al.* [2013]
2010–2011	Iran	75				100	89.3	57.3					5.3	8.0		AD	Goudarzi *et al.* [2013]

(continued)

Table 1. Continued

Year of isolation	Country	Number of clinical isolates	Percentage of clinical isolates resistant to											Predominant *C. difficile* types (method different from PCR-ribotyping)	Susceptibility method	References
			CFs						FQs							
			CTT	FOX	CRO	CTX	CLI	ERY	CIP	MXF	GAT	RIF	MTZ	VAN		
2010–2012	China	60		86.67			73.3			30.0		1.7	0.0	0.0	tr017, tr065, tr014, tr012 (TRST)	AD	Dong *et al.* [2013]
2011–2012	US	317					19			21			0.0	0.0	078, PA01, PA04	Etest	Varshney *et al.* [2014]
2011–2012	Europe	953					49.62			39.99		13.40	0.11	0.87	027, 001/072, 078,014	AI	Freeman *et al.* [2015]
2012	Poland	83					27.7	85.5	100	83.1		18.0	0.0	0.0	027, 176	Etest	Lachowicz *et al.* [2015]
2011–2013	Japan	159					69	70		62	71	1.2	0.0	0.0	018, 369, 014, 002	Etest	Senoh *et al.* [2015]
2012–2013	Japan	130			49		59		99				0.0	0.0	ST2, ST17, ST8 (MLST)	AD	Kuwata *et al.* [2015]
2013–2014	Australia	440			18.2		84.3			3.4			0.0	0.0	014, 002	AD	Knight *et al.* [2015]
2014	Israel	208								66			18	47	027	Etest	Adler *et al.* [2015]
2014–2015	Czech Republic	20					10	100	100	100		65	0.0	0.0	176	Etest	Krutova *et al.* [2015]
–	Zimbabwe	23				100	100	43.5	100				0.0	0.0		DD	Simango and Ulandi [2014]

CFs, cephalosporins; FQs, fluoroquinolones; CTT, cefotetan; FOX, cefoxitin; CRO, ceftriaxone; CTX, cefotaxime; CLI, clindamycin; ERY, erythromycin; CIP, ciprofloxacin; MXF, moxifloxacin; GAT, gatifloxacin; RIF, rifampin; MTZ, metronidazole; VAN, vancomycin; PCR, polymerase chain reaction; PFGE, pulsed field gel electrophoresis; TRST, tandem repeat sequence typing; MLST, multi locus sequence typing; AD, agar dilution; BD, broth dilution; AI, agar incorporation; DD, disk diffusion.

resistant (79%), whereas resistance to third-generation CFs [ceftriaxone (CRO) and cefotaxime (CTX)] is present in a lower number of isolates (38%). Similarly, resistance to ciprofloxacin (CIP), a second-generation FQ, is very common in *C. difficile* (99% of the strains tested), while resistance to moxifloxacin (MXF) and gatifloxacin (GAT), fourth-generation FQs, have been detected in 34% of the strains analyzed for these antibiotics.

The most common antibiotic susceptibility methods used for *C. difficile* are the agar dilution (AD) and the epsilometer test (Etest), a commercially available gradient diffusion system for quantitative antibiotic susceptibility testing. The Clinical and Laboratory Standards Institute (CLSI) indicates AD as the reference method for *C. difficile* and underlines that other techniques may be used as long as equivalence to the reference methods is established [Clinical and Laboratory Standards Institute, 2012]. The disadvantages of AD approach are the laborious, time-consuming steps required to prepare testing plates, particularly when the number of compounds to be tested is high or when only a limited number of bacteria are to be analyzed. For these reasons, most laboratories use Etest for routine (Table 1).

Mechanisms of resistance

Cephalosporins. Resistance to CFs is still uncharacterized in *C. difficile*, although most of strains are resistant to these antibiotics (Table 1). *C. difficile* overgrowth seems to occur after CFs therapy, as reported in several studies [Ambrose *et al.* 1985; de Lalla *et al.* 1989; Impallomeni *et al.* 1995]. *C. difficile* is often described as 'constitutively resistant' to CFs, but the variable minimum inhibitory concentration (MIC) values to the different CFs suggest that resistance to these antibiotics may be strain-dependent. β-Lactam antibiotic resistance is caused mainly by two mechanisms: antibiotic-degrading enzymes, β-lactamases, and modification of target sites, penicillin-binding proteins (PBPs). Genome analysis of *C. difficile* 630 (accession number AM180355.1) shows a number of coding sequences (CDSs) potentially involved in this resistance (Table 2). These CDSs strains are present also in other *C. difficile* strains, with identity ranging between 73% and 100%. Genomic comparison and functional analysis of

Table 2. *C. difficile 630* CDSs potentially involved in resistance to cephalosporins.

Locus-tag in *C. difficile* 630	Product
CD630_03440	Putative β-lactamase-like protein
CD630_04580	Putative β-lactamase
CD630_04640	Putative β-lactamase-like hydrolase
CD630_04700	β-lactamase-inducing penicillin-binding protein
CD630_04710	Penicillinase transcriptional regulator
CD630_05150	D-alanyl-D-alanine carboxypeptidase, S11 peptidase family
CD630_05270	Putative β-lactamase-like hydrolase
CD630_05480	Putative penicillin-binding peptidase
CD630_06550	Putative β-lactamase-like protein
CD630_07810	Putative penicillin-binding protein
CD630_08290	Putative metallo-β-lactamase superfamily protein
CD630_08950	Metallo-β-lactamase superfamily exported protein
CD630_11480	Putative penicillin-binding protein
CD630_12290	Peptidoglycan glycosyltransferase
CD630_12910	Penicillin-binding protein
CD630_13740	Putative β-lactamase-inhibitor protein II
CD630_14690	Putative cell surface protein; putative penicillin-binding protein cwp20
CD630_16270	D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein)
CD630_18020	Putative hydrolase, metallo-β-lactamase superfamily
CD630_21410	Serine-type D-Ala-D-Ala carboxypeptidase
CD630_24980	Putative sporulation-specific penicillin-binding protein
CD630_26560	Stage V sporulation protein D (sporulation-specific penicillin-binding protein)
CD630_27420	Putative hydrolase β-lactamase-like
CD630_31960	Putative penicillin-binding protein
CD630_36510	Putative metallo-β-lactamase-like hydrolase

Table 3. Genetic elements involved in *C. difficile* antibiotic resistance.

Antibiotic	Resistance mechanism	Genetic element	Gene	References
MLS_B	Ribosomal methylation	Tn*5398* and Tn*5398*-like	*erm*B	Farrow *et al.* [2001]; Brouwer *et al.* [2011]; Spigaglia *et al.* [2005]; Spigaglia *et al.* [2011]
		Tn*6194*	*erm*B	Wasels *et al.* [2013]; He *et al.* [2010, 2013]
		Tn*6215*	*erm*B	Goh *et al.* [2013]; Wasels *et al.* [2015]
		Tn*6218*	*ermAB/cfr*	Dingle *et al.* [2014]
Tetracycline	Ribosomal protection	Tn*5397*	*tet*M	Roberts *et al.* [2001, 2011]
		Tn*916*-like	*tet*M	Sebaihia *et al.* [2006]; Brouwer *et al.* [2011, 2012]; Spigaglia *et al.* [2005, 2007]
		Tn*6164*	*tet*44	Corver *et al.* [2012]
Chloramphenicol	Chloramphenicol acetyltransferase	Tn*4453*a and Tn*4453*b	*cat*D	Wren *et al.* [1988, 1989]

C. difficile strains showing different phenotypes will be necessary to clarify the role of these potential β-lactam interacting genes.

MLS_B antibiotics. Ribosomal methylation is the most widespread mechanism of resistance to the antibiotics of the macrolide–lincosamide–streptograminB (MLS_B) family in *C. difficile* (Table 3). Erythromycin ribosomal methylases (*erm*) genes of class B commonly mediate resistance to these antibiotics, even if other *erm* genes have rarely been detected in *C. difficile* isolates [Roberts *et al.* 1994; Spigaglia *et al.* 2005; Schmidt *et al.* 2007]. Tn*5398* is a mobilizable non-conjugative element of 9.6 kb length containing two copies of *erm*B genes [Farrow *et al.* 2001]. This element is able to transfer *in vitro* from *C. difficile* to *Staphylococcus aureus* and to *Bacillus subtilis* [Hächler *et al.* 1987; Mullany *et al.* 1995]. Since Tn*5398* does not have genes encoding a recombinase, other conjugative transposons present in the donor genome could provide integration/excision functions to transfer the element to the recipient strain [Mullany *et al.* 2015]. The element could integrate into the recipient chromosome either by homologous recombination or by using a site-specific recombinase of the recipient. Another possibility is that a portion of the donor genome containing Tn*5398* is transferred to the recipient and integrates by homologous recombination [Wasels *et al.* 2015b].

*erm*B-containing elements different from Tn*5398* have been found in the majority of *C. difficile* strains resistant to MLS_B. A total of 17 genetic organizations of these elements, denominated E1–E17, have been identified by a PCR-mapping method designed on the genetic organization of Tn*5398* [Farrow *et al.* 2001; Spigaglia *et al.* 2005, 2011]. The most frequent genetic organization detected among European *C. difficile* clinical isolates in 2005 was the E4 [Spigaglia *et al.* 2011]. A recent analysis has demonstrated that elements E4 are related to Tn*6194*, a conjugative transposon firstly identified in *C. difficile* 2007855 [He *et al.* 2010, 2013; Wasels *et al.* 2013]. This element has a conjugative region related to that of Tn*916*, a large family of conjugative elements widely spread in Gram-positive and Gram-negative bacteria, and an accessory region that is related to Tn*5398*. Transfer of Tn*6194* from *C. difficile* to *Enterococcus faecalis* has been demonstrated *in vitro* [Wasels *et al.* 2014].

Tn*6215* is a peculiar element conferring resistance to MLS recently characterized in *C. difficile* CD80 [Goh *et al.* 2013]. Interestingly, this mobilizable transposon of about 13 kb in length can be transferred between *C. difficile* strains by a conjugation-like mechanism and by phage ΦC2 transduction. Furthermore, very recent data suggest that both Tn*6215* and Tn*5398* can be transferred to the *C. difficile* recipient strain *C. difficile* CD13 by a transformation-like mechanism [Wasels *et al.* 2015b].

It has been demonstrated that the *in vitro* maintenance of *erm*B-containing elements in

C. difficile genome has a cost on the fitness of the bacterium [Wasels *et al.* 2013]. Nonetheless, these elements are widespread in the *C. difficile* population, suggesting that, regardless of the burden that an element imposes on fitness, other factors (i.e. the capability of transfer and the intrinsic genetic characteristics of the strains) are involved in the successful spreading of an element among *C. difficile* isolates.

Several *C. difficile erm*-negative strains resistant to both ERY and CLI or only to ERY have been identified [Spigaglia and Mastrantonio, 2004; Pituch, *et al.* 2006; Ratnayake *et al.* 2011; Spigaglia *et al.* 2011]. Alterations in the 23S rDNA or ribosomal proteins (L4 or L22) have been found in some of these strains, but the presence of the same changes in susceptible isolates excludes their role in resistance [Spigaglia *et al.* 2011]. Furthermore, resistant *erm*-negative strains treated with reserpine and carbonyl cyanide m-chlorophenyl hydrazone (CCCP), two pump inhibitors, do not show any reduction in MICs, suggesting that resistance is not mediated by efflux mechanisms [Spigaglia *et al.* 2011]. Tn*6218* are nonconjugative Tn*916*-related elements recently identified in *C. difficile* strains [Dingle *et al.* 2014]. These elements carry multiple accessory genes conferring resistance to clinically relevant antibiotics, including *cfr* and *erm*AB genes. In particular, the multidrug resistance (MDR) gene *cfr*, present in a wide range of Gram-positive and Gram-negative species, could have a role in *C. difficile* resistance to MLS_B in the absence of *erm* genes.

Fluoroquinolones. Resistance to FQs in *C. difficile* is due to alterations in the quinolone-resistance determining region (QRDR) of either GyrA or GyrB, the DNA gyrase subunits [Ackermann *et al.* 2001, 2003; Dridi *et al.* 2002; Drudy *et al.* 2006, 2007]. Several amino acidic substitutions have been identified in both GyrA and/or GyrB (Table 4), but almost all *C. difficile* FQs-resistant strains show the substitution Thr82Ile in GyrA [Ackermann *et al.* 2001; Dridi *et al.* 2002; Spigaglia *et al.* 2008a, 2011; Kuwata *et al.* 2015]. *In vitro* experiments have demonstrated the MXF and levofloxacin (LE) exposure induce high frequency of selection for GyrA and GyrB drug-resistant mutants in previously susceptible strains [Spigaglia *et al.* 2009]. Therefore, it can be hypothesized that during the first stages of antibiotic treatment, when the concentration of drug in the intestine is still not inhibitory, a subpopulation of *C. difficile* may be able to acquire substitutions conferring resistance to FQs. Interestingly, a recent study has shown that Thr82Ile in GyrA has not detectable cost on the fitness of *C. difficile*, suggesting that this substitution can be maintained in the bacterial population even in the absence of antibiotic selective pressure [Wasels *et al.* 2015a].

Antibiotics for treatment of CDI

Metronidazole

Metronidazole (MTZ) is a nitroaromatic prodrug that need the reduction of the 5-nitro group of the imidazole ring to become cytotoxic to bacterial cells [Goldman, 1982] and it is considered the first choice for mild to moderate CDI [Debast *et al.* 2014; Lyras and Cooper, 2015].

Although the percentage of *C. difficile* strains resistant to MTZ is, in general, low (Table 1), several studies have emphasized treatment failure after treatment with MTZ [Musher *et al.* 2005; Pépin *et al.* 2005a; Vardakas *et al.* 2012]. An elevated geometric mean of MICs to MTZ have recently been observed in RT027 (1.1–1.42 mg/l), RT001/072 (0.65 mg/l), RT106 (0.65 mg/l), RT356 (0.61 mg/l) and in the nontoxigenic RT010 (1.5 mg/l), compared with the values of the other RTs (0.13–0.41 mg/l) [Moura *et al.* 2013; Freeman *et al.* 2015]. Furthermore, a number of *C. difficile* strains with MICs >2 mg/l, the EUCAST epidemiological cut-off (ECOFF) for MTZ (see http://mic.eucast.org/Eucast2/), have been reported in both humans and animals, as shown in Table 5. In particular, a very recent study reports the spread of strains RT027 with reduced susceptibility to MTZ in Israel, where they cause severe infections and a wide outbreak in 2013 in Jerusalem [Adler *et al.* 2015].

Heteroresistance seems to be a preresistance stage in *C. difficile*, as part of the population acquires the capacity of growth in presence of an antibiotic [Falagas *et al.* 2008; Peláez *et al.* 2008]. *In vitro* analysis suggest that subinhibitory concentrations of MTZ could have a role in selecting and maintaining colonies with increased MICs [Peláez *et al.* 2008; Moura *et al.* 2013]. Since mean concentration of MTZ in the feces of patients ranges from 0.8 to 24.2 μg/g [Bolton and Culshaw, 1986] it has been hypothesized that the concentrations achieved in the colon could be insufficient for the treatment of CDI due to strains with higher MICs [Brazier *et al.* 2001;

Table 4. Amino acid substitutions detected in *C. difficile* clinical isolates resistant to fluoroquinolones or rifamycins.

Antibiotic	Target	Amino acid substitution	References
Fluoroquinolones	**DNA gyrase subunits**	**GyrA**	
		Thr82Ile	Ackermann *et al.* [2001]; Dridi *et al.* [2002]; Spigaglia *et al.* [2008]; Kuwata *et al.* [2015]
		Thr82Val	Ackermann *et al.* [2001]; Dridi *et al.* [2002]; Spigaglia *et al.* [2008]; Kuwata *et al.* [2015]; Liao *et al.* [2012]
		Asp71Val	Dridi *et al.* [2002]; Walkty *et al.* [2010]; Liao *et al.* [2012]
		Ala118Thr	Dridi *et al.* [2002]
		Val43Asp	Carman *et al.* [2009]
		Asp81Asn	Huang *et al.* [2009]; Liao *et al.* [2012]
		Ala384Asp	Mac Aogáin *et al.* [2015]
		GyrB	
		Asp426Asn	Dridi *et al.* [2002]; Spigaglia *et al.* [2008]; Liao *et al.* [2012]
		Asp426Val	Spigaglia *et al.* [2008]
		Arg447Lys	Walkty *et al.* [2010]; Liao *et al.* [2012]
		Glu466Val	Liao *et al.* [2012]
		Arg377Gly	Liao *et al.* [2012]
		Ser416Ala	
		GyrA/GyrB	
		Thr82Ile/Ser416Ala	Spigaglia *et al.* [2008]; Liao *et al.* [2012]
		Thr82Ile/Ser366Ala	Huang *et al.* [2009]; Kuwata *et al.* [2015]
		Thr82Val/Asp426Val	Huang *et al.* [2009]; Liao *et al.* [2012]
		Thr82Ile/Ser366Ala and Asp426Val	Walkty *et al.* [2010]; Kuwata *et al.* [2015]
		Thr82Ile/Asp426Asn	Walkty *et al.* [2010]; Kuwata *et al.* [2015]
		Thr82Ile/Leu444Phe	Walkty *et al.* [2010]
		Thr82Ile/Asp426Val	Spigaglia *et al.* [2011]
		Thr82Ala/Ser366Ala and Gln434Lys	Kuwata *et al.* [2015]
Rifamycins	**RNA polymerase**	**RpoB**	
		His502Tyr	O'Connor *et al.* [2008]; Pecavar *et al.* [2012]
		His502Arg	O'Connor *et al.* [2008]
		Arg505Lys	O'Connor *et al.* [2008]; Curry *et al.* [2009]; Miller *et al.* [2011]; Spigaglia *et al.* [2011]; Pecavar *et al.* [2012]
		His502Asn	Miller *et al.* [2011]; Pecavar *et al.* [2012]
		Asp492Asn	Pecavar *et al.* [2012]
		Asp492Val	Pecavar *et al.* [2012]
		His502Leu	Pecavar *et al.* [2012]
		Ser550Phe	Pecavar *et al.* [2012]
		Ser550Tyr	Pecavar *et al.* [2012]
		Ser448Thr and Arg505Lys	O'Connor *et al.* [2008]; Curry *et al.* [2009]
		Asp492Asn and Arg505Lys	O'Connor *et al.* [2008]
		His502Asn and Arg505Lys	O'Connor *et al.* [2008]; Curry *et al.* [2009]; Miller *et al.* [2011]; Spigaglia *et al.* [2011]; Pecavar *et al.* [2012]
		Arg505Lys and Ile548Met	O'Connor *et al.* [2008]; Curry *et al.* [2009]; Pecavar *et al.* [2012]
		Ser498Thr and Arg505Lys	Curry *et al.* [2009]; Miller *et al.* [2011]
		Leu487Phe and His502Tyr	Pecavar *et al.* [2012]
		His502Tyr and Pro496Ser	Carman *et al.* [2009]

Table 5. Characteristics of *C. difficile* strains with reduced susceptibility to metronidazole from 16 studies published between 2012 and 2015.

Antibiotic	Origin	Year of isolation	Country	Number of strains	PCR-ribotypes	MIC value (mg/l)	MICs after strains manipulation (mg/l)	References
Metronidazole								
	Human							
		2002–2007	Taiwan	2		>2		Chia *et al.* [2013]
		2009	Canada	1	027	>32	8	Lynch *et al.* [2013]
		2007–2010	Texas	36		32		Norman *et al.* [2014]
		2010–2011	Iran	4		32–64		Goudarzi *et al.* [2013]
		2011–2012	Europe	1	106	8		Freeman *et al.* [2015]
		2014	Israel	38	027	>2		Adler *et al.* [2015]
	Animal							
	swine	-	Spain	4	078	≥256		Peláez *et al.* [2013]
	dog	-	Sweden	1		64	2–8	Wetterwik *et al.* [2013]
	swine	2007–2010	Texas	21		32		Norman *et al.* [2014]
	zebra	-	Spain	1		≥256	< 2	Álvarez-Pérez *et al.* [2014]
	dog	2007–2013	Italy	14	010 078	3–≥256	0.25–32	Spigaglia *et al.* [2015]
Vancomycin								
	Human							
		2002–2007	Taiwan	5		>2		Chia *et al.* [2013]
		2010–2011	Iran	2		4		Goudarzi *et al.* [2013]
		2010–2012	China	2		8		Dong *et al.* [2013]
		2005–2010	Taiwan	2		4		Liao *et al.* [2012]
		2014	Israel	98	027	>2		Adler *et al.* [2015]

MIC, minimum inhibitory concentration; PCR, polymerase chain reaction.

Baines *et al.* 2008; Moura *et al.* 2013]. Although the clinical relevance of reduced susceptibility to MTZ is still not completely understood, a recent study suggest a potential impact of decreased susceptibility to MTZ of strains RT027 on the pathophysiology of recurrent CDI [Richardson *et al.* 2015].

Detection of strains with reduced susceptibility to MTZ can be problematic. In fact, this resistance is often unstable and laboratory manipulation of strains frequently results in MIC decrease towards a susceptibility range [Peláez *et al.* 2008; Lynch *et al.* 2013]. Experimental methodology may affect the magnitude of measured metronidazole MICs for *C. difficile*. The overall data reported in a recent study suggest the agar incorporation method (AIM) [Freeman *et al.* 2005] as the method of choice to detect strains with reduced susceptibility to metronidazole compared with the Etest and the AD [Moura *et al.* 2013]. Differences in the media used (Schaedlers broth and Wilkins–Chalgren agar for AIM and Brucella broth/agar for both Etest and AD) and in the duration of the precultured period (24 h for AIM and 48 h for both Etest and AD) seems to affect MIC determination [Baines *et al.* 2008; Moura *et al.* 2013]. Metronidazole susceptibility breakpoint for *C. difficile* defined by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) are not equivalent: the first is defined as ≥32 mg/l, the second >2 mg/l [Clinical and Laboratory Standards Institute, 2015] (see also http://www.eucast.org/clinical_breakpoints/). Methodological differences and different interpretation categories may cause discrepancies in results, influencing therapeutic decision and comparison of data. For these reasons, international committees are currently cooperating with the intention of harmonizing susceptibility testing and international breakpoints.

In contrast to other pathogens, such as *Helicobacter pylori* and *Bacteroides fragilis*, nitroimidazole (*nim*) genes conferring resistance to MTZ [Gal and Brazier, 2004] have not been identified in *C. difficile* [Moura *et al.* 2014]. *C. difficile* mechanism of resistance to MTZ is still not completely understood. Data obtained in recent studies on strains RT027 and RT010 suggest that this resistance is a multifactorial

process involving alterations in different metabolic pathways, such as activity of nitroreductases, iron uptake and DNA repair [Chong *et al.* 2014; Moura *et al.* 2014].

Furthermore, recent evidences obtained *in vitro* seem to demonstrate that sub-inhibitory concentrations of MTZ are able to enhance the biofilm production of strains RT010, susceptible or with reduced susceptibility to this antibiotic, suggesting a possible role of biofilm in *C. difficile* resistance to MTZ [Vuotto *et al.* 2015].

Vancomycin

Vancomycin, the first-line antibiotic for moderate to severe CDI [Debast *et al.* 2014; Jarrad *et al.* 2015], consists of a glycosylated hexapeptide chain and crosslinked aromatic rings by aryl ether bonds, with a poor absorption in the gastrointestinal tract [Yu and Sun, 2013]. Its mechanism of action results in inhibition of the biosynthesis of peptidoglycan, an essential component of the bacterial cell wall envelope [Perkins and Nieto, 1974]. Resistance to VAN has frequently observed in Enterococci and Staphylococci, but it is not so largely diffused in *C. difficile* (Table 1), although a number of strains with reduced susceptibility to VAN (MICs range >2–16 mg/l) have recently been described (Table 5).

The mechanism of resistance in *C. difficile* is still unclear. Several Tn*1549*-like elements have been found in *C. difficile* [Brouwer *et al.* 2011, 2012]. Differently from the original Tn*1549* element described in *E. faecalis*, the Tn*1549*-like elements of *C. difficile* do not have a functional *van*B operon. Recently, a *van*G-like gene cluster, homologous to the cluster found *in E. faecalis*, have been described in a number of *C. difficile* isolates but, although this cluster is expressed, it is not able to promote resistance to VAN [Ammam *et al.* 2012, 2013]. A recent study has demonstrated that the amino acid change Pro108Leu in the MurG of VAN-resistant mutants is obtained *in vitro* [Leeds *et al.* 2014]. Since MurG converts lipid I to lipid II during the membrane-bound stage of peptidoglycan biosynthesis, this substitution may affect VAN activity. Biofilm formation could be also probably involved in VAN-resistance. *C. difficile* within biofilms have been found more resistant to high concentrations of VAN (20 mg/l) and biofilm formation seems to be induced in the presence of subinhibitory and inhibitory concentrations of the antibiotic [Dapa *et al.* 2013].

The clinical significance of reduced susceptibility to VAN remains to be determined, since the fecal concentration of this antibiotic is very high, ranging between 520 and 2200 mg/l [Young *et al.* 1985].

Rifamycins and fidaxomicin

An increased rate of treatment failure and recurrence of infection have been associated with MTZ and VAN treatment [Vardakas *et al.* 2012], therefore other therapy options for CDI have been proposed in the recent years.

Rifamycins, in particular rifaximin (RFX), have recently been prosed as 'chaser therapy' for the treatment of relapsing CDI [Oldfield *et al.* 2014], while fidaxomicin (FDX) is a bactericidal new narrow spectrum macrocyclic antibiotic that is used for the management of CDI with high risk for recurrences [Chaparro-Rojas and Mullane, 2013]. Both RIFs and FDX are inhibitors of bacterial transcription but they have different RNA polymerase (RNAP) target sites. FDX binds to the 'switch region' of RNAP, a target site that is adjacent to the RIF target but does not overlap [Mullane and Gorbach, 2011; Srivastava *et al.* 2011].

Susceptibility to rifampin (RIF) by either Etest or AD correlated completely with susceptibility to RFX [Miller *et al.* 2011b]. Thus, rifamycin class susceptibility in *C. difficile* can be assessed by testing susceptibility to RIF, a rifamycin that is related to RFX. Data extrapolated from recent studies (Table 1) indicate that 11% of *C. difficile* clinical isolates are resistant to RIF and the rate of overall resistance appears to be rising [Huang *et al.* 2013; Rodríguez-Pardo *et al.* 2013; Eitel *et al.* 2015; Terhes *et al.* 2014]. *C. difficile* clinical isolates resistant to RIF have been detected in 17/22 countries participating in a recent pan-European surveillance and, in particular, high percentages of resistance (between 57% and 64%) have been observed in Italy, Czech Republic, Denmark and Hungary [Freeman *et al.* 2015]. Prior exposure to RIFs has been reported to be a risk factor for RIF-resistant *C. difficile* [Curry *et al.* 2009; Miller *et al.* 2011b] and resistant *C. difficile* strains may emerge even during therapy [Johnson *et al.* 2009; Carman *et al.* 2012]. RIFs are commonly used as antituberculosis (TB) agents. Interestingly, all strains

belonging to the emergent RT046 isolated in Poland from patients affected by TB and with a prolonged RIF therapy have been found highly resistant to these antibiotics [Obuch-Woszczatynski *et al.* 2013]. Different SNPs within the encoding gene for the β-subunit of the RNA polymerase (*rpo*B) have been identified in strains resistant to RIF (Table 4). Among the amino acid substitutions identified, Arg505Lys is the most common particularly in strains RT027 [Miller *et al.* 2011; Spigaglia *et al.* 2011; Carman *et al.* 2012; Pecavar *et al.* 2012].

Fidaxomicin provides cure rates not inferior to VAN and is associated with a significantly lower rate of recurrence of CDI associated with strains non-RT027 [Louie *et al.* 2011]. Furthermore, it has a minimal impact on the composition of indigenous fecal microbiota, in particular Bacteroides species [Tannock *et al.* 2010; Louie *et al.* 2012], with a high local concentration in the gut and feces (1225.1 μg/g after 10 days of therapy) [Goldstein *et al.* 2012; Sears *et al.* 2012]. Reduced susceptibility to FDX is very rare and only one *C. difficile* clinical isolate with a MIC of 16 mg/l has been described [Goldstein *et al.* 2011]. *In vitro* analysis has demonstrated the presence of mutations in *rpo*B or CD22120, which encodes a homolog to the MDR-associated transcriptional regulator MarR, in *C. difficile* mutants resistant to FDX [Leeds *et al.* 2014]. Interestingly, FDX retains activity against RIF-resistant strains since mutations causing resistance to FDX arise in *rpo*B gene at distinct loci compared to those causing resistance to RIFs [Optimer Pharmaceuticals, Inc., 2011].

Other antibiotics

Tetracycline

Recent papers indicate that *C. difficile* resistance to tetracycline (TET) varies among countries, from 2.4% to 41.67%, and is not so widespread among *C. difficile* clinical isolates [Dong *et al.* 2013; Pirš *et al.* 2013; Lachowicz *et al.* 2015; Norman *et al.* 2014; Simango and Uladi, 2014; Zhou *et al.* 2014]. In this pathogen, resistance is commonly due to protection of the ribosomes from the action of antibiotic (Table 3). The most widespread *tet* class in *C. difficile* is *tet*M, usually found on conjugative Tn*916*-like elements [Spigaglia *et al.* 2005; Mullany *et al.* 2012; Dong *et al.* 2014]. The Tn*916*-like family is responsible for the spread of antibiotic resistance (usually referred to TET but also to MLS_B and other antibiotics) to many important pathogens [Roberts and Mullany, 2011]. In *C. difficile*, the best-known element of this family is Tn*5397*, a 21 kb element able to transfer between *C. difficile* and *B. subtilis* or *E. faecalis in vitro* [Mullany *et al.* 1990; Jasni *et al.* 2010]. Tn*5397* differs from Tn*916* for the presence of a group II intron and for a different excision/insertion module. In fact, Tn*916* contains two genes, *xis*Tn and *int*Tn, encoding an excisionase and a tyrosine integrase, whereas Tn*5397* has a *tnd*X gene that encodes a large serine recombinase [Roberts *et al.* 2001]. Furthermore, Tn*916* inserts into multiple regions of the *C. difficile* genome [Mullany *et al.* 2012], while Tn5397 inserts DNA predicted filamentation processes induced by cAMP (Fic) domain [Wang *et al.* 2006].

Tn*916*-like elements may have different genetic organizations and carry different *tet*M alleles [Spigaglia *et al.* 2005, 2006]. A peculiar Tn*916*-element, containing both *tet*M and *erm*B, has been detected in the clinical isolate cd1911 [Spigaglia *et al.* 2007]. This element is nonconjugative and probably originated from the combination of one or more plasmids and a Tn*916*-like element.

Although *tet*M is the predominant class in *C. difficile,* other *tet* genes have been identified. In particular, the copresence of both *tet*M and *tet*W have been described in *C. difficile* isolates from humans and animals [Spigaglia *et al.* 2008b; Fry *et al.* 2012].

Furthermore, other integrative mobile genetic elements probably have a role in resistance to TET. An interesting element of 106 kb, the Tn*6164*, has been identified in *C. difficile* strain M120, a RT078 isolate [Corver *et al.* 2012]. This transposon is composed by parts of other elements from different bacteria, particularly from *Thermoanaerobacter sp.* and *Streptococcus pneumoniae*. Even if M120 is susceptible to TET and streptomycin, Tn*6164* contains *tet*(44) and *ant*(6)-Ib predicted to confer resistance to these antibiotics, respectively. An analysis of data from patients indicate that mortality was more common in patients infected with strains RT078 containing Tn*6164* compared with those infected with strains without this element. Although preliminary, these data are indicative of a possible association between Tn*6164* and higher virulence of strains RT078.

Chloramphenicol

Resistance to chloramphenicol (CHL) is not so common in *C. difficile* and only 3.7% of European clinical isolates have been found resistant to this antibiotic [Freeman *et al.* 2015]. *C. difficile* resistance to CHL is usually conferred by a *cat*D gene, encoding for a chloramphenicol acetyltransferase [Wren *et al.* 1988, 1989] (Table 3). The *cat*D gene is located on the transposons Tn*4453*a and Tn*4453*b, structurally and functionally related to the *Clostridium perfringens* mobilizable element Tn*4451* [Lyras *et al.* 1998]. The conjugative transposon Tn*6104*, recently described, contains elements closely related to Tn*4453*ab and Tn*4451* but instead of a *cat*D gene it has genes predicted to encode for transcriptional regulator, a two-component regulatory system, an ABC transporter, three sigma factors and a putative toxin–antitoxin system [Brouwer *et al.* 2011]. The role of these genes is not clear and remains to be determined.

MDR in *C. difficile*

All of the most common RTs, including the hypervirulent RT027 and RT078, are associated to resistance (Table 1) and many of these strains are MDR. An analysis performed during the first European prospective survey of *C. difficile* infections, indicated that 55% of resistant clinical isolates were MDR in 2005 [Spigaglia *et al.* 2011]. Results from 13 studies published between 2012 and 2015, indicate that MDR patterns include resistance to CLI, FQs, ERY and CFs (Table 6). Interestingly, resistance to multiple antibiotics characterized recently emerged epidemic RTs. Resistance to ERY, MXF, CIP and RIF has been observed in strains RT176, a type closely related to RT027, recently circulating in Poland and the Czech Republic [Obuch-Woszczatynski *et al.* 2014; Krutova *et al.* 2015]. RT356 is a MDR type predominant in Italy, genetically related to RT018, another type common in this country [Spigaglia *et al.* 2010, 2015]. Resistant to CLI, ERY, MXF and RIF characterized almost all of the strains belonging to RT356 and RT018 in Italy [Spigaglia *et al.* 2015], while strains RT018 described in Korea and Japan show resistance only to CLI, ERY and MXF [Kim *et al.* 2012; Senoh *et al.* 2015]. The long use of RIFs in Italy, more than 20 years [Salix Pharmaceuticals, Ltd, 2003], could explain the spread of this resistance in Italian isolates RT018. Although not defined as hypervirulent, RT018 has peculiar virulent traits and strains RT018 have been demonstrated to be highly transmissible, with a transmission index tenfold higher compared with that of strains RT078 [Baldan *et al.* 2015]. Old age (≥65 years), severe pulmonary comorbidity, previous use of FQs, and infection by RT018 have been associated as significant risk factors for complicated infections [Bauer *et al.* 2011].

Conclusions

CDI is a growing concern for global public health. CDI has become the most common healthcare-associated infection in US hospitals and *C. difficile* is recognized as the most frequent cause of hospital-acquired gastrointestinal infections in Europe. Paralleling the increased CDI incidence, an increased morbidity and mortality was also observed and associated with the emergence and spread of the *C. difficile* hypervirulent type RT027. CDI due to strains RT027 is characterized by an increased incidence and severity, by being refractory to traditional therapy, and by a greater risk of relapse. Recently, additional types, highly virulent, have been reported to cause severe infections with poor outcomes. *C. difficile* adaptable capability and genome plasticity has determined an increase of isolates resistant to multiple antibiotics. The majority of epidemic clinical isolates are currently characterized as MDR. In particular, a wide range of mobile elements and alterations of antibiotic targets mediate resistance to the MLSB antibiotics and FQs, which are significantly associated with CDI. Furthermore, a decreased susceptibility to first-line antibiotics for therapy, in particular MTZ, and to those used for CDI recurrences, such as RIFs, may have a role in the low rate of response to treatment reported over recent years. Recent studies support the maintenance of antibiotic resistances in *C. difficile* population, regardless of the burden imposed by the acquisition of genetic elements/mutations conferring resistance and the decrease of antibiotics pressure. These data may partially explain the persistence of 'old' resistances (such as resistance to CLI) and the rapid diffusion of 'new' resistances, such as resistance to FQs, in *C. difficile* strains. The situation becomes more complex considering that antibiotic resistance in this pathogen can be a multifactorial phenomenon, which can involve more than one mechanism and alterations in various components. The rapid evolution of antibiotic resistance and the several mechanisms involved emphasize the need for a careful monitoring of *C. difficile* population to identify new phenotypic and genotypic characteristics. Effective antimicrobial stewardship,

Table 6. Antibiotic resistance patterns of MDR *C. difficile* clinical isolates from 13 studies published between 2012 and 2015.

Year of isolation	Country	Number of MDR clinical isolates (%)	*C. difficile* PCR-ribotypes associate to MDR	Pattern of resistance (number of clinical isolates)							References
2000–2009	Korea	94 (-)	001, 018, 017	CLI	ERY	CIP				(86)	Lee *et al.* [2014]
2008–2009	US and Canada	22 (27.5%)	027	CLI	MXF	RIF				(22)	Tenover *et al.* [2012]
2009–2010	Poland	7 (70%)	046	CLI	MXF	ERY	RIF			(7)	Obuch-Woszczatynski *et al.* [2013]
2008–2010	Poland	17 (100%)	176, 027	CLI	ERY	MXF	CIP	GAT		(17)	Obuch-Woszczatynski *et al.* [2014]
2012	Poland	71 (85.5%)	027, 176, 012, 046	ERY	MXF	CIP	RIF			(15)	Lachowicz *et al.* [2015]
				ERY	MXF	IMP				(21)	
				CLI	ERY	CIP	IMP			(2)	
2010–2011	Croatia	7 (30%)	001	CLI	ERY	CIP	LVX	GAT	MXF	(7)	Novak *et al.* [2014]
2010–2011	Iran	36(48%)		CLI	ERY	CTX	MTZ			(3)	Goudarzi *et al.* [2013]
				CLI	ERY	CTX	VAN			(2)	
				CLI	ERY	CTX				(30)	
				CLI	CTX	VAN				(1)	
2010–2012	China	44 (73.3%)	tr017, tr065, tr014,tr012, tr46, tr039, trsh2	CLI	FOX	CIP				(14)	Dong *et al.* [2013]
				CIP	FOX	TET				(1)	
				CLI	FOX	CIP	MXF			(4)	
				CLI	FOX	CIP	RIF			(1)	
				CLI	FOX	CIP	TET			(9)	
				CLI	FOX	CIP	TET	MXF		(13)	
				CLI	CIP	TET	MXF			(1)	
				CLI	CIP	TET				(1)	
2011–2013	Japan	96 (-)	018, 369	CLI	ERY	MXF	GAT			(86)	Senoh *et al.* [2015]
				CLI	ERY	GAT				(11)	
2012–2013	Japan	51 (39.2%)	ST17, ST81	CLI	CIP	CRO				(51)	Kuwata *et al.* [2015]
2012–2013	Italy	61 (71%)	356, 018, 126, 027, 046	CLI	ERY	MXF	RIF			(48)	Spigaglia *et al.* [2015]
				CLI	ERY	MXF				(11)	
				CLI	ERY	RIF				(2)	
2014–2015	Czech Republic	13 (65)	176	ERY	MXF	CIP	RIF			(13)	Krutova *et al.* [2015]
–	Zimbabwe	23 (100)		CLI	ERY	CIP	CTX			(23)	Simango and Ulandi [2014]

MDR, multidrug resistance; CLI, clindamycin; ERY, erythromycin; CIP, ciprofloxacin; MXF, moxifloxacin; RIF, rifampin; GAT, gatifloxacin; IMP, imipenem; LVX, levofloxacin; CTX, cefotaxime; MTZ, metronidazole; VAN, vancomycin; FOX, cefoxitin.

implementation of infection control programs, and the development of alternative therapies are also necessary to prevent and contain the spread of resistance and to ensure an efficacious therapy for CDI.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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