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Original article

DISTRIBUTION OF *CLOSTRIDIOIDES DIFFICILE* RIBOTYPES ISOLATED FROM PATIENTS IN NORTH MACEDONIA - UPDATE

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Abstract

As one of the major hospital pathogens *Clostridioides difficile* strains are constantly a subject of typing. PCR ribotyping is the standard molecular typing method for this bacterium in Europe.

The aim of this study was to determine the distribution of *C. difficile* ribotypes isolated from patients in North Macedonia.

Eighty isolates of *C. difficile*, isolated from the same number of patients` fecal samples being sent to the Institute of Microbiology and Parasitology in Skopje in the period from 2016 to 2020 in order to diagnose *C. difficile* infection, were included.

PCR ribotyping was performed by using primers and protocols described by Bidet. The final determination of the ribotypes was done by using the software BioNumerics.

We determined the presence of 20 ribotypes. The most common ribotype was 001/072 represented with 32 (40%) isolates, followed by 014/020 represented with 10 (12,5%) isolates and ribotypes 002, 017 and 027 represented by 5 (6,2%) isolates each. All other ribotypes were represented by less than 4 isolates. All ribotype 001/072 isolates originated from patients of the "Mother Teresa" Clinical Center.

Unlike in most of the European countries where the hypervirulent ribotype 027 is the dominant one, for a prolonged period of time 001/072 has been the dominant *C. difficile* ribotype isolated from patients in our country. Considering that most of the isolates of this ribotype had originated from Surgical and Internal Diseases Clinics in the "Mother Teresa" Clinical Center, we might assume that it is the endemic *C. difficile* strain there.

Keywords: *Clostridioides difficile,* PCR ribotyping, North Macedonia

Introduction

Clostridioides difficile is one of the major hospital pathogens and the number of healthcare-associated infections are on a constant rise[1].

The primary goal of the typing of *C. difficile* isolates is to follow the spread of the strains in a certain environment. It can be a group of patients in a specific hospital, or it can be a much larger group like a whole population in a specific country or region, but also it is very popular nowadays to follow specific genotypes of *C. difficile* that are found in some animal species[2,3].

In recent years, typing of *C. difficile* isolates has been done almost exclusively with molecular methods[4]. One of the few phenotyping methods that are still being used is the

resistotyping, where the main goal is to detect resistant strains and to determine the optimal therapeutic protocols[5,6].

Many methods of molecular typing of *C. difficile* have been introduced in the last twenty years and all of them differ in their discriminatory power. A large study[4] published a few years ago compared 7 molecular typing methods in terms of their discriminatory power against *C. difficile* strains. The method used in this study, PCR ribotyping, took the fifth place. Molecular typing of the strains of this pathogen "bloomed" with the emergence of hypervirulent epidemic strains like BI/NAP- 1/027. The results of the three then most common typing methods (REA, PFGE and PCR ribotyping) were used to obtain this term in order to eliminate confusion.

After all, PCR ribotyping is still the standard and most commonly used typing method in Europe. Hence, taking into consideration our country's geoposition and the need to survey the distribution and movement of the strains not only within the country, but also in the region, choosing this method was inevitable. This is an opportunity for the potential inclusion of our country in the European distribution map of *C. difficile* ribotypes[7,8].

Over the last 10 years, strains of *C. difficile* from patients isolated at the Institute of Microbiology and Parasitology in Skopje were ribotyped twice[9,10]. Still, the number of isolates was low, and the time frame of their collection was not long enough to make firmer conclusions.

The aim of this study was to determine the distribution of *C. difficile* ribotypes isolated from patients in North Macedonia, which would help in the investigation of the epidemic pathways of these hospital pathogens, and would also result in the inclusion of our country in the European maps representing such data.

Materials and methods

Eighty isolates of *C. difficile*, isolated from the same number of patients` fecal samples sent to the Institute of Microbiology and Parasitology in Skopje in the period from 2016 to 2020, in order to diagnose *C. difficile* infection, were included in this study.

In order to explore them better, the locations where the fecal samples had been sent from, were divided into five groups: Surgical Clinics, Internal Diseases Clinics, Clinic for Children's Diseases, Clinic for Infectious Diseases, as well as samples sent from private health institutions (PHI) and hospitals outside the Clinical Center "Mother Teresa".

Cultivation of the samples: Fecal samples were planted directly on CCFA agar (Oxoid), as well as on Columbia blood agar (Oxoid) after applying the alcohol shock method on the samples. Planted plates were put in anaerobic incubation for 48 hours at 37^{o} C.

Colonies with the characteristic look after the cultivation were finally identified with the automated system Vitek 2 (Biomerieux). Isolates were kept safe for further investigation in form of spores collected on a cotton swab (after subcultivation to a monoculture on a blood agar plate and incubation of five days minimum).

PCR Ribotyping: In *C. difficile* PCR ribotyping is based on amplification of intergenic spacer region (ITS) between 16S and 23S rRNA genes. Since this operon can be found in several copies in *C. difficile* genome and also copies differ in ITS lengths, a pair of primers can produce different combinations of bands ranging from 200 to 700 bp[11,12,13]. These bands can be visualized on agarose gel. Combinations of the bands can be analyzed either visually or by using a specific software. In this study BioNumerics, Applied Maths software is used. It enables analyzing, saving and exchange of data in a standardized way.

PCR ribotyping has been described by many authors[11-14]. Most of the laboratories nowadays are using primers and protocol described by Bidet *et al*.[14], which are also used in this study. This method gives a combination of bands which are comparable. PCR ribotype is

defined as a group of strains with identical combinations of bands. One single band differently positioned means a different ribotype.

A very large collection of strains of various origins is kept at the Anaerobe Reference Center in Cardiff, UK. It consists of more than 200 ribotypes marked with numbers (001, 027, 106…)[15]. In order to improve the interlaboratory reproducibility of this method, sets of such reference strains are being used. Isolates that could not correspond to any of the reference strains ribotypes in this study, were named using internal nomenclature. In our case, such ribotypes are labeled with SLO, using the internal nomenclature of the laboratory in Maribor, Slovenia, where the ribotyping of the isolates took place.

Results

In the period of 2016-2020, 1380 fecal samples from patients suspected of *C. difficile* infection (CDI) were sent to the Institute of Microbiology and Parasitology in Skopje in order to diagnose CDI. *C. difficile* was detected in 182(13%) of samples. According to inclusion criteria for involving one isolate per patient and after discarding the isolates that failed to be subcultivated, 80 *C. difficile* isolates were collected for this study.

These isolates originated from patients from: the Clinic for Infectious Diseases (10 out of 442 sent samples), Clinic for Children's Diseases (7 out of 413 sent samples), Internal Diseases Clinics (31 out of 256 sent samples), Surgical Clinics (22 out of 70 sent samples), and from private health institutions and other hospitals outside the Clinical Center "Mother Teresa" (10 out of 199 sent samples) (Table1).

Table 1. Origin of the isolates

Origin of the fecal samples		Collected isolates	Total samples sent (Percentage of positive samples)
Surgical Clinics	Number	22	70
	%	27.5%	(31.4%+)
Internal Diseases Clinics	Number	31	256
	%	38.8%	(12.1%+)
Clinic for Children's Diseases	Number	7	413
	%	8.7%	(1.7%+)
Clinic for Infectious Diseases	Number	10	442
	%	12.5%	(2.3% +)
PHI and hospitals outside Clinical Center "Mother Theresa"	Number	10	199
	%	12.5%	(5%+)

After PCR ribotyping had been done, we detected the presence of 20 ribotypes: 001/072, 002, 003, 005, 012, 014/020, 015, 017, 023, 027, 046, 070, 255/258, SLO 046, SLO 047, SLO 069, SLO 110, SLO 120, SLO 160 and SLO 187 (Table 2).

The most common ribotype was 001/072 represented with 32 (40%) isolates, followed by 014/020 represented with 10 (12,5) isolates and ribotypes 002, 017 and 027 represented by 5 (6,2%) isolates each. Ribotypes 005, 255/258, SLO 046 and SLO 047 were represented by 3 (3,7%) isolates each. All other ribotypes were represented by one isolate (1.2%) only.

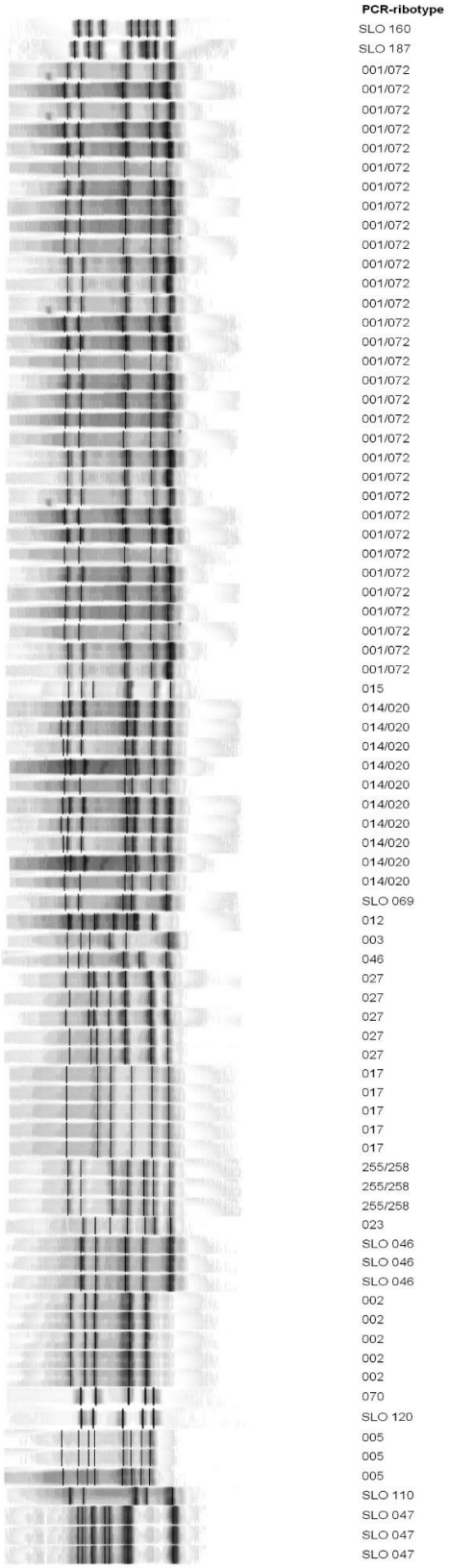

Fig. 1. Dendrogramic representation of the detected ribotypes from eighty isolates of *C. Difficile*

Table 2. *C. difficile* ribotypes detected

Ribotypes of C. difficile		Number (N)	%
1	001/072	32	40.00%
2	002	5	6.25%
3	003	1	1.25%
4	005	3	3.75%
5	012	1	1.25%
6	014/020	10	12.50%
7	015	1	1.25%
8	017	5	6.25%
9	023	1	1.25%
10	027	5	6.25%
11	046	1	1.25%
12	070	1	1.25%
13	255/258	3	3.75%
14	SLO 046	3	3.75%
15	SLO 047	3	3.75%
16	SLO 069	1	1.25%
17	SLO 110	1	1.25%
18	SLO 120	1	1.25%
19	SLO 160	1	1.25%
20	SLO 187	1	1.25%
Total		80	100%

The analysis of *C. difficile* ribotypes was made in terms of the place of origin of the fecal samples, considering the 5 defined locations mentioned before, as well as 6 groups of detected ribotypes (Table 3).

Table 3. Distribution of detected *C. difficile* ribotypes

Origin of the fecal samples		Ribotypes					
		001/072	014/020	002	017	027	[1]others
Surgical Clinics	Number	16	2	0	2	0	2
	%	50%	20%	0%	40%	0%	8.70%
Internal Diseases Clinics	Number	13	1	4	2	4	7
	%	40.63%	10%	80%	40%	80%	30.43%
Clinic for Children's Diseases	Number	1	0	1	0	0	5
	%	3.13%	0%	20%	0%	0%	21.74%
Clinic for Infectious Diseases	Number	2	5	0	0	0	3
	%	6.25%	50%	0%	0%	0%	13.04%
PHI and hospitals outside Clinical Center "Mother Theresa"	Number	0	2	0	1	1	6
	%	0%	20%	0%	20%	20%	26.09%
Total	Number	32	10	5	5	5	23
	%	40%	12.50%	6.25%	6.25%	6.25%	28.75%

[1]others= 003, 005, 012, 015, 023, 046, 070, 255/258, SLO 046, SLO 047, SLO 069, SLO 110, SLO 120, SLO 160, SLO 187

As illustrated in Table 3, the analysis of the distribution of detected *C. difficile* ribotypes showed the following:

- Ribotype 001/072 - half of the isolates belonging to this ribotype or 16(50%) originated from samples sent from Surgical Clinics, followed by 13(40.6%) originating from Internal Diseases Clinics. One isolate originated from the Clinic for Children's Diseases and two from the Clinic for Infectious Diseases. In none of the samples sent from PHI and hospitals outside the Clinical Center "Mother Teresa" this ribotype was detected.

- Ribotype 014/020 - 5(50%) detected isolates of this ribotype originated from samples from the Clinic for Infectious Diseases, followed by 2(20%) from Surgical Clinics and 1 from Internal Diseases Clinics. It can be seen that that two (20%) of this group of isolates originated from PHI and hospitals outside the Clinical Center "Mother Teresa".
- Ribotype 002 - Eighty percent of the isolates from this ribotype (total of 4) were detected from samples from Internal Diseases Clinics, followed by 1 isolate from the Clinic for Children's Diseases. In samples from other defined locations, this ribotype was not detected.
- Ribotype 017 - Equal amount or 2 each (40% each) from this ribotype isolates originated from samples from Surgical Clinics and from Internal Diseases Clinics. One isolate from this ribotype was also found in the samples received from PHI and hospitals outside the Clinical Center "Mother Teresa".
- Ribotype 027 - 4(80%) isolates of this ribotype originated from samples from Internal Diseases Clinics. The remaining isolate (20%) from ribotype 027 was collected from samples from PHI and hospitals outside Clinical Center "Mother Teresa".
- All other less common ribotypes (003, 005, 012, 015, 023, 046, 070, 255/258, SLO 046, SLO 047, SLO 069, SLO 110, SLO 120, SLO 160, SLO 187) were analyzed in a group. Isolates belonging to this group of ribotypes were collected from samples from all defined locations: 7(30.4%) from Internal Diseases Clinics, 6(26.1%) from PHI and hospitals outside the Clinical Center "Mother Teresa", 5(21.7%) from the Clinic for Children's Diseases, 3(13%) from the Clinic for Infectious Diseases and 2(8.7%) from the Surgical Clinics.

Discussion

The number of fecal samples from CDI suspected patients that had been sent from each of the five defined locations in this study was not proportional to the number of collected isolates. The reason for this might be that at the Clinic for Infectious Diseases and the Clinic for Children's Diseases diarrheal syndromes with various etiology are much more common. Therefore, the number of fecal samples sent from those clinics is much larger than the number of isolates. However, the big difference between *C. difficile* isolation percentages from the samples sent from the Clinic for Infectious Diseases (2.3%) and the Clinic for Children's Diseases (1.7%), compared to those from the Surgical Clinics (31.4%), implied certain selectivity in sending the samples and insufficient awareness of CDI, thus resulting in subdiagnosis of this infection.

PCR ribotyping of all eighty isolates of *C. difficile* isolated at the Institute of Microbiology and Parasitology, Faculty of Medicine in Skopje, between 2016 and 2020, revealed that at least 20 ribotypes had been circulating in our country in that period. We can indeed notice that ribotype 001/072 is the most dominant. Knowing that in the past (2013-2014) and (2015-2016) isolates of this bacterium from our country were ribotyped[9,10], and that we had almost similar results, we can now assume that these findings are not unexpected. Namely, ribotype 001/072 is quite common in European countries, more precisely it is the second most frequent among isolates in a large pan-European study[7] found in 11%.

Apart from North Macedonia, this ribotype is also dominant in neighboring Bulgaria, which implies a possible geographical connection of the strains, but it is also prevalent in more distant countries on the opposite borders of Europe, such as Spain, Finland and Slovakia[7].

Although very common, this ribotype has been mentioned in the group of so-called hypervirulent ribotypes; however, in some studies it is designated as an epidemic strain[16]. In our study, the vast majority (90%) of the isolates from ribotype 001/072 originated from patients hospitalized at the Surgical Clinics and Internal Diseases Clinics, with 16 and 13 cases, respectively. None of the isolates from this ribotype originated from patients outside

the Clinical Center "Mother Teresa" (fifth group). This, as well as the fact that it was the most common ribotype over an extended period of more than 5 years, suggests that this strain may be endemic in the Clinical Center "Mother Teresa" in Skopje. To prove such a claim, much more extensive studies are needed. For example, data are also needed on the distribution of *C. difficile* ribotypes among strains isolated from inanimate environmental surfaces in the "Mother Theresa" Clinical Center.

The second most common ribotype in this study was 014/020 represented with 10 (12.5%) isolates. This ribotype is in third place in the above-mentioned pan-European study, with 10 % of the isolates. It is the most common one in France and Sweden[7]. Half of the isolates of the ribotype 014/020 in our study originated from patients of the Clinic for Infectious Diseases. Unlike the dominant ribotype 001/072, this ribotype was isolated from patients outside the Clinical Center "Mother Theresa" in 20% of cases.

The hypervirulent ribotype 027, which is the dominant one in Europe with 19% of isolates in the last large pan-European study[7] and in North America and Australia[8], in our study was found in 5 isolates (6.24%). This ribotype, after the large epidemics it caused in the USA and Canada (after which it was studied in detail), was detected only in some Western European countries[7]. In recent years, it has been prevalent in Central and Eastern Europe. It is the most common ribotype in Germany, Austria, Romania and Poland[7]. In another study[9], the results showed that in neighboring Serbia, between 2011 and 2015, out of more than 100 examined *C. difficile* isolates, ribotype 027 was determined in more than 90%. Fortunately, in our country this percentage is much smaller. In our study 4(80%) of the isolates belonging to this ribotype originated from the Internal Diseases Clinics and one isolate originated from a patient outside the "Mother Teresa" Clinical Center.

In some studies[17,18] ribotype 017 has been noted as the dominant one in Asia. In a pan-European study from 2013[7], it is not one of the prevalent ones, although in some articles[19] it is recognized as the dominant one. In our study 5(6,25%) of the isolates belonged to 017 and 4 of these originated from patients from Surgical and Internal Diseases Clinics (2 each).

Ribotype 002 was also found in 5 isolates in this study. It is characterized with a greater sporulation ability according to some studies[20]. It is also one of the more common types in Europe, but still, it is not the dominant one in any country[7]. Majority (80%) of the isolates from this ribotype in our study originated from the Internal Diseases Clinics.

All other ribotypes detected in this study were represented by less than five isolates. In this group of 23 isolates, the largest percentage (30%) originated from the Internal Diseases Clinics, and even 26% originated from patients outside the "Mother Teresa" Clinical Center, which was a significantly higher percentage than in any other group of ribotypes.

It can be noted that ribotype 078, which in some studies has been designated as hypervirulent and which is also the most common one isolated from domestic and wild animals and from retail meat[21-23], was not detected in our study. This ribotype is one of the most common types in neighboring Greece[7].

Conclusion

Ribotype 001/072 is the dominant *C. difficile* ribotype isolated from patients in our country over a long period of time. Considering that most of the isolates of this ribotype originated from Surgical and Internal Diseases Clinics in the "Mother Teresa" Clinical Center, we might assume that it is the endemic *C. difficile* strain in our largest clinical center. However, to prove such a claim requires ribotyping of *C. difficile* isolates isolated from the non-living environment at the same location. PCR ribotyping is a very useful typing method

for collecting epidemiological data to determine local and global spreading pathways of this nosocomial pathogen.

Conflict of interest statement. None declared.

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