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Kachrimanidou, M.; Metallidis, S.; Tsachouridou, O.; Harmanus, C.; Lola, V.; Protonotariou, E.; ... ; Kuijper, E.

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Original Article

Predominance of *Clostridioides difficile* PCR ribotype 181 in northern Greece, 2016–2019

Melina Kachrimanidou [a, *], Symeon Metallidis [b], Olga Tsachouridou [b], Celine Harmanus [c], Vassiliki Lola [a], Efthymia Protonotariou [d], Lemonia Skoura [d], Ed Kuijper [c]

[a] First Department of Microbiology, Aristotle University of Thessaloniki, Medical School, Thessaloniki, Greece
[b] First Internal Medicine Department, Infectious Diseases Division, AHEPA Hospital, Medical School, Aristotle University of Thessaloniki, Greece
[c] Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, And National Institute of Public Health, Bilthoven, the Netherlands
[d] Department of Microbiology, AHEPA University Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece

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ABSTRACT

Objectives: The epidemiology of *Clostridioides difficile* infection (CDI) has undergone many changes since the beginning of this century and continues to evolve based on recent studies. Here, we performed a molecular analysis of *C. difficile* isolates in northern Greece across 10 health-care facilities, spanning from 2016 to 2019.

Methods: 221 *C. difficile* isolates were cultured from stool samples of hospitalized patients with diarrhea and screened by PCR for the presence of the toxin A (*tcdA*), toxin B (*tcdB*), the binary toxin (*cdtA* and *cdtB*) genes and the regulating gene of *tcdC*. PCR ribotyping of the cultured isolates was performed by a standardized protocol for capillary gel-based PCR ribotyping and an international database with well-documented reference strains.

Results: Thirty-five different PCR ribotypes were identified. The most common RTs identified were: 181 (36%, 80/221), 017 (10%, 21/221), 126 (9%, 19/221), 078 (4%, 9/221) and 012 (4%, 8/221). Notably, the predominant RT181, with toxin profile $tcdA^+$ $tcdB^+$ $cdtA^+$ $cdtB^+$, was identified in seven out of ten participating hospitals.

Conclusions: Multiple *C. difficile* ribotypes have been circulating in the northern Greece region with RTs 181 (closely related to 027), 017, 126 and 078 being predominant.

1. Introduction

Clostridioides difficile infection (CDI) has become a worldwide public health problem causing high mortality and a large disease burden. CDI is a toxin-mediated disease with a wide range of clinical presentations from mild self-limiting diarrhea to life-threatening pseudomembranous colitis, toxic megacolon, bowel perforation and sepsis [1,2]. The major *C. difficile* virulence factors are large clostridial toxins designated enterotoxin A (TcdA) and cytotoxin B (TcdB), encoded by *tcdA* and *tcdB* genes which are co-locatecd in a 19.6 kb pathogenicity locus region (PaLoc) [3]. In addition to toxins A and B, some *C. difficile* isolates can produce a binary toxin, encoded by *cdtA* and *cdtB* genes, but it's exact role in the pathogenesis of CDI is unknown [4].

The epidemiology of CDI has undergone many changes since the beginning of this century and continues to evolve based on molecular typing and analysis studies. The distribution of the most common ribotypes (RTs) has changed and new RTs have emerged with a wide diversity of genotypes in different countries in Europe [5]. However, there is limited data on the molecular characterization of *C. difficile* isolates in Greece, especially using longitudinal multicenter studies.

C. DEFINE, a multicenter study which aimed to assess point-prevalence of CDI in Greece, revealed a considerable prevalence of CDI in Greek hospitals ranging between 3.9 and 5.6 per 10,000 patient bed-days, and between 14.3 and 17.0% of hospitalized patients with diarrhea, respectively [6]. Our study is the first study of molecular characterization of *C. difficile* isolates in Greece across a large timeframe and attempts to cover a gap in knowledge in our

* Corresponding author. 1st Department of Microbiology, Medical School, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece.
E-mail address: mkachrim@gmail.com (M. Kachrimanidou).

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region and other RTs for 027-lineage [7].

In this study, we performed a molecular analysis of *C. difficile* isolates in northern Greece across 10 health-care facilities, spanning from 2016 to 2019. We report on toxin gene profiles and PCR ribotypes. This study will help better understand the epidemiology of CDI in northern Greece and thus better control and prevent CDI.

2. Materials and methods

2.1. Study samples and case definitions

This is a retrospective study including in-patients with diarrhea of all ages from 10 hospitals (7 general hospitals and one long-term care facility in Thessaloniki, one general hospital in Chalkidiki and Veroia, respectively) across northern Greece from 2016 to 2019. Only 1 sample per patient was included. A total of 419 diarrheal stool samples of hospitalized patients with suspected CDI were investigated by anaerobic culture on a selective *C. difficile* medium at the laboratory in the Microbiology Department of Aristotle University Medical School (CDI Reference Centre). These specimens were selected from patients with presumptive CDI, defined as patients who produced three or more loose stools (Bristol stool type 5 to 7) for at least two consecutive days, who were receiving or had received antibiotics in the preceding 6 weeks. Clinical data and data on CDI treatment were not collected.

2.2. Bacterial culture and identification

All stool samples underwent culture for *C. difficile*. Each specimen was pretreated using the alcohol-shock method; industrial methylated spirits ([IMS] 0.5 ml) was added to a 0.5 ml fecal sample, and the sample was vortex mixed for 10 s every 15 min and incubated at room temperature for 1 h. A loopful was then cultured onto modified Brazier's cycloserine-cefoxitin-egg yolk (CCEY) agar (CCEY agar base containing cycloserine-cefoxitin supplement and 5% defibrinated horse blood), and the plates were incubated anaerobically at 37 °C for up to 5 days. A single colony was subcultured onto a Columbia blood agar (CBA) plate and incubated for 48 h, after which colonies giving the characteristic odor and fluorescence under UV illumination were obtained. Suspected colonies were further confirmed by amplification of the *gluD* gene encoding glutamate dehydrogenase [8], and the gene for 16SrRNA [9]. For long-term storage, isolates were homogenized in nutrient broth containing 10% glycerol and stored at −80 °C.

2.3. DNA isolation

DNA was extracted from single colony from sub-cultures incubated anaerobically for 48 h on CBA. A few (4–5) colonies were suspended in TE (Tris-EDTA) buffer (Sigma-Aldrich Co., Ltd., Gillingham, United Kingdom) and heated at 100 °C for 10 min. Debris was removed by centrifugation at 13,500 rpm for 2 min, and the supernatant was removed for use. DNA was stored at −20 °C.

2.4. Toxin gene profiling

All isolates were screened by PCR for the presence of the toxin A (*tcdA*), toxin B (*tcdB*) genes, the binary toxin (*cdtA* and *cdtB*) genes and the regulating gene of *tcdC*. The oligonucleotide primers were used to detect the *tcdA* [10], *tcdB*, and *tcdC* genes within the pathogenicity locus operon (PaLoc) [11,12]. The absence of the PaLoc was demonstrated using primers lok1 and lok3 [13].

2.5. PCR-ribotyping

All PCR ribotyping of the cultured isolates described in the present study was performed at the Department of Medical Microbiology, National Reference Laboratory for *C. difficile*, Leiden University Medical Center, Leiden, the Netherlands. PCR ribotyping was performed by a standardized protocol for capillary gel-based PCR ribotyping and an international database with well-documented reference strains [14].

3. Results

From the 419 diarrheal stool samples, 221 *C. difficile* strains were isolated. Of the total of 221 patients, 122 were female (55%) and 99 were male (45%). The age of patients ranged from 60 days to 95 years and 73,8% of the patients were older than 60 years. The distribution of the 221 isolated *C. difficile* strains per clinical site were as follows: 89 cases from long-term care facility (Thessaloniki), 82 cases from AHEPA Hospital (Thessaloniki), 33 cases from PAPANIKOLAOU Hospital (Thessaloniki), 5 cases from PAPAGEORGIOU Hospital (Thessaloniki), 4 cases from GENIMMATA Hospital (Thessaloniki), 2 cases AGIOS PAVLOS Hospital (Thessaloniki), 2 cases from AGIOS DIMITRIOS Hospital (Thessaloniki), 2 cases from General Hospital of Chalkidiki, 1 case from General Hospital of Veroia, and 1 case from HIPPOKRATEION Hospital (Thessaloniki). Not all hospitals contributed cases throughout the three years period.

3.1. Ribotype distribution

Thirty-five different PCR ribotypes were identified; the most common were RT181 (36%, 80/221), RT017 (10%, 21/221), RT126 (9%, 19/221), RT078 (4%, 9/221) and RT012 (4%, 8/221). The RT181 assignment in the Webribo database is A1-33 (https://webribo.ages.at). Notably, the predominant RT181 was identified in seven out of ten participating hospitals in northern Greece (Table 1).

The remaining RTs were detected sporadically and were represented by up to six isolates. The RT profiles of 14 isolates (6%, 14/221), differed from each other and did not match any of the reference RTs available in this study (provided by the Leiden Reference laboratory).

3.2. Toxin genes profile

Of the 221 *C. difficile* isolates, 11 were non-toxigenic (all the tested virulence genes and regulating gene were negative). The predominant toxin genes profile was $tcdA^+$ $tcdB^+$ $cdtA^+$ $cdtB^+$, which corresponds to 51% of all isolates. The toxin profiles of all isolates are shown in Table 1. PCR toxin gene testing confirmed all PCR ribotype 181 isolates to be positive for *tcdA, tcdB* and *cdtA, cdtB.*

4. Discussion

The key findings of this study are: 1) CDI is common in northern Greece hospitals; 2) key RTs are RT181, RT017, RT126 and RT078; 3) RT027 was not detected in our region. These data provide important new insights on the epidemiology of CDI in Greece given that molecular reports and particularly multicenter studies of *C. difficile* are scarce in our region.

In our study, RT181, RT017, RT126 and RT078 were the most prevalent RTs identified. A 027-like ribotype 181 has been recently recognized causing a large outbreak in a 180-bed Rehabilitation Centre in Greece. Genomic analysis revealed that the outbreak strain belonged to Clade 2, sequence type (ST) 1 and had a 18bp deletion in *tcdC* at position 311 together with a single nucleotide deletion at position 117, similar to RT027 [15]. Interestingly, the

Table 1
Toxin-encoding genes profile and RT types of the 221 isolated *Clostridioides difficile* strains in Greece (2016–2019). The distribution of RTs for the 7 hospitals that had the predominant RT181 is also shown.

RT	No. of strains	Toxin gene pattern					Hospitals						
		tcdA$^+$ *tcdB*$^+$ *cdtA-cdtB*$^-$	*tcdA*$^-$*tcdB*$^+$*cdtA-cdtB*$^-$	*tcdA*$^+$*tcdB*$^+$*cdtA-cdtB*$^+$	*tcdA*$^-$*tcdB*$^+$*cdtA-cdtB*$^+$	*tcdA*$^-$*tcdB*$^-$ *cdtA-cdtB*$^-$	A	B	C	D	E	F	G
RT001	**5**	5					2		2		1		
RT002	**1**	1					1						
RT005	**3**	3					1		2				
RT009	**2**					2	2						
RT010	**2**					2	1		1				
RT012	**8**	8					3	1	2	2			
RT014	**5**	5					2	1	2				
RT015	**1**	1							1				
RT017	**21**		21				15	2	4				
RT020	**4**	4					3		1				
RT021	**1**	1					1						
RT036	**3**			3					3				
RT039	**5**					5	3	1	1				
RT046	**6**	6					2		4				
RT070	**4**	4					1	1	1	1			
RT073	**1**	1					1						
RT076	**2**	2					1	1					
RT078	**9**				9		6	3					
RT110	**1**	1											
RT126	**19**			19			9	5	3		1		
RT181	**80**			80			9	9	56		2	2	2
RT198	**1**			1					1				
RT202	**5**	5					3	1	1				
RT207	**1**	1					1						
RT307	**1**					1		1					
RT024	**1**	1					1						
RT050	**1**	1					1						
RT063	**1**					1				1			
RT087	**1**	1						1					
RT106	**5**	5					3	1		1			
RT119	**1**		1						1				
RT131	**2**			2				2					
RT137	**2**	2						1	1				
RT220	**1**	1					1						
RT369	**1**		1				1						
UNKNOWN	**14**						8	2	4				

Hospital A: AXEPA; B: PAPANIKOLAOU; C: REHABILITATION CENTER; D: PAPAGEORGIOU; E: GENNIMATA; F: AGIOS DIMITRIOS; G: AGIOS PAVLOS.

current study confirms the prominence of the newly identified ribotype RT181 in our region, given that this RT was found in 70% of hospitals that participated in our study. To date this RT has not been linked with an outbreak of CDI in another country, except for Romania [16].

The second and third most common ribotypes in the current study, RT017 and RT126 respectively, were already known to be circulating in our region since 2014–2015, based on a previous study conducted in a single tertiary hospital. In that report, *C. difficile* toxin A-negative PCR ribotype 017 was the predominant type followed by RT126 [17]. Historically, RT017 was initially reported in Asia, but has now been reported worldwide, including countries such as Poland, Korea, Japan, Bulgaria and Argentina, where it has been associated with severe disease [18–21]. The RT017, with its unique toxin profile and unusual global prevalence, has been overshadowed by the global outbreak of the ribotype 027 lineage. The toxigenic PCR ribotype 126, regarded as a hypervirulent clone, is phylogenetically close to hypervirulent RT078 and differ by only one band by electrophoresis in PCR-ribotyping profile [22]. Presence and detection of RT126 is important, because severe CDI cases are associated with RT126 or RT078-like clones [23].

Less frequently, we encountered RT078 which has been associated with similarly severe disease manifestations as RT027. It is one of the prevalent ribotypes in European hospitals, generally characterized by the presence of all toxin genes *tcdA*$^+$ *tcdB*$^+$ *cdtA*$^+$ *cdtB*$^+$, and considered to be associated with complicated infection [8,24]. Epidemiological studies reported the PCR-ribotype 078 as the predominant type in pig, cattle and horse species worldwide, and also reported an increase in its prevalence in humans in different countries [25,26]. Notably, isolates from humans and pigs were found to be highly genetically related [27]. Comparisons of strains have shown that animals and humans can be colonized by identical *C. difficile* clones or strains that cluster in the same lineage. Therefore, it is suggested that *C. difficile* should be considered as a zoonotic pathogen and the interspecies transmission between animals and humans is possible with animals as a reservoir for humans [28]. These findings highlight the importance of a comprehensive One Health perspective in monitoring and controlling *C. difficile* infection. Interestingly, transcontinental transmission of RT078 through animal export has also been reported [29].

The virulent PCR ribotype 027 that has caused multiple outbreaks throughout Europe was not observed in our study. Our results corroborate previous findings that countries with low prevalence of ribotype 027 have higher overall ribotype diversity among *C. difficile* isolates, highlighting the diverse epidemiology of *C. difficile* across the continent [5]. Of note, Davies and colleagues reported that ribotypes 078, 126 and 017 were among the 10 most commonly isolated ribotypes from southern European countries (Greece, Italy, Portugal and Spain) [5]. Moreover, except RT181 the spectrum of ribotyping profiles identified belong to the human RTs occurring in Europe [8].

4.1. Limitations

Our study has several limitations. First, not all *C. difficile* isolates used in this study were collected at the same period. CDI epidemiology is dynamically changing; thus, the distributions of genotypes may have shown slight bias during data analysis. Second, limited clinical data were available and thus no firm conclusions can be made about the virulence of RT181. Though the microbiological data suggest that RT181 has potential characteristics of a hypervirulent RT, we need clinical evidence to confirm this. Finally, this was a retrospective study involving relatively small numbers of cases recruited from some sites. Therefore, further investigations are required to clarify the differences of various genotypes of *C. difficile* in different regions and confirm the molecular characteristics of *C. difficile* isolates in northern Greece. Nevertheless, this study is an important step towards improving the surveillance of CDI in our region.

5. Conclusions

In conclusion, this is the first multi-center study of *C. difficile* isolates in Greece, which elucidates the molecular epidemiological characteristics of *C. difficile* in northern Greece across a large timespan. Multiple *C. difficile* ribotypes have been circulating in the northern Greece region with RT181, RT017, RT126 and RT078 being the predominant RTs. Although no RT027 was found, a significant number of potentially similarly virulent RT181 isolates were present in our cohort. Toxin profile $A^+B^+CDT^+$ is the main type in our geographic area. Our data provide evidence of the unfavorable epidemiological situation in Greek health-care facilities.

Declaration of competing interest

None of the authors has a conflict of interest.

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