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Antimicrobial susceptibility and mechanisms of resistance of Greek *Clostridium difficile* clinical isolates

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ABSTRACT

Objectives: This study examined the antimicrobial susceptibility and resistance mechanisms of *Clostridium difficile* recovered in Greek hospitals during 2012–2015.
Methods: *C. difficile* isolates ($n = 88$) were collected from clinically-confirmed *C. difficile* infection from symptomatic patients in 10 Greek hospitals. Minimum inhibitory concentrations (MICs) of various antimicrobial agents were determined by Etest. Isolates were typed by multilocus sequence typing (MLST). Toxin and resistance genes were detected by PCR. Chromosomal mutations in *gyrA*, *gyrB* and *rpoB* were identified by PCR and sequencing. The genetic environment of resistance genes was characterised by Illumina sequencing.
Results: The 88 *C. difficile* isolates comprised 27 sequence types (STs), with ST37 ($n = 26$) and ST11 ($n = 21$) being the most prevalent. All isolates were susceptible to vancomycin and metronidazole, with variable resistance rates to other antimicrobials. Of the 88 isolates, 45.5% were multidrug-resistant and the majority belonged to ST11 and ST37. The presence of chromosomal mutations in *gyrA*, *gyrB* and *rpoB* was mainly observed in high-risk clones such as ST11 and ST37. The antimicrobial resistance genes *ermB*, *mefA*, *msrA* and *tetM* were identified at different prevalences and combinations. Additionally, *cfrB* and *cfrC* were identified for the first time in Greece and were carried by a Tn*6218* transposon and a novel plasmid, respectively.
Conclusions: To our knowledge, this is the first study examining the resistance profiles and respective mechanisms of *C. difficile* recovered in Greek hospitals. Gut commensals such as *C. difficile* may serve as hubs for further transfer of antimicrobial resistance genes.

1. Introduction

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus carried asymptomatically in the gut of ca. 7% of healthy human adults [1]. In addition, the micro-organism is a major cause of pseudomembranous colitis as well as antibiotic-associated diarrhoea and colitis [2]. The pathogenicity of *C. difficile* is mainly mediated by two toxins, namely toxin A (potent enterotoxin) and toxin B (potent cytotoxin), encoded by the *tcdA* and *tcdB* genes, respectively [3]. The TcdA and TcdB toxins play a crucial role in the pathogenesis of *C. difficile* infection (CDI). In addition, some *C. difficile* isolates produce a multidomain, actin ADP-ribosylating binary toxin (CDT) encoded by the *cdtA* and *cdtB* genes [4]. CDT is thought to modify actin in a manner that facilitates bacterial adhesion [5].

According to the guidelines of the Infectious Diseases Society of America (IDSA) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), fidaxomicin and vancomycin are antimicrobial agents recommended for the treatment of severe CDI, whilst metronidazole is usually recommended as oral antibiotic treatment of initial CDI in mild/moderate disease [6,7]. However, the emergence of antimicrobial resistance in *C. difficile* is associated with the acquisition of genes encoding resistance to different antibiotics or with accumulation of genomic mutations altering antibiotic target sites [8,9]. Resistance genes usually are located on mobile genetic elements (MGEs) such as transposons or plasmids. MGEs constitute a large proportion of the *C. difficile* genome (ca. 11%), indicating that conjugation,

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transduction and transformation are important mechanisms for *C. difficile* to acquire antimicrobial resistance genes [10]. In *C. difficile*, the *ermB* gene, which encodes a 23S rRNA methyltransferase and confers resistance to macrolides, lincosamides and streptogramin B (MLS_B) antibiotics, is transferred by transposons Tn*5398* and Tn*6215* [10]. Furthermore, *tetM*, which is the predominant gene in *C. difficile* encoding resistance to tetracyclines, is usually carried on Tn*5397* and Tn*916* transposons [10]. Thus, emergence of antimicrobial resistance in association with antibiotic usage can lead to the selection and further dissemination of high-risk clones such as PCR ribotypes 017 and 078. In addition, Clostridia may act as a pool for the further spread of resistance determinants in opportunistic Gram-positive or Gram-negative bacteria [11]. Therefore, surveillance of the antimicrobial susceptibility and resistance mechanisms of *C. difficile* isolates is of significant importance.

Previous studies have shown that the rates of antimicrobial resistance vary in different geographic regions and are associated with local antibiotic policies [10]. However, data on antimicrobial resistance of *C. difficile* of Greek origin are extremely limited. Therefore, in the present study the antimicrobial susceptibility, resistance mechanisms and multilocus sequence typing (MLST) of *C. difficile* clinical isolates recovered in Greek hospitals during 2012–2015 were investigated.

2. Materials and methods

2.1. Collection and confirmation of C. difficile isolates

C. difficile clinical isolates collected from laboratory-confirmed CDI cases from symptomatic patients treated in 10 Greek hospitals during 2012–2015 were stored at −80 °C in nutrient broth containing 10% glycerol and were sent to the Microbiology Department of the University Hospital of Larissa (UHL) (Larissa, Greece) for further analysis. Duplicate isolates from the same patient were excluded. In UHL, a subculture of each isolate was performed on Columbia blood agar plates incubated anaerobically for 48 h at 35 °C. Identification of colonies was based on Gram stain, characteristic odour and fluorescence under ultraviolet illumination.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of clindamycin, moxifloxacin, vancomycin, metronidazole, tetracycline and rifampicin were determined by Etest (bioMérieux, Marcy l'Étoile, France). MICs of clindamycin and tetracycline were interpreted according to Clinical and Laboratory Standards Institute (CLSI) breakpoint criteria [12], whereas for moxifloxacin, vancomycin, metronidazole and rifampicin the interpretation of MICs was done according to the epidemiological cut-off values proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/). To further clarify resistance to clindamycin, MICs of erythromycin were determined by Etest and were interpreted using breakpoints previously described by Spigaglia et al. [9]. *Bacteroides fragilis* ATCC 25289 and *C. difficile* ATCC 700057 were used as quality control strains. An isolate was defined as multidrug-resistant (MDR) when it exhibited resistance to at least three different classes of antibiotics.

2.3. DNA extraction

Genomic DNA was prepared from *C. difficile* grown on blood agar incubated anaerobically for 48 h. A few colonies were emulsified in TE [Tris–ethylene diamine tera-acetic acid (EDTA)] buffer and were heated at 100 °C for 10 min. Debris was removed by centrifugation at 13 500 rpm for 2 min and the supernatant was removed. DNA was stored at −20 °C.

2.4. Multilocus sequence typing (MLST) of C. difficile isolates

Isolates were typed by MLST performed by sequencing of seven gene loci (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA* and *tpi*) as described previously [13]. The data for *C. difficile* alleles and sequence types (STs) were obtained using the MLST database (http://pubmlst.org/cdifficile).

2.5. Toxin gene detection

Genes encoding *C. difficile* toxins were detected by PCR using specific primers for *tcdA*, *tcdB*, *cdtA* and *cdtB* [14,15]. The *tcdA* primer pair amplifies a 369-bp amplicon for toxin A-positive isolates and a 110-bp amplicon for isolates containing a deletion in the *tcdA* gene [14].

2.6. Detection of antimicrobial resistance genes

All isolates, independent of their phenotype, were tested for the presence of *ermB*, *mphC*, *msrA*, *mefA*, *lnuB*, *cfrB* and *cfrC* genes conferring resistance to clindamycin and/or erythromycin and for the presence of *tetM* and *tetW* genes conferring resistance to tetracyclines by PCR using specific primers as described previously [16,17].

2.7. Detection of fluoroquinolone and rifamycin resistance mechanisms

All isolates were examined for the presence of mutations mediating fluoroquinolone resistance in the *gyrA* and *gyrB* genes. Both genes were amplified by PCR as described previously [18].

Furthermore, all isolates were tested for mutations in the *rpoB* gene encoding the β-subunit of the RNA polymerase as described by Curry et al. [19]. Both strands of the PCR products were sequenced using an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Sequences were compared using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

2.8. Illumina sequencing

Genomic DNA of selected *C. difficile* isolates was sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Sequencing, assembling of the reads and sequence analysis were performed as described previously [20].

2.9. Nucleotide sequence accession nos

The nucleotide sequences of *cfrB*- and *cfrC*-carrying regions from isolates Cd-14Lar and Cd-13Lar have been assigned GenBank accession nos. **MH229775** and **MH229772**, respectively. The *ermB*-carrying sequence from isolate Cd-14Lar has been assigned GenBank accession no. **MH229774**.

3. Results

3.1. Frequency of antimicrobial resistance in C. difficile

During 2012–2015, a total of 88 *C. difficile* isolates were randomly collected from 10 Greek hospitals. Five hospitals (N1–N5) were located in Northern Greece ($n = 35$ isolates; 39.8%) and five hospitals (C1–C5) were in Central Greece ($n = 53$ isolates; 60.2%). Antimicrobial susceptibilities, in terms of MIC range and MIC_{50} and MIC_{90} values (MICs required to inhibit 50% and 90% of

Table 1
Susceptibility of *Clostridium difficile* isolates ($n = 88$) to various antimicrobial agents.

Antimicrobial agent	MIC (mg/L)			
	Range	MIC_{50}	MIC_{90}	GM
Erythromycin (R, ≥8)	0.094–256	256	256	34.14
Clindamycin (R, ≥8)	0.016–256	12	256	19.85
Moxifloxacin (R, ≥4)	1–32	1	32	13.85
Rifampicin (R, ≥0.004)	0.002–32	0.002	32	0.084
Tetracycline (R, ≥16)	0.016–256	3	24	1.516
Vancomycin (R, ≥2)	0.016–2	0.38	0.75	0.324
Metronidazole (R, ≥2)	0.016–3	0.19	0.5	0.188

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MICs required to inhibit 50% and 90% of the isolates, respectively; GM, geometric mean; R, resistance breakpoint (in mg/L).

the isolates, respectively) are presented in Table 1. All isolates were susceptible to vancomycin and metronidazole. The rate of resistance of *C. difficile* to moxifloxacin reached 71.6% ($n = 63$). Regarding resistance to other antimicrobials, 58 (65.9%), 54 (61.4%), 32 (36.4%) and 21 (23.9%) isolates exhibited resistance to erythromycin, clindamycin, rifampicin and tetracycline, respectively (Table 2). Of the 88 *C. difficile* isolates, 40 (45.5%) were MDR exhibiting resistance to erythromycin, clindamycin and moxifloxacin, and 28 of these were also resistant to rifampicin.

3.2. Population structure of C. difficile

The population structure of *C. difficile* studied by MLST is shown in Table 2. The *C. difficile* isolates comprised 27 STs, with ST37 ($n = 26$) and ST11 being the most prevalent ($n = 21$); both clones were equally distributed during the study period. The majority of ST37 isolates were recovered from hospitals located in Central Greece ($n = 21$), whilst only 5 of the 35 isolates collected in Northern Greece belonged to ST37. ST37, which is associated with PCR ribotype 017, was initially identified in CDI outbreaks in Asia [21,22]. Of the 21 ST11 isolates, 14 were collected in hospitals from Central Greece [C1 ($n = 1$), C2 ($n = 9$), C4 ($n = 2$) and C5 ($n = 2$)], 6 were collected in hospital N1, whereas only 1 was collected in hospital N2. ST11, which is associated with PCR ribotype 078, is the dominant genotype of *C. difficile* spreading in North America and Europe [23,24]. Moreover, 24 of the isolates were distributed in ST35 ($n = 5$), ST42 ($n = 4$), ST46 ($n = 4$), ST236 ($n = 3$), ST2 ($n = 2$), ST16 ($n = 2$), ST110 ($n = 2$) and ST147 ($n = 2$). The remaining isolates belonged to unique STs (Table 2).

3.3. Molecular detection of C. difficile toxin genes

Of the 88 *C. difficile* isolates, 60 were positive for both *tcdA* and *tcdB* by PCR, 27 had a partial deletion in *tcdA* ($A^{-d}B^{+}$) and the remaining isolate was negative for *tcdA* but positive for *tcdB* ($A^{-}B^{+}$). The majority of $A^{-d}B^{+}$ belonged to ST37 ($n = 26$), whilst 1 isolate was assigned to ST11. The $A^{-}B^{+}$ isolate belonged to ST207.

The *cdtA/B* gene encoding the binary toxin was detected in 24 strains. Eighteen were $A^{+}B^{+}$ isolates, from which eleven were assigned to ST11, two to ST35 and five isolates were distributed in distinct STs. In addition, six $A^{-d}B^{+}$ isolates belonging to ST37 were binary toxin-positive. The remaining *C. difficile*, including the ST207 $A^{-}B^{+}$ isolate, were all binary toxin-negative (CDT^{-}). In summary, five toxigenic profiles were revealed in this study: $A^{+}B^{+}CDT^{-}$ ($n = 42$); $A^{+}B^{+}CDT^{+}$ ($n = 18$); $A^{-d}B^{+}CDT^{-}$ ($n = 21$); $A^{-d}B^{+}CDT^{+}$ ($n = 6$); and $A^{-}B^{+}CDT^{-}$ ($n = 1$).

3.4. Antimicrobial resistance genes in C. difficile

All *C. difficile* were examined for the presence of antimicrobial resistance genes. Of the 54 clindamycin-resistant *C. difficile* isolates, 52 were found to carry the *ermB* gene, either alone or

Table 2
Characteristics of the *Clostridium difficile* isolates.

MLST	No. of strains	Hospital (no. of strains)	No of *C. difficile* strains resistant to:						
			ERY (R, ≥8)	CLI (R, ≥8)	MFX (R, ≥4)	RIF (R, ≥0.004)	TET (R, ≥16)	VAN (R, ≥2)	MTR (R, ≥2)
ST2	2	N2 (2)	–	–	–	–	–	–	–
ST3	1	N1 (1)	–	–	–	–	–	–	–
ST7	1	N2 (1)	–	–	–	–	–	–	–
ST11	21	C2 (9), N1 (6), C4 (2), C5 (2), C1 (1), N2 (1)	16	14	21	3	6	–	–
ST12	1	C2 (1)	1	1	1	–	–	–	–
ST14	1	C2 (1)	–	–	–	–	–	–	–
ST15	1	C3 (1)	1	1	–	–	–	–	–
ST16	2	N1 (1), C3 (1)	–	–	–	–	–	–	–
ST19	1	C2 (1)	–	–	–	–	–	–	–
ST35	5	N1 (2), C2 (2), N2 (1)	5	5	3	–	2	–	–
ST37	26	C2 (14), C1 (3), C4 (2), C3 (2), N5 (2), N1 (1), N2 (1), N3 (1)	26	26	26	26	10	–	–
ST42	4	C2 (2), N4 (1), N1 (1)	4	3	3	1	1	–	–
ST46	4	C2 (2), N1 (2)	–	–	3	–	–	–	–
ST54	1	N5 (1)	1	1	–	–	–	–	–
ST90	1	N2 (1)	–	–	–	–	–	–	–
ST92	1	C1 (1)	–	–	1	1	1	–	–
ST93	1	N2 (1)	1	1	–	–	–	–	–
ST94	1	N3 (1)	–	–	1	–	–	–	–
ST110	2	C4 (2)	1	1	–	1	–	–	–
ST135	1	C2 (1)	–	–	–	–	–	–	–
ST147	2	N1 (1), N2 (1)	1	1	2	–	1	–	–
ST160	1	C1 (1)	–	–	–	–	–	–	–
ST193	1	C3 (1)	–	–	1	–	–	–	–
ST207	1	N4 (1)	1	–	–	–	–	–	–
ST214	1	N4 (1)	–	–	–	–	–	–	–
ST236	3	C5 (1), N2 (1), N5 (1)	–	–	1	–	–	–	–
ST326	1	N1 (1)	–	–	–	–	–	–	–
	88		58	54	63	32	21	0	0

MLST, multilocus sequence typing; ERY, erythromycin; CLI, clindamycin; MFX, moxifloxacin; RIF, rifampicin; TET, tetracycline; VAN, vancomycin; MTR, metronidazole; R, resistance breakpoint (in mg/L).

combined with other MLS_B-related resistance genes (Table 3). All *ermB*-positive isolates also exhibited resistance to erythromycin. Of note was that the presence of *ermB* was strongly associated with ST37*C. difficile*. In addition, *cfrB* ($n = 6$) and *cfrC* ($n = 2$), with or without the *ermB* gene, were identified in one ST12 and seven ST37*C. difficile* isolates (Table 3). Furthermore, one ST37*C. difficile* was positive for the *mefA* gene combined with the *ermB* gene. In summary, five resistance gene combinations were revealed among clindamycin-resistant isolates: *ermB* ($n = 44$); *ermB* + *cfrB* ($n = 6$); *ermB* + *cfrC* ($n = 1$); *ermB* + *mefA* ($n = 1$); and *cfrC* ($n = 1$). Moreover, one clindamycin-resistant isolate was found to be negative for all the tested genes. Finally, one ST42*C. difficile* susceptible to clindamycin but resistant to erythromycin was positive for the *msrA* gene, which is responsible for resistance to macrolides and streptogramin B. On the other hand, five erythromycin-resistant but clindamycin-susceptible *C. difficile* isolates were negative for all of the tested genes. None of the isolates being both clindamycin- and erythromycin-susceptible were found to be positive for genes associated with resistance to these drugs.

All tetracycline-resistant *C. difficile* ($n = 21$) were found to carry the *tetM* gene. The *tetM* gene was also found in 16*C. difficile* exhibiting intermediate resistance level to tetracycline (Table 3).

3.5. Mechanisms of resistance to fluoroquinolones and rifamycins

To investigate the mechanisms of the observed high frequency of resistance to fluoroquinolones, all *C. difficile* were examined for the presence of amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *gyrB* genes. Sequencing of the QRDRs revealed the presence of amino acid substitutions in 58 of 63 moxifloxacin-resistant (MOX-R) *C. difficile* (Table 3). Twelve different patterns of amino acid substitutions were identified among the MOX-R *C. difficile*: (i) GyrA:T82I ($n = 33$); (ii) GyrA:T82I; GyrB:S416A ($n = 11$); (iii) GyrB:S416A ($n = 5$); (iv) GyrA:T82V ($n = 1$); (v) GyrB:D426N ($n = 1$); (vi) GyrB:D426G ($n = 1$); (vii) GyrB:R436I ($n = 1$); (viii) GyrB:S416A + D426N ($n = 1$); (ix) GyrB:S366V + S416A + D426N ($n = 1$); (x) GyrA:T82I; GyrB:S366A ($n = 1$); (xi) GyrA:T82I; GyrB:S416A + D426N ($n = 1$); and (xii) GyrA:T82I; GyrB:D426N + R436I ($n = 1$). The presence of a GyrA:T82I pattern was mainly found in ST37 isolates, whilst the GyrA:T82I; GyrB:S416A and GyrB:S416A patterns were strongly associated with *C. difficile* ST11. The five remaining MOX-R *C. difficile*, where no mutations in the QRDRs of *gyrA* and *gyrB* were identified, belonged to ST11, ST12, ST42 and ST236. A lack of amino acid substitutions in the QRDRs of *gyrA* and *gyrB* was observed in 23 of 25 moxifloxacin-susceptible *C. difficile*. In the two remaining isolates, which belonged to ST90 and ST93, GyrB included the S366A or D426S substitutions.

Sequencing of *rpoB* gene revealed the presence of amino acid substitutions H502N and R505K in 27 of 32 rifampicin-resistant (RIF-R) *C. difficile* isolates (Table 3). The presence of H502N and R505K mutations was strongly associated with *C. difficile* ST37 (PCR ribotype 017). The five RIF-R *C. difficile* where no mutations in the *rpoB* were identified were assigned to ST11 ($n = 3$), ST42 ($n = 1$) and ST110 ($n = 1$). Amino acid substitutions in the *rpoB* were not observed in any rifampicin-susceptible isolates ($n = 56$).

3.6. Genetic background of resistance genes

To our knowledge, the *cfrB* and *cfrC* genes are reported for the first time in Greek isolates. To examine the genetic environment of these resistance genes, *C. difficile* isolates Cd-13Lar and Cd-14Lar

Table 3
Molecular analysis of resistance in the studied *Clostridium difficile* isolates.

MLST	No. of strains	No. of *C. difficile* strains positive for:						Amino acid substitutions (no. of strains) in:		
		ermB	*msrA*	*mefA*	*cfrB*	*cfrC*	*tetM*	GyrA	GyrB	RpoB
ST2	2	–	–	–	–	–	–	–	–	–
ST3	1	–	–	–	–	–	–	–	–	–
ST7	1	–	–	–	–	–	–	–	–	–
ST11	21	14	–	–	–	–	13	T82I (12)	S416A (16) D426G (1) S416A + D426N (1) S366V + S416A + D426N (1)	–
ST12	1	–	–	–	–	1	–	–	–	–
ST14	1	–	–	–	–	–	–	–	–	–
ST15	1	–	–	–	–	–	–	–	–	–
ST16	2	–	–	–	–	–	–	–	–	–
ST19	1	–	–	–	–	–	–	–	–	–
ST35	5	5	–	–	–	–	2	T82I (3)	–	–
ST37	26	26	–	1	6	1	18	T82I (26)	S366A (1)	H502N + R505K (26)
ST42	4	3	1	–	–	–	1	T82I (2)	D426N + R436I (1)	–
ST46	4	–	–	–	–	–	–	–	D426N (1) R436I (1)	–
ST54	1	1	–	–	–	–	–	–	–	–
ST90	1	–	–	–	–	–	–	–	D426S (1)	–
ST92	1	–	–	–	–	–	1	T82I (1)	–	H502N + R505K (1)
ST93	1	1	–	–	–	–	–	–	S366A (1)	–
ST94	1	–	–	–	–	–	–	T82V (1)	–	–
ST110	2	1	–	–	–	–	–	–	–	–
ST135	1	–	–	–	–	–	–	–	–	–
ST147	2	1	–	–	–	–	1	T82I (2)	S416A + D426N (1)	–
ST160	1	–	–	–	–	–	–	–	–	–
ST193	1	–	–	–	–	–	–	–	S416A (1)	–
ST207	1	–	–	–	–	–	1	T82I (1)	–	–
ST214	1	–	–	–	–	–	–	–	–	–
ST236	3	–	–	–	–	–	–	–	–	–
ST326	1	–	–	–	–	–	–	–	–	–
	88	52	1	1	6	2	37	48	27	27

MLST, multilocus sequence typing.

were analysed by whole-genome sequencing (WGS). Analysis of WGS data of Cd-14Lar revealed that *cfrB* was part of the transposon Tn*6218*. Tn*6218* was previously described from three *C. difficile* (ST5 and ST385) isolates [25]. In these isolates, Tn*6218* was inserted into the toxin A (*tcdA*) and B (*tcdB*) gene-carrying pathogenicity locus (PaLoc). However, in isolate Cd-14Lar, Tn*6218* was found in a different position of the *C. difficile* chromosome. In addition, de novo assembly obtained a unique contig containing *ermB* for *C. difficile* isolate Cd-14Lar. Sequence analysis showed that Cd-14Lar harboured a 12.8-kb plasmidic fragment containing *ermB* integrated into the *C. difficile* chromosome. The plasmidic fragment (nucleotides 105 759–117 579 in GenBank accession no. **MH229774**), which exhibited limited similarity with previously described structures, consisted of two copies of *ermB* as well as genes encoding replication, mobilisation and stabilisation proteins.

Finally, sequence analysis indicated the presence of a *cfrC*-carrying plasmid (pCd-13Lar) in *C. difficile* isolate Cd-13Lar. Plasmid pCd13-Lar, which is 6961 bp in size, was composed of genes encoding proteins for plasmid replication (*rep*), mobilisation (*mob*) and stability (*relB*/*dniJ*). The *cfrC* gene was highly similar (99%) to the respective gene originally found in plasmid pTx40 from a *Campylobacter coli* isolate [26] and was localised upstream of the *mob* gene. Similar to pTx40, the *aphA3* gene conferring resistance to aminoglycosides was found next to the *cfrC* gene.

4. Discussion

CDI is a growing threat to global public health. Antimicrobial therapy plays a central role in the development of CDI, and antibiotics are the most common risk factor for CDI development [27]. The risk of CDI is increased if *C. difficile* is resistant to the antimicrobial agent used [5]. One of the proposed theories behind the major reported outbreaks was that fluoroquinolone-resistant *C. difficile* strain 027 was circulating at the same time that the use of fluoroquinolones was common in Canadian hospitals [28]. *C. difficile* is known to be resistant to multiple antibiotics such as erythromycin, lincomycin, tetracycline, fluoroquinolones, etc. that are commonly used in the treatment of bacterial infections in clinical settings [10]. Rates of antimicrobial resistance in *C. difficile* vary in different geographic regions [10]. In a recent comparative, cross-sectional, multicentre study including isolates from representative regions of Germany, Ghana, Tanzania and Indonesia, the rate of resistance to macrolides and moxifloxacin varied significantly (15.2–75.9% for macrolides and vs. 0–65.5% for moxifloxacin) [29]. On the other hand, data from the last *Clos*ER study conducted between 2011 and 2014 in 28 European countries showed that 0.2% and 0.1% of isolates were resistant to metronidazole and vancomycin, respectively; moxifloxacin and clindamycin resistance was widespread, with rates reaching 36.9% and 71%, respectively, whilst the rate of rifampicin resistance was 16.9% [30]. To our knowledge, the current study is the first to examine the resistance profiles and respective resistance mechanisms of *C. difficile* recovered in Greek hospitals. The findings showed that *C. difficile* remained fully susceptible to vancomycin and metronidazole, the first lines for the treatment of CDI, while exhibiting increased resistance levels to fluoroquinolones, erythromycin, clindamycin, rifampicin and tetracycline. These data indicate that resistance to moxifloxacin and rifampicin was twofold higher than in the *Clos*ER study [30]. Resistance to fluoroquinolones and rifampicin was mediated by the presence of chromosomal mutations in the QRDRs of the *gyrA* and *gyrB* genes and in the *rpoB* gene, respectively. The presence of chromosomal mutations in these genes was highly associated with high-risk clones such as ST11 and ST37, being the most prevalent in this study. Interestingly, most of the observed amino acid substitution patterns in the QRDRs of *gyrA* and *gyrB* have been previously identified among fluoroquinolone-resistant *C. difficile* belonging to different PCR ribotypes such as 001, 012 and 176 [9,31] and derived from different geographical areas [18,32]. However, the role of novel amino acid substitutions (S366V, D426S, D426G and R436I in GyrB) in the development fluoroquinolone resistance needs to be evaluated in further studies. Furthermore, the H502N and R505K amino acid substitution pattern identified in the *rpoB* gene is the most common in RIF-R *C. difficile* of several PCR ribotypes [9,33], including 017. PCR ribotype 017 has attracted attention in Latin America, Asia and Europe [21,22,34].

Furthermore, resistance to clindamycin and tetracycline was associated with the presence of various resistance genes such as *ermB* and *tetM*. Presence of the *ermB* and *tetM* genes in MDR *C. difficile* recovered from hospitals in European countries has been previously reported [9,17,35,36]. However, the most interesting finding was detection of the resistance genes *cfrB* and *cfrC*, which were identified for the first time in Greek isolates and confer resistance to clindamycin but also to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics [37]. The *cfrC* gene was carried by a novel plasmid, indicating the ability of the resistance gene to be transferred to other clones or species. In agreement with the current results, previous studies have shown the presence of resistance genes in Clostridia colonising the human gut [10,17,25]. Thus, gut commensals such as *C. difficile* may serve as hubs for the further transfer of resistance genes. The fact that *C. difficile* spores can survive for long time in the environment poses a real threat for the further spread of antimicrobial resistance.

In a previous study conducted in 2005 in 14 European countries, 48 (15.2%) out 316 isolates were resistant to four different classes of antibiotics, including erythromycin, clindamycin, moxifloxacin and rifampicin [9]. However, in the current study a higher proportion of isolates (31.8%) shared the same resistance phenotype. The fact that the rate of MDR *C. difficile* was significantly higher in Greece might mirror antibiotic consumption. In Greek hospitals, antibiotics are extensively used for the treatment of other infectious agents such as multidrug-resistant, including to carbapenems, Enterobacteriaceae, which are endemic in these settings [38]. In addition, the high rate of MDR *C. difficile* indicates that acquisition of antimicrobial resistance is important for the emergence and persistence of specific epidemic clones over time in hospital settings. Therefore, proper use of antibiotics as well avoidance of the overuse of these agents should be pursued.

Finally, these findings contribute to the current knowledge on antimicrobial resistance and resistance mechanisms of *C. difficile*. The rapid evolution of antimicrobial resistance, combined with the changing epidemiology of CDI, emphasises the need for implementation of effective antimicrobial stewardship and infection control practices.

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Competing interests

None declared.

Ethical approval

Not required.

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