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Distribution of *Clostridium Difficile* Ribotypes in Macedonian Patients and their Antimicrobial Susceptibility

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Abstract

BACKGROUND: *Clostridium difficile* is a major nosocomial pathogen. In Europe, this bacterium is mostly characterised by PCR ribotyping. Most of the *Clostridium difficile* infections (CDI) are treated with vancomycin or metronidazole, although prolonged antibiotic use is considered as one of the main risk factors for CDI.

AIM: This study aimed to detect the presence of various *C. difficile* ribotypes in hospitalised patients and to investigate their toxigenicity and antibiotic susceptibility.

MATERIAL AND METHODS: All stool samples obtained from each patient were inoculated on Columbia blood agar and cycloserine cefoxitine fructose agar (CCFA) for isolation of *C. difficile*. Glutamate dehydrogenase and toxins A and B were investigated by immunochromatographic tests. Final confirmation of the isolates was performed by Vitek 2 and MALDI-TOF. A total of 21 isolates were collected for further investigation. PCR ribotyping was performed as described by Janezic and Rupnik. PCR ribotype profiles were analysed using software (Bionumerics, Applied Maths). Antibiotic susceptibility was determined by E-tests for metronidazole, vancomycin, tetracycline, clindamycin, erythromycin, imipenem, ciprofloxacin and moxifloxacin.

RESULTS: About 48% of *C. difficile* isolates belonged to ribotype 001/072. So, this ribotype was the most common ribotype in this study. The remaining 52% of *C. difficile* isolates consisted of 10 different ribotypes: 017, SLO 160, SLO 187, SLO 120, 255/258, 014/020, 046, 002, 070 and 027. Furthermore, 20 (95.2 %) out of 21 isolates of *C. difficile* were toxigenic. Toxins A and B were detected simultaneously in 90.5 % of *C. difficile* isolates. Two isolates from the ribotype 017 were toxin B positive only. Treatments with any of the following antimicrobials: clindamycin, erythromycin, ciprofloxacin and moxifloxacin (as well as many other antibiotics), could be a risk factor for CDI due to the high resistance of the strains in this study. About 90% of the strains from the most common ribotype 001/072 have MICs for clindamycin and erythromycin > 256 μg/ml.

CONCLUSION: All strains isolated are highly resistant to ciprofloxacin. All strains were susceptible to vancomycin (median MIC was 0.63 μg/ml) and metronidazole (median MIC was 0.084 μg/ml), so these two antimicrobials remain optimal treatment option for CDI.

Introduction

Clostridium difficile is one of the most common causes of infections in hospitalised patients, especially in those with long term hospital stay [1], [2]. Although in many underdeveloped countries, *Clostridium difficile* infection (CDI) was under-diagnosed for a very long time, during the last decade, a significant increase in its prevalence was detected [3]. *C.difficile* infection (CDI) can turn even into hospital outbreak very frequently [4].

Many typing methods are involved in the investigation of the mode of spreading of CDI, but molecular methods are used almost exclusively, due to their higher discriminative power [5]. PCR ribotyping is the most widely used typing method for *C. difficile* in Europe, although some other sequencing-based molecular methods are used in many other countries worldwide [6]. PCR ribotyping was also used for characterisation of the hypervirulent *C. difficile* strains that have caused a large outbreak in Canada in 2002 [6]. Since 2005, the most frequent hypervirulent *C. difficile* strain (027/NAP1/BI) has

been identified in many patients from the USA and Europe [7]. Ribotype 027 is known as a strain which produces toxins A, B and binary toxin, as well as a strain with the increased ability for sporulation and high antimicrobial resistance [9]. In recent years some other hypervirulent strains, like ribotypes 017 and 078, have also emerged. Those two ribotypes have been involved in many serious outbreaks recently, which were quite rare in the past [7], [8], [9], [10].

Metronidazole and vancomycin are the most effective antimicrobial agents for the effective treatment of CDI so far. In the last decade, the emergence of reduced susceptibility to both of these drugs has been reported [11]. It is crucial to monitor the susceptibility of *C. difficile* isolates to antimicrobial agents, not only for selecting the optimal treatment option but also for risk assessment of acquiring CDI in the future. It is well known that using many of the broad-spectrum antimicrobials is a major risk factor for acquiring CDI like antibiotic-associated diarrhoea or pseudomembranous colitis [1].

This study aimed to detect the presence of *C. difficile* ribotypes in hospitalised patients and to investigate their antibiotic susceptibility and their toxigenicity.

Material and Methods

A group of 21 strains of *C. difficile* were isolated from stool samples obtained from patients with symptoms of CDI, hospitalised in different clinics within the Mother Theresa Clinical Centre in Skopje, Macedonia, in the period from 2016 to 2018. All stool samples were tested for glutamate dehydrogenase (GDH) and both *C.difficile* toxins: A and B, with immunochromatographic tests (Mascia Brunelli). Both of these quick tests were performed according to the manufacturer's instructions. The stool samples were also inoculated on selective CCFA agar (Oxoid) and incubated for 48 hours in an anaerobic atmosphere. At the same time, an alcohol shock test was performed with subsequent inoculation on Columbia agar (Oxoid) and incubation under the same conditions. *Clostridium difficile* colonies were identified by their typical appearance and smell. They were further microscopically confirmed by Gram staining. Final confirmation was performed by Vitek 2 (Biomerieux) and MALDI-TOF (Bruker). The 21 *Clostridium difficile* isolates were collected for their further typing and antimicrobial susceptibility testing.

PCR Ribotyping

As a molecular method, PCR ribotyping is based on the amplification of intergenic spacer region (ITS) between 16S and 23S rDNA and was performed as described by Janezic and Rupnik [12], [13[. PCR ribotype profiles of *C. difficile* isolates were analysed by software (Bionumerics, Applied Maths).

Antimicrobial Susceptibility Testing

Antibiotic susceptibility of all *C. difficile* isolates was investigated through the determination of the minimal inhibitory concentrations (MICs) obtained by performing the E test (Biomerieux). One McFarland turbidity standard bacteria suspension was prepared and inoculated on Mueller-Hinton agar supplemented with 5% sheep blood. Antimicrobial strips (Biomerieux) were applied at every single plate, and they were incubated at the same conditions as for the primary isolation. For that purpose, the following antimicrobial agents were used: metronidazole 0.016 - 256 µg/ml, vancomycin 0.016 - 256 µg/ml, tetracycline 0.016 - 256 µg/ml, clindamycin 0.016 – 256 µg/ml, erythromycin 0.016 - 256 µg/ml, imipenem 0.002 - 32 µg/ml, ciprofloxacin 0.002-32 µg/ml and moxifloxacin 0.002-32 µg/ml. The susceptibility of the isolates was analysed according to CLSI M100-S25 and EUCAST v. 8.0, 2018.

Table 1: Interpretation criteria for antimicrobial susceptibility of *C. difficile* isolates

	VAN**	MTZ**	TC*	EM*	CM*	CI*	MX*	IP*
S (µg/ml)	≤ 2	≤ 2	≤ 4	-	≤ 2	≤ 2	≤ 2	≤ 4
I (µg/ml)	-	-	8	-	4	4	4	8
R (µg/ml)	> 2	> 2	≥ 16	≥8	≥ 8	≥8	≥ 8	≥ 16

*Interpretation based on CLSI M100-S25; **Interpretation based on The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 8.0, 2018.

Chi-square and Fisher's exact tests were used for testing the differences between proportions, and p-value less than 0.05 was considered statistically significant.

Isolation of the strains, toxins determination, Vitek 2 confirmations and antimicrobial susceptibility tests were performed at the Institute of Microbiology and Parasitology, Medical Faculty Skopje, Macedonia. MALDI-TOF confirmations and PCR ribotyping were performed at the National Laboratory of Health, Environment and Food in Maribor, Slovenia.

Results

During the study period, 21 *C. difficile* isolates were obtained from hospitalised patients from 7 to 80 years of age. 16 out of 21 *C. difficile* isolates were detected in old patients at the age above 60. 13 (62%) of the patients were female. All isolates were distributed in four different clinics. The total number of *C. difficile* isolates belonged to 11 PCR ribotypes (Table 2 and Figure 1).

Table 2: Distribution of *Clostridium difficile* ribotypes

Clinic	11 different *C. difficile* ribotypes	Number of particular *C. difficile* ribotype	Toxins in *C. difficile* ribotypes
Clinic for pediatric diseases	001/072	1	A and B
	002	1	A and B
	SLO 120	1	A and B
	001/072	3	A and B
Clinic for internal diseases	014/020	1	A and B
	SLO160	1	A and B
	255/258	1	A and B
Surgery clinic with ICU	001/072	5	A and B
	017	1	B only
	001/072	1	A and B
	017	1	B only
Clinic for infectious diseases	027	1	A and B
	046	1	None
	070	1	A and B
	SLO 187	1	A and B

ICU- Intensive care unit.

The relatedness of *C. difficile* isolates is shown on the dendrogram in Figure 1. It presents the similarity of the band's distribution after the electrophoresis.

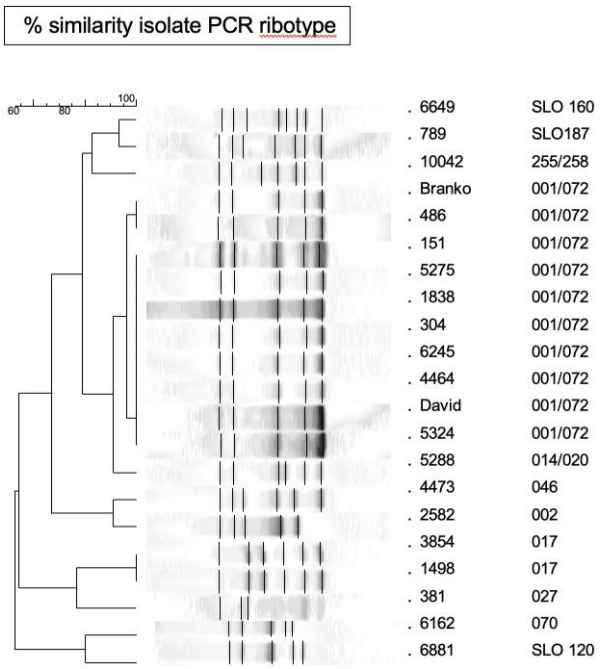

Figure 1: Dendrogramic representation of the twenty-one isolate of Clostridium difficile characterised by PCR ribotyping

Antimicrobial susceptibility of the isolates can be observed by comparing the MICs from Table 3 to the interpretation criteria shown in Table 1.

Table 3: Minimal inhibitory concentrations (μg/ml) of the eight antimicrobials towards the isolates of Clostridium difficile

C. difficile Ribotype	Mtz	Van	Tc	Cm	Em	Ip	Ci	Mx
001/072	0.047	0.75	1.5	> 256	> 256	> 32	> 32	> 32
001/072	< 0.016	1	2	> 256	> 256	> 32	> 32	> 32
001/072	< 0.016	0.38	2	> 256	> 256	> 32	> 32	> 32
001/072	0.094	0.50	0.047	3	1	4	> 32	0.25
001/072	0.047	0.50	2	> 256	> 256	6	> 32	> 32
001/072	0.023	0.38	1.5	> 256	> 256	4	> 32	> 32
001/072	0.094	0.75	1	> 256	> 256	4	> 32	> 32
001/072	0.094	1	3	> 256	> 256	6	> 32	> 32
001/072	0.032	0.50	2	> 256	> 256	4	> 32	> 32
001/072	< 0.016	0.75	2	> 256	> 256	> 32	> 32	> 32
002	0.047	0.38	0.094	6	1.5	> 32	> 32	0.5
014/020	0.094	0.38	0.094	4	0.75	3	>32	0.5
017	0.032	0.38	8	>256	>256	>32	>32	0.5
017	0.023	0.75	8	>256	>256	>32	>32	>32
027	0.047	0.50	0.064	3	>256	>32	>32	>32
046	0.047	0.38	0.064	>256	>256	4	>32	0.38
070	0.25	0.75	0.094	6	1	>32	>32	0.5
SLO120	0.19	0.50	0.125	6	1	4	>32	0.5
SLO160	0.19	1.5	0.125	12	>256	4	>32	0.5
SLO187	0.023	0.50	0.064	4	1	4	>32	0.5
255/258	0.064	0.75	0.094	4	1	3	>32	0.75

Mtz-Metronidazole; Van-Vancomycin; Tc-Tetracycline; Cm-Clindamycin; Em-Erythromycin; Ip-Imipenem; Ci-Ciprofloxacin, Mx-Moxifloxacin.

Discussion

About 76.2% of *C. difficile* isolates were detected in patients above 60 years of age. Older age in patients is a statistically significant risk factor for *C. difficile* infection ($p < 0.05$). The isolation rate of *Clostridium difficile* was not statistically different between male and female patients ($p > 0.05$), which is consistent with many other studies [14]. *Clostridium difficile* infection was identified at all clinics included in the study. This finding is not unusual since most of the patients in these clinics are constantly receiving broad-spectrum antibiotics, which is considered as a major risk factor for acquiring CDI.

C. difficile ribotype 001/072 is the most frequent PCR ribotype in our patients ($p < 0.05$). This finding is similar to the results obtained in the previous study [15]. 50% of *C.difficile* isolates with this ribotype were detected at the Surgery clinic and the Intensive care unit related to it. This high percentage of 001/072 ribotype suggests that it may be an intrahospital ribotype of *Clostridium difficile* at the Surgery clinic. But due to the low number of isolates, this suggestion should be confirmed with the larger number of isolates and by performing additional typing methods in the future. Unlike the results obtained in one of our neighbouring countries based on the same type of investigation [15], [16], where 027 [15] was revealed as the most common *C. difficile* ribotype, in our study this ribotype was detected in one patient only.

All isolates except the one in this study were toxigenic. Non-toxigenic strains are not capable of causing a symptomatic disease [17]. Both *C. difficile* toxins (A and B) were detected in 90% of isolates. Only two isolates belonging to the ribotype 017 were toxin B positive, but toxin A negative. Some authors have emphasised that toxin B can not only act like cytotoxin but also like enterotoxin [18]. So, patients infected with *C. difficile* strains toxin B positive, toxin A negative, could be able to develop identical clinical symptoms as patients infected with *C. difficile* toxin A and B positive strains [19]. *C. difficile* strains positive for toxin A only has not been confirmed so far [20].

Treatments with any of the following antimicrobials: clindamycin, erythromycin, ciprofloxacin and moxifloxacin, could be a risk factor for CDI due to the high resistance of the strains in this study. Ninety per cent of the strains from the most common ribotype 001/072 have MICs for clindamycin and erythromycin > 256 μg/ml. All strains isolated are highly resistant to ciprofloxacin. Resistance to moxifloxacin is somewhat lower, but it is still very common in the most dominant ribotype 001/072. Nine out of ten strains are highly resistant to this antibiotic. All *C. difficile* isolates were susceptible to tetracycline except two of them belonging to the ribotype 017 which revealed intermediate susceptibility.

All strains were susceptible to vancomycin

(median MIC was 0.63 µg/ml) and metronidazole (median MIC was 0.084 µg/ml), so these two antimicrobials remain an optimal treatment option for CDI (Table 2). There is an emergence of resistance of *C. difficile* strains to these two antibiotics globally [11], so it should be necessary to monitor the susceptibility of all the isolates continuously in the future.

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References

1. Kelly CP, LaMont JT. Clostridium difficile - more difficult than ever. N Engl J Med. 2008; 359:1932-40. https://doi.org/10.1056/NEJMra0707500 PMid:18971494

2. Laffan AM, Bellantoni MF, Greenough WB, et al. Burden of Clostridium difficile-associated diarrhea in a long-term care facility. J Am Geriatr Soc. 2006; 54:1068-73. https://doi.org/10.1111/j.1532-5415.2006.00768.x PMid:16866677

3. European Centre for Disease Prevention and Control. Point prevalence survey of healthcare associated infections and antimicrobial use in European acute care hospitals. Stockholm: ECDC; 2013.

4. Birgand G, Blankaert K, Carbonne A, et al. Investigation of a large outbreak of Clostridium difficile PCR-ribotype 027 infections in northern France, 2006-2007 and associated clusters in 2008-2009. Euro Surveill. 2010; 15(25):19597. https://doi.org/10.2807/ese.15.25.19597-en PMid:20587362

5. Killgore G, Thompson A, Johnson S, et al. Comparison of seven techniques for typing international epidemic strains of Clostridium difficile: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J Clin Microbiol. 2008; 46(2):431-7. https://doi.org/10.1128/JCM.01484-07 PMid:18039796 PMCid:PMC2238077

6. Van Steenbergen, J., Debast, S., Van Kregten, et al. Isolation of Clostridium difficile ribotype 027, toxinotype III in the Netherlands after increase in C. difficile-associated diarrhoea. Euro Surveill. 2005; 10(28):2745. https://doi.org/10.2807/esw.10.28.02745-en

7. Hensgens MP, Goorhuis A, Notermans D, et al. Decrease of hypervirulent Clostridium difficile PCR ribotype 027 in the Netherlands. Euro Surveill. 2009; 14. https://doi.org/10.2807/ese.14.45.19402-en PMid:19941791

8. Akerlund T, Persson I, Unemo M, et al. Increased sporulation rate of epidemic Clostridium difficile Type 027/NAP1. J Clin Microbiol. 2008; 46:1530-3. https://doi.org/10.1128/JCM.01964-07 PMid:18287318 PMCid:PMC2292905

9. McDonald LC, Killgore GE, Thompson A, et al. An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med. 2005; 353: 2433-41. https://doi.org/10.1056/NEJMoa051590 PMid:16322603

10. Dawson LF, Valiente E, Wren BW. Clostridium difficile--a continually evolving and problematic pathogen. Infect Genet Evol. 2009; 9:1410-7. https://doi.org/10.1016/j.meegid.2009.06.005 PMid:19539054

11. Barkin JA, Sussman DA, Fifadara N, et al. Clostridium difficile Infection and Patient-Specific Antimicrobial Resistance Testing Reveals a High Metronidazole Resistance Rate. Dig Dis Sci. 2017; 62:1035. https://doi.org/10.1007/s10620-017-4462-9 PMid:28116592

12. Janezic S, M Rupnik. Molecular typing methods for Clostridium difficile: pulsed-field gel electrophoresis and PCR ribotyping. Methods Mol Biol. 2010; 646:55-65. https://doi.org/10.1007/978-1-60327-365-7_4 PMid:20597002

13. Janežič S, Štrumbelj I, Rupnik M. Use of modified PCR ribotyping for direct detection of Clostridium difficile ribotypes in stool samples. Journal of Clinical Microbiology. 2011; 49(8):3024-5. https://doi.org/10.1128/JCM.01013-11 PMid:21632902 PMCid:PMC3147761

14. Esteban-Vasallo MD, Pellicer SN, Domínguez-Berjón MF, Caballero MC, Asensio Á, Saravia G, Astray-Mochales J. Age and gender differences in Clostridium difficile-related hospitalization trends in Madrid (Spain) over a 12-year period. European Journal of Clinical Microbiology & Infectious Diseases. 2016; 35(6):1037-44. https://doi.org/10.1007/s10096-016-2635-7 PMid:27056555

15. Rupnik M, Tambic Andrasevic A, Trajkovska Dokic E, et al. Distribution of Clostridium difficile PCR ribotypes and high proportion of 027 and 176 in some hospitals in four South Eastern European countries. Anaerobe. 2016; 42:142-144. https://doi.org/10.1016/j.anaerobe.2016.10.005 PMid:27751937

16. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, Drudy D, Fitzpatrick F, Wiuff C, Brown DJ, Coia JE. Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. Eurosurveillance. 2008; 13(31):18942.

17. Poxton IR, McCoubrey J, Blair G. The pathogenicity of Clostridium difficile. Clin Microbiol Infect. 2001; 7:421-7. https://doi.org/10.1046/j.1198-743x.2001.00287.x PMid:11591205

18. Pothoulakis C, Lamont JT. Microbes and microbial toxins: paradigms for microbial-mucosal interactions II. The integrated response of the intestine to Clostridium difficile toxins. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2001; 280(2):G178-83. https://doi.org/10.1152/ajpgi.2001.280.2.G178 PMid:11208538

19. Drudy D, Harnedy N, Fanning S, Hannan M, Kyne L. Emergence and control of fluoroquinolone-resistant, toxin A-negative, toxin B-positive Clostridium difficile. Infect Control Hosp Epidemiol. 2007; 28: 932-40. https://doi.org/10.1086/519181 PMid:17620240

20. Lyras D, O'Connor JR, Howarth P, et al. Toxin B is essential for virulence of Clostridium difficile. Nature. 2009; 458:1176-9. https://doi.org/10.1038/nature07822 PMid:19252482 PMCid:PMC2679968