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Antibiotic susceptibility and resistance profiles of Romanian *Clostridioides difficile* isolates

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Abstract

This study investigated the antibiotic susceptibility patterns and genetic resistance markers of 35 C. difficile strains isolated from patients with C. difficile infection. Vancomycin, metronidazole, tigecycline, teicoplanin, rifampicin, moxifloxacin, cefotaxime, tetracycline, erythromycin, clindamycin, chloramphenicol, linezolid and imipenem MICs were determined for toxigenic strains belonging to PCR ribotypes (PR) 012 (2), 014 (4), 017 (3), 018 (2), 027 (17), 046 (2), 087 (3) and 115 (2). Results showed vancomycin, metronidazole, tigecycline and teicoplanin to be active against all isolates. High resistance rates were noticed against cefotaxime (n = 35), clindamycin (n = 33), imipenem (n = 31), moxifloxacin (n = 25), erythromycin (n = 25) and rifampicin (n = 22). Linezolid-resistance was found in three isolates (PR 017/2, PR 012/1), showing complex resistance (7-9 antibiotics). PR 012, 017, 018, 027 and 046 isolates (n = 26) were resistant to 5-9 antibiotics. Twelve resistance profiles (2-9 antibiotics) were detected. Rifampicin-moxifloxacin-cefotaxime-erythromycin-clindamycin-imipenem-resistance was predominant, being expressed by 18 strains (PR 027/17, PR 018/1). PCR results suggested tetracycline-resistance to be induced by the gene tetM. Three tetM-positive isolates (PRs 012, 046), were also tndX-positive, suggesting the presence of a Tn5397-like element. Only two MLSB-resistant strains (PR 012) had the ermB gene and chloramphenicol-resistance determinant catD was not detected, leaving room for further investigating resistance mechanisms. Multidrug resistance could be attributed to most analysed strains, underlining, once more, the impact of wide-spectrum antimicrobial over prescription, still a tendency in our country, on transmission of antimicrobial resistance and emergence of epidemic C. difficile strains generating outbreaks.

***Keywords**: C. difficile, antibiotic susceptibility, resistance profile PCR ribotype*

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Introduction

Clostridioides difficile is an anaerobic, Gram positive spore-forming bacillus, considered the etiologic agent of hospital - or community-acquired post-antibiotic diarrhoea and pseudomembranous colitis (PMC) [1]. The bacterium is ubiquitous in the environment: spores can be found in soil, lake waters or sediments, but may also colonize the large intestine of pets (dogs, cats) or farm animals [2, 3]. It was first discovered in the gut microbiota of healthy newborns in 1935 [4]. Most *C. difficile* strains are toxin-producing, but there are also non-toxigenic, clinically irrelevant strains. Toxin A (TcdA – enterotoxin) and toxin B (TcdB – cytotoxin) are the main virulence factors, although important epidemic strains also produce a third type – the binary toxin (*C. difficile* toxin – CDT) [5]. The main risk factors for colonization with toxigenic *C. difficile* and associated disease are considered to be hospitalisation in acute or long-term care units, advanced age (≥ 65 years) [1, 6] and prolonged treatment with wide-spectrum antibiotics like clindamycin, cephalosporins or fluoroquinolones, to which many *C. difficile* strains are non-susceptible [2, 6-10]. *C. difficile* infection (CDI) may also be community-associated, targeting both paediatric (> 2 years) and adult patients [2, 11], through contact with spore-contaminated surfaces, infected persons, or as zoonotic infections [3]. Many clinical isolates in Europe, including those belonging to PCR ribotypes (PR) 001, 012, 017, 018, 027 or 078, are considered multi-drug resistant (MDR) [12, 13]. Resistance patterns often target antibiotics from the Macrolide-Lincosamide-Streptogramin B (MLS_B) family, tetracycline, chloramphenicol, rifaximin and fluoroquinolones, especially in hospital settings with high rates of clindamycin, rifampin or fluoroquinolone administration. Clindamycin administration was historically defined as a major risk factor for CDI [7]. Presently, it is suggested to represent a lower risk of infection with the major epidemic type PR 027 emerged in the last decades, compared to fluoroquinolones [10, 14]. However, high levels of MLS_B-resistance are still being reported for other epidemic PRs, such as 001, 017 or 078 [12]. Although MICs may vary, *C. difficile* strains are considered constitutively resistant to cephalosporins, through mechanisms that are insufficiently known at this moment [10].

Antibiotic resistance is frequently transmitted between bacterial strains through mobile genetic elements. The best described genetic determinant was considered class B erythromycin ribosomal methylase gene (*erm*B), harboured by Tn*5398*, Tn*5398*-like or other transposons [10, 16, 17]. Resistance to tetracycline is induced, in most cases, by a ribosome protection protein (RPP) – TetM [18] – encoded by the *tet*M gene, carried by Tn*5397* or other Tn*916*-like transposons [10, 19]. Tn*5397*, first identified in *C. difficile* strains [19], also carries the gene *tnd*X, encoding a protein responsible for the insertion and excision of the element [20]. *tnd*X is used as a marker for the detection of Tn*5397* in *C. difficile* strains [21]. In *C. difficile*, tetracycline-resistance may also be mediated by gene *tet*W, sometimes co-present with *tet*M [22, 23]. Chloramphencol-resistance may be induced by chloramphenicol acetyltransferase, encoded by the *cat*D gene, specific to Tn*4453a* or Tn*4453b* transposons [10, 17]. CDI has represented a considerable burden for public health in North America, Asia and Europe in the last decades [6, 24].

In Romania, high rates persisted since first outbreaks occurred in 2011, with PR 027 as prevalent among the strain types causing outbreaks [25-27]. One of the main causes for the persistence of high CDI rates may be the over prescription of wide spectrum antibiotics, still an issue in our country. However, antimicrobial susceptibility or resistance patterns of *C. diffi-*

cile strains involved in Romanian infections are insufficiently described at this moment. For this purpose, the antibiotic susceptibility of *C. difficile* strains, isolated from Romanian patients with antibiotic-associated diarrhoea, was analysed in the present study. Molecular mechanisms of resistance were also investigated.

Materials and Methods

In vitro antibiotic susceptibility testing

The study was based on the analysis of 35 non-duplicate toxigenic *C. difficile* strains isolated between 2013 and 2017 from patients with antibiotic-associated diarrhoea confirmed as *C. difficile* infection by rapid immunochromatographic toxin A/B screening tests and toxigenic culture. PRs of the isolates were identified in previous studies [25, 28]. Seventeen strains belonging to PR 027 were included. The rest of the analysed isolates belonged to 7 other PRs, as follows: 017 (n = 3), 087 (n = 3), 014 (n = 4), 046 (n = 2), 018 (n = 2), 012 (n = 2) and 115 (n = 2). Antibiotic susceptibility profiles and MICs were determined using Etest (bioMérieux, France), according to manufacturer's instructions. Susceptibility to the following antibiotics was tested: vancomycin (VAN), metronidazole (MTR), tigecycline (TGC), teicoplanin (TEC), rifampicin (RIF), moxifloxacin (MFX), cefotaxime (CTX), tetracycline (TCY), erythromycin (ERY), clindamycin (CLI), chloramphenicol (CHL), linezolid (LNZ) and imipenem (IPM). Bacterial suspensions of 1.0 McFarland density, prepared from 24-48h cultures, were inoculated on Brucella blood agar plates (BBA Agar, bioMérieux, France). After 48h plate incubation in anaerobiosis, MIC values were recorded and interpreted using EUCAST breakpoints or epidemiological cut-off (ECOFF) MIC values for *C. difficile*, CLSI breakpoints for Gram positive anaerobes and literature references as guidance [9, 29-31].

PCR detection of genetic determinants of antibiotic resistance

The presence of genes known to mediate resistance to TCY, MLS_B family and CHL was also analysed [10]. DNA samples were extracted from *C. difficile* colonies grown on Columbia blood agar (COS Agar plates, bioMérieux, France), using a resin extraction kit (InstaGene Matrix, Bio-Rad, USA), according to the manufacturer's instructions. The presence of antibiotic resistance genes *tet*M, *erm*B and *cat*D was simultaneously tested, using a multiplex PCR protocol, as previously described [17]. The reaction mix contained specific pairs of primers (5'→3'): TETMd (TGGAATTGATTTATCAACGG) and TETMr (TTCCAACCATACAATCCTTG) for *tet*M, [31], E5 (CTCAAAACTTTTTAACGAGTG) and E6 (CCTCCCGTTAAATAATAGATA) for *erm*B and CL1 (ATACAGCATGACCGTTAAAG) / CL2 (ATGTGAAATCCGTCACATAC) for *cat*D [17]. The PCR reaction was performed with 0,3-0,6 pmol/µl of primers, a ready-to-use mix containing Taq polymerase (Qiagen Multiplex PCR, USA) and 5 µl of DNA sample, in a final volume of 50µl. The amplification parameters were set according to the above mentioned protocol [17]. Simplex PCR was performed for the detection of the *tet*W gene, using the WRC1/WRC2 pair of primers previously published [23]. Another PCR protocol was used to determine the presence of gene *tnd*X, a Tn*5367*-like marker, with the specific set of primers TNDX1 and TNDX2, described by Spigaglia *et al.* [21]. The PCR products were verified with conventional gel electrophoresis, using 1.5% agarose gels stained with ethidium bromide. The expected amplification products were fragments of: 1080 bp (*tet*M), 711 bp (*erm*B) and 500 bp (*cat*D) in the multiplex PCR, 457 bp (*tet*W) and 1600 bp (*tnd*X) for the other PCR protocols.

Results

In vitro antibiotic susceptibility

Results showed VAN, MTR and TGC to be highly active against the analysed group of isolates: geometric mean (GM) MIC was 0.415 µg/ml for VAN, 0.11 µg/ml for MTR and 0.021 µg/ml for TCG, as presented in Table 1. The highest MTR MIC values – 0.75 µg/ml and 1 µg/ml – were found in strains belonging to PR 027, among the analysed PRs [data not shown]. TEC susceptibility breakpoints or ECOFF values were not available for *C. difficile* or Gram positive anaerobes [29]. However, the low MICs resulted (GM MIC = 0.082 µg/ml) suggest the glycopeptide to be active against the analysed *C. difficile* strains (Table 1, Figure 1)

Antibiotic resistance was noticed especially in isolates belonging to PRs 012, 017, 018, 027 and 046 (n = 26), which were non-susceptible to 5-9 antibiotics, while PRs 014, 087 and 115 (n = 9) showed less complex resistance profiles (2-3 antibiotics). None of the isolates was susceptible to CTX (Figure 1). MICs ranged from 64 to 256 µg/ml, most strains being highly resistant (Table 2). The analysed group of isolates consisted of both susceptible and non-susceptible strains to the remaining antibiotics (RIF, MFX, TCY, ERY, CLI, CHL, LNZ and IPM). Susceptibility and MIC values are summarised in Table 1.

A high resistance rate, represented by strains from all the ribotypes included in the study, resulted against CLI (33/35), followed by resist-

Table 1. Susceptibility (MIC values) of the analysed group of *C. difficile* isolates (n = 35) to 13 antibiotics.

Antibiotic	Breakpoint (S) [a,b] (µg/ml)	Susceptible / Resistant isolates (n)	Geometric mean MICs (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC range
VAN	≤ 2	35 / 0	0.415	0.38	0.75	0.125 – 1
MTR	≤ 2	35 / 0	0.11	0.094	0.75	0.016 – 1
TGC	≤ 0.25	35 / 0	0.021	0.023	0.032	< 0.016 – 0.047
TEC [c]	-	-	0.082	0.064	0.25	0.016 – 0.75
RIF	≤ 0.004	13 / 22	1.358	> 32	> 32	< 0.002 – > 32
MFX	≤ 4	10 / 25	17.139	> 32	> 32	0.38 – > 32
CTX	≤ 16	0 / 35	> 256	> 256	> 256	64 – > 256
TCY	≤ 0.25	27 / 8	0.21	0.064	12	0.016 – 32
ERY	≤ 2	10 / 25	85.721	> 256	> 256	0.25 – > 256
CLI	≤ 2	2 / 33 [d]	11.835	6	> 256	1 – > 256
CHL	≤ 8	31 / 4 [e]	5.858	4	16	1 – > 256
LNZ	≤ 4	32 / 3	2.162	2	4	0.5 – 32
IPM	≤ 4	4 / 31 [f]	25.072	> 32	> 32	1.5 – > 32

Abbreviations: VAN – vancomycin, MTR – metronidazole, TGC – tigecycline TEC – teicoplanin, RIF – rifampicin, MFX – moxifloxacin, CTX – cefotaxime, TCY – tetracycline, ERY – erythromycin, CLI – clindamicin, CHL – chloramphenicol, LNZ – linezolid, IPM – imipenem; S – susceptible, R – resistant, I – intermediary.

a) Breakpoints define susceptible isolates.

b) VAN, MTR – EUCAST clinical breakpoints for *C. difficile*; TGC, RIF, MFX, ERY – EUCAST epidemiological cut-off values (ECOFF) for *C. difficile* [42]; CTX, CLI, CHL, IPM – CLSI breakpoints for susceptible Gram positive anaerobes [41]; LNZ – published sources [2, 21].

c) Breakpoints or ECOFF MICs are not given for TEC. However, the antibiotic could be considered active against the strains, as the low MICs obtained might suggest.

d) $R = \sum_{R\ (n = 26)\ ,\ I\ (n = 7)}$;

e) $R = \sum_{R\ (n = 3),\ I\ (n = 1)}$.

f) $R = \sum_{R\ (n = 26),\ I\ (n = 5)}$;

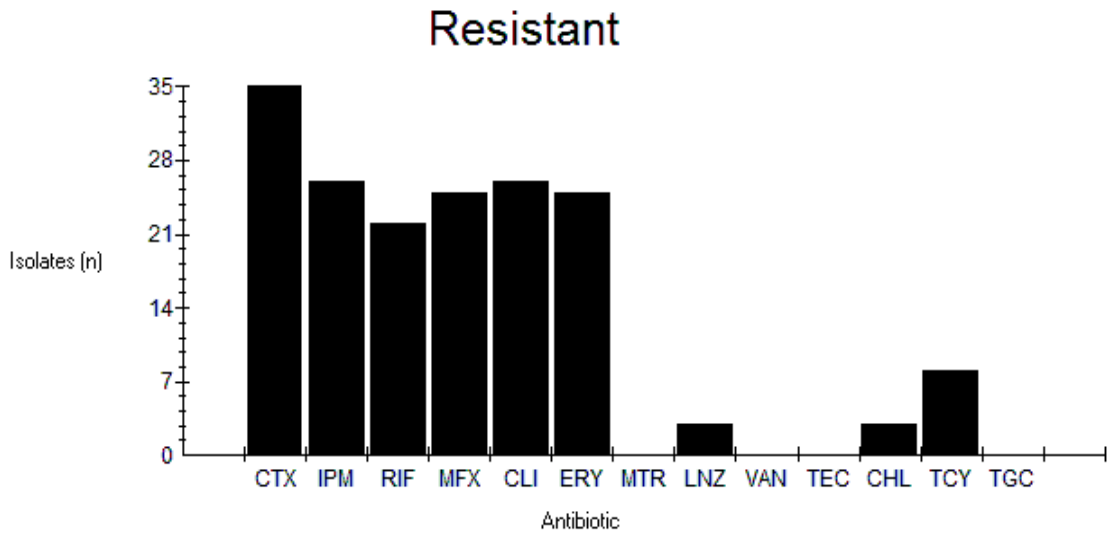

Figure 1. Antibiotic resistance rates of the analysed *C. difficile* isolates to the antibiotics tested: CTX – cefotaxime, IPM – imipenem, RIF – rifampicin, MFX – moxifloxacin, CLI – clindamycin, ERY – erythromycin, MTR – metronidazole, LNZ – linezolid, VAN – vancomycin, TEC – teicoplanin, CHL – chloramphenicol, TCY – tetracycline, TGC – tigecycline.

Table 2. Geometric mean MIC values resulted for PCR ribotypes

PCR Ribotype (n)	Geometric mean MICs (µg/ml)												
	VAN	MTR	TGC	TEC	RIF	MFX	CTX	TCY	ERY	CLI	CHL	LNZ	IPM
012 (2)	0.38	0.089	0.033	0.089	0.002	1.732	> 256	11.314	> 256	> 256	8	8	> 32
014 (4)	0.555	0.078	0.019	0.121	< 0.002	0.892	>256	0.05	1.456	6.447	2.913	1.316	19.596
017 (3)	0.416	0.08	0.029	0.198	> 32	> 32	>256	15.119	58.148	92.304	80.635	8.32	> 32
018 (2)	0.612	0.078	0.027	0.75	> 32	> 32	> 256	0.375	> 256	6.928	3.464	2.449	11.314
027 (17)	0.361	0.137	0.018	0.047	> 32	> 32	> 256	0.072	> 256	6.14	4.476	1.747	30.935
046 (2)	0.534	0.063	0.027	0.108	0.002	0.38	> 256	13.856	> 256	> 256	11.314	1.732	> 32
087 (3)	0.33	0.115	0.02	0.047	0.002	20.966	184.608	0.059	0.63	2.08	4.579	1.5	6.604
115 (2)	0.612	0.154	0.023	0.19	< 0.002	1.061	181.019	0.055	1.732	6	4.243	2.449	6.928

Abbreviations: VAN – vancomycin, MTR – metronidazole, TGC – tigecycline, TEC – teicoplanin, RIF – rifampicin, MFX – moxifloxacin, CTX – cefotaxime, TCY – tetracycline, ERY – erythromycin, CLI – clindamicin, CHL – chloramphenicol, LNZ – linezolid, IPM – imipenem.

ance to IPM (31/35), MFX (25/35), ERY (25/35) and RIF (22/35) (Table 3). CLI and ERY were simultaneously inactive against 25 isolates: PRs 012, 017, 018, 027, 046 (Table 3). In 6 of these isolates, representing PRs 012 (2/2), 017 (2/3) and 046 (2/2), both MLS_B antibiotics showed MICs above 256 µg/ml [data not shown]. High resistance to MFX and RIF was noticed in PRs 027, 018 and 017, while PRs 012, 014, 046 and 115 were susceptible to both antibiotics (Table 3). LNZ-resistance was found in PR 017 (2/3) and PR 012 (1/2). The respective strains were also resistant to CHL (16 - > 256 µg/ml) (Table 3). IPM-resistance was noted in all PRs. Antibiotic GM MIC values resulted for the PRs are presented in Table 2.

In total, 12 resistance profiles, including 2-9 classes of antibiotics, were generated, as shown

Table 3. Phenotypic and genotypic antibiotic resistance profiles of the analysed *C. difficile* isolates

PCR Ribotype	Resistance profiles	No. of antibiotic classes	No. of isolates	*tet*M/*erm*B/*cat*D genotype	*tet*W	*Tn5397* marker (*tnd*X)
012	CTX-TCY-ERY-CLI-IPM	5	1	+/+/–	–	–
	CTX-TCY-ERY-CLI-CHL-LNZ-IPM	7	1	+/+/–	–	+
014	CTX-CLI	2	1	–/–/–	–	–
	CTX-CLI-IPM	3	3	–/–/–	–	–
017	RIF-MFX-CTX-TCY-CLI-IPM	6	1	+/–/–	–	–
	RIF-MFX-CTX-TCY-ERY-CLI-CHL-LNZ-IPM	9	2	+/–/–	–	–
018	RIF-MFX-CTX-ERY-CLI-IPM	6	1	–/–/–	–	–
	RIF-MFX-CTX-TCY-ERY-CLI-IPM	7	1	+/–/–	–	–
027	RIF-MFX-CTX-ERY-CLI-IPM	6	17	–/–/–	–	–
046	CTX-TCY-ERY-CLI-IPM	5	1	+/–/–	–	+
	CTX-TCY-ERY-CLI-CHL-IPM	6	1	+/–/–	–	+
087	MFX-CTX	2	1	–/–/–	–	–
	MFX-CTX-CLI	3	1	–/–/–	–	–
	MFX-CTX-IPM	3	1	–/–/–	–	–
115	CTX-CLI	2	1	–/–/–	–	–
	CTX-CLI-IPM	3	1	–/–/–	–	–
Total	12		35			

Abbreviations: RIF – rifampicin, MFX – moxifloxacin, CTX – cefotaxime, TCY – tetracycline, ERY – erythromycin, CLI – clindamicin, CHL – chloramphenicol, LNZ – linezolid, IPM – imipenem; + = gene detected; – = gene not detected.

in Table 3. PR 027 isolates (17/17) showed a prevalent profile – RIF-MFX-CTX-ERY-CLI-IPM (Figure 2). The LNZ-resistant isolates belonging to PR 017 expressed identical 9-antibiotic-resistance profiles (RIF-MFX-CTX-TCY-ERY-CLI-CHL-LNZ-IPM), while the third isolate showed a 6-antibiotic-profile (RIF-MFX-CTX-TCY-CLI-IPM). Resistance to CTX, TCY, ERY, CLI and IPM coincided in PR 012 and PR 046 strains. Both PR 018 strains were non-susceptible to RIF, MFX, CTX, ERY, CLI and IPM. One also showed TCY-resistance. PR 087 isolates (3/3) were resistant to MFX and CTX, but were RIF-susceptible, although in other PRs tested, MFX- and RIF-resistance were associated. Two of them also expressed resistance to a third antibiotic – CLI or IPM (Table 3). PRs 014 (4/4) and 115 (2/2) were resistant to CTX and CLI. Three representatives of PR 014 and one PR 115 strain were also resistant to IPM (Table 3).

Molecular test results

PCR results showed presence of TCY-resistance gene *tet*M in all the 8 phenotipically resistant isolates. Three of these strains, belonging to PR 046 (2/2) and PR 012 (1/2), were also positive for gene *tnd*X, specific toTn*5397*-like transposons [20]. None of the analysed isolates showed presence of the *tet*W gene. Both *tet*M and *erm*B were detected in two isolates belonging to PR 012, phenotypically resistant to TCY and MLS_B (Table 3). The rest of the MLS_B-resistant isolates were *erm*B-negative. None of the CHL-resistant strains, belonging to PRs 012 (1/2), 017 (2/3) and 046 (1/2), were positive for *cat*D, suggesting that other molecular mechanisms are generating resistance (Table 3). Phenotypic TCY-, MLS_B-, or CHL-susceptible isolates did not show presence of genetic resistance determinants investigated in the study. Only three isolates showed phenotypic and genotypic concordance regarding susceptibility to TCY, MLS_B and CHL (Table 3).

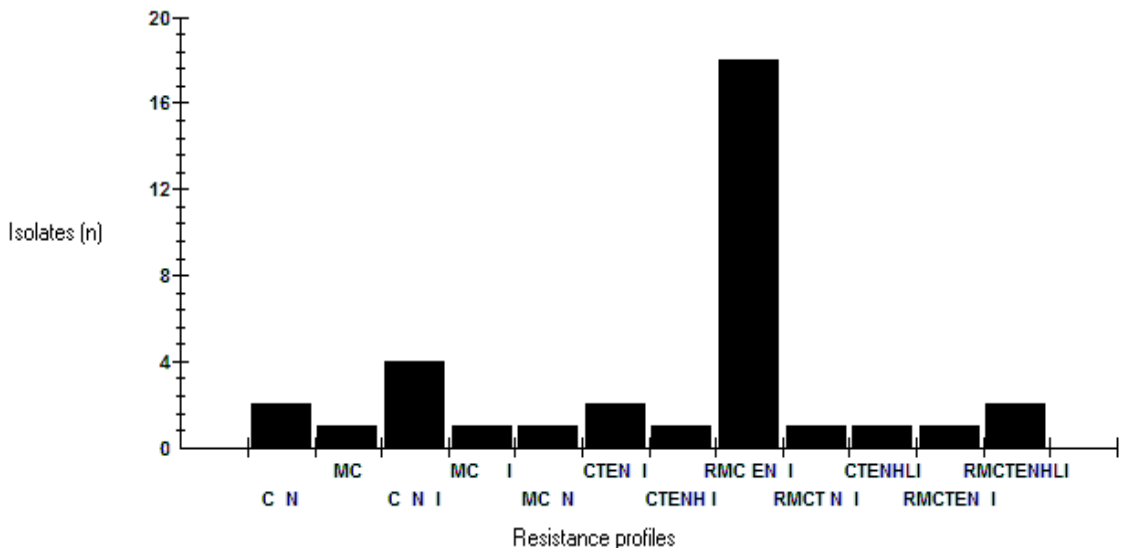

Figure 2. Distribution of antibiotic resistance profiles resulted for the studied group of isolates: CN – CTX-CLI; MC – MFX-CTX; CNI – CTX-CLI-IPM; MCI – MFX-CTX-IPM; MCN – MFX-CTX-CLI; CTENI – CTX-TCY-ERY-CLI-IPM; CTENHI – CTX-TCY-ERY-CLI-CHL-IPM; RMCENI – RIF-MFX-CTX-ERY-CLI-IPM; RMCTNI – RIF-MFX-CTX-TCY-CLI-IPM; CTENHLI – CTX-TCY-ERY-CLI-CHL-LNZ-IPM; RMCTENI – RIF-MFX-CTX-TCY-ERY-CLI-IPM; RMCTENHLI – RIF-MFX-CTX-TCY-ERY-CLI-CHL-LNZ-IPM.

Discussion

In the context of insufficient antibiotic susceptibility data available at this moment to describe Romanian *C. difficile* strains causing CDI, the present study was focused on characterizing the susceptibility patterns of toxigenic isolates belonging to some of the PRs identified in Romanian CDI outbreaks or in sporadical cases: 012, 014, 017, 018, 027, 046, 087 and 115 [25, 28].

In vitro antibiotic susceptibility test results showed the studied group to be highly susceptible to VAN, MTR, TGC and TEC. Some of the PR 027 strains showed higher MIC values of MTR compared to other PRs, although the respective strains were classified as susceptible [29, 30] and the GM MIC (0.137 μg/ml) suggests high overall susceptibility levels. However, the method used in the study to determine antibiotic susceptibility of *C. difficile* is considered to have lower accuracy in detecting reduced susceptibility to MTR, compared to agar dilution based methods [33]. Recent reports show European PR 027 strains to have elevated MTR MICs [12, 15]. Therefore reduced MTR-susceptibility could be present among Romanian strains as well. The analysed PR 027 strains were highly susceptible to VAN (GM MIC = 0.361 μg/ml). High levels of *in vitro* activity of MTR and VAN against the PR 027 isolates do not guarantee the successful outcome of the antibiotic treatment of infections generated by these strains. PR 027 is known to have high recurrence potential [34]. Previous reports [33] suggested that the lack of correspondence between *in vitro* and *in vivo* antibiotic activity may be explained by pharmacokinetic properties for MTR – high upper gastrointestinal absorption leading to low concentration in the intestine [35] – or physiological characteristics ensuring survival of the strains. Recent *in vivo* studies demonstrated the competitive advantage of PR 027 over other ribotypes, the authors suggesting an increased potential of PR 027 strains in using limited environmental resources [36]. Re-colonization of the patient through contact with spore-contaminated surfaces could be another cause of relapse.

A high level of antimicrobial resistance, with heterogeneous resistance profiles, was characteristic for the studied group. A predominant profile – RIF-MFX-CTX-ERY-CLI-IPM – was, however, noticed. It was characteristic to PR 027 (17/17), but one PR 018 strain also expressed this pattern. Strains belonging to PRs 012, 017, 018, 027 and 046, previously described as MDR [10, 13], showed wide antibiotic resistance, especially PR 017, resistant to antibiotics belonging to 9 classes.

MIC results of CTX, a third-generation cephalosporin (64 – > 256 μg/ml), are in accordance with other reports in suggesting that the activity level of different cephalosporins may vary between individual strains, although this class of antibiotics is considered constitutively inactive against *C. difficile* [10]. The activity of other antibiotics – RIF, MFX, TCY, ERY, CLI, CHL, LNZ and IPM – was heterogeneous within the group, with both susceptible and non-susceptible strains. In most isolates, a previously reported phenotypic association between the activities of RIF and fourth-generation fluoroquinolone MFX [13] was noticed: PRs 017, 018 and 027 were highly resistant, while PRs 012, 046 and 115 were susceptible to both antibiotics. Exceptionally, isolates belonging to PR 087 were susceptible to RIF and resistant to MFX. Overall, high RIF- and MFX-resistance rates were characteristic for isolates belonging to different PRs. This finding represents a starting point for further analysis, in order to determine molecular patterns of rifampin- and fluoroquinolone-resistance in Romanian *C. difficile* isolates.

Moderate resistance rates were observed for TCY and CHL. MLS_B antibiotics were predominantly inactive. All ribotypes were resistant to CLI. Among the CLI-resistant strains, 25 were

also highly resistant to ERY (PRs 012, 017, 018, 027 and 046), coinciding with previous European study results [12, 15]. Particularly, PR 027 was mostly associated with high ERY-resistance (GM MIC > 256 µg/ml) and moderate CLI-resistance (GM MIC = 6.14 µg/ml), while many European reports described PR 027 strains as highly resistant to CLI [12, 15]. LNZ-resistance, previously reported in studies performed in Europe [12, 31], was detected here in strains belonging to PR 017 and PR 012, in association with high TCY, MLS_B- and CHL-resistance. The resistance noticed against IPM (GM MIC = 25.072 µg/ml) could be reflecting the tendency to frequently prescribe carbapenem antibiotics in the country. Similar results, showing high levels of resistance in different PRs, were previously reported in Europe [15, 37].

Molecular test results suggest TCY-resistance to be mediated by *tet*M in the analysed *C. difficile* strains. The gene *tet*W, reported in other studies as a co-determinant [22, 23], was not detected. Nevertheless, the possibility of other genetic mechanisms contributing to tetracycline-resistance in the analysed group is not to be ruled out. The presence of Tn*5397*-like specific gene *tnd*X in three of the *tet*M-positive strains belonging to PRs 012 and 046, suggests that TCY-resistance was delivered through such an element, commonly transmitted between species of bacterial pathogens including *C. difficile* [21]. Only two strains (PR 012) showed presence of both *tet*M and *erm*B genes, although all TCY-resistant strains were also phenotypically resistant to MLS_B. The co-presence of *tet*M and *erm*B and the absence of *tnd*X in one of the two strains suggest the possibility of a Tn*916*-like element carrying both resistance genes to be integrated [38]. For the rest of the isolates, MLS_B-resistance could not be explained by *erm*B-encoded ribosomal metilation. In the last years, *erm*B-negative *C. difficile* strains phenotypically resistant to MLS_B, including PR 027 strains, have been described [10, 13]. None of the analysed CHL-resistant isolates showed evidence of *cat*D-mediated resistance, requiring further investigation of the molecular mechanisms of resistance. Heterogeneity of phenotypic and genotypic susceptibility to TCY, MLS_B and CHL was noticed in different strains belonging to the same ribotype (e.g. PRs 017, 046, 087), suggesting that antibiotic resistance is acquired through horizontal transmission of genetic determinants between individual strains and is not necessarily ribotype-dependent. LNZ-resistance, previously reported in studies performed in Europe [12, 31], was detected in strains belonging to PR 017, in association with high MLS_B- and CHL-resistance. However, the absence of genes *erm*B and *cat*D in these strains allows speculating that other molecular determinants, such as MDR genes, could be inducing resistance [31, 39].

In conclusion, the clinical isolates analysed in this study have high levels of antimicrobial resistance, especially to wide-spectrum antibiotics representing cephalosporins, MLS_B, rifampins, fluoroquinolones and carbapenems, considered risk factors for development of *C. difficile* infection, along with advanced age and hospitalization [1, 8, 10, 40]. Multi-drug resistance may be attributed to the majority of isolates, resistant to at least five classes of antibiotics [13]. Tetracycline-resistance was attributed to the *tet*M gene. As most MLS_B-resistant isolates were *erm*B-negative and no chloramphenicol-resistant isolates showed presence of *cat*D, molecular resistance mechanisms against these antibiotics remain unclear and are to be analysed more thoroughly in future studies. The findings in the present study support the assertion that extensive administration of rifampin, carbapenems such as imipenem, fluoroquinolones, cephalosporins or MLS_B [6, 15, 21, 40, 41], could involve high risk of CDI development and emergence of outbreaks, especially with epidemic PRs 027 or 017. In contrast, taking into consideration antibiotics

demonstrated to be highly active against *C. difficile*, when selecting treatment for various types of infections, could contribute to minimising the risk of CDI spread.

This study underlines, once again, the impact of over prescription of wide-spectrum antibiotics on transmission of antibiotic resistance and emergence of highly epidemic *C. difficile* strains generating outbreaks. In our country, reckless administration of antibiotics has been an issue for many years, contributing to high incidence of infections caused by *C. difficile* in hospital settings or in the community and also making CDI prevention difficult.

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List of abbreviations

PMC – pseudomembranous colitis;
CDI – *C. difficile* infection;
TcdA – toxin *C. difficile* A (enterotoxin);
TcdB – toxin *C. difficile* B (cytotoxin);
CDT – *C. difficile* toxin (binary toxin);
PR – PCR ribotype;
MDR – multi-drug resistant;
MLS_B – Macrolide-Lincosamide-Streptogramin B;
*erm*B – class B erythromycin ribosomal methylase gene;
RPP – ribosome protection protein;
*tet*M – tetracycline resistance protein class M (TetM) gene;
*tet*W - tetracycline resistance protein class W (TetW) gene;
*tnd*X – conjugative transposon site-specific recombinase (TndX) gene;
*cat*D – chloramphenicol acetyltransferase (CatD) gene;
VAN – vancomycin;
MTR – metronidazole;
TGC – tigecycline;
TEC – teicoplanin;
RIF – rifampicin;
MFX – moxifloxacin;
CTX – cefotaxime;
TCY – tetracycline;
ERY – erythromycin;
CLI – clindamycin;
CHL – chloramphenicol;
LNZ - linezolid;
IPM – imipenem;
ECOFF - epidemiological cut-off;
S – susceptible;
I – intermediary;
R – resistant;
CN – CTX-CLI;
MC – MFX-CTX;
CNI – CTX-CLI-IPM;
MCI – MFX-CTX-IPM;
MCN – MFX-CTX-CLI;
CTENI – CTX-TCY-ERY-CLI-IPM;
CTENHI – CTX-TCY-ERY-CLI-CHL-IPM;
RMCENI – RIF-MFX-CTX-ERY-CLI-IPM;
CTENHLI – CTX-TCY-ERY-CLI-CHL-LNZ-IPM;
RMCTNI – RIF-MFX-CTX-TCY-CLI-IPM;
RMCTENI – RIF-MFX-CTX-TCY-ERY-CLI-IPM;
RMCTENHLI – RIF-MFX-CTX-TCY-ERY-CLI-CHL-LNZ-IPM.

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