Journal of Medical Microbiology (2016), **65**, 1158–1163 DOI 10.1099/jmm.0.000334

Distribution of PCR ribotypes among recent *Clostridium difficile* isolates collected in two districts of Hungary using capillary gel electrophoresis and review of changes in the circulating ribotypes over time

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Received 23 May 2016
Accepted 16 August 2016

Following the first description of a *Clostridium difficile* case caused by ribotype 027 in Hungary in 2007, the rapid spread of *C. difficile* infection in different hospitals within the country was observed. The aim of this pilot study was to investigate the distribution of different PCR ribotypes among inpatient and outpatient isolates obtained in two geographically different parts of Hungary. One hundred and ninety-two toxigenic *C. difficile* isolates collected between 1 October and 1 December 2014 were PCR ribotyped using capillary gel electrophoresis and the database of WEBRIBO (http://webribo.ages.at), which allows the automatic analysis and comparison of capillary-sequencer-based PCR ribotyping data. Altogether, 31 different known ribotypes were found, and 16 isolates showed a novel banding pattern, not included in the current library. Besides the dominance of 027 (33.3 %) among all isolates, there were differences in its presence among isolates obtained from the two regions (45.8 % in the central region and 20.8 % in the south-east region, respectively), whereas the second most prevalent ribotype 036 (19.8 %) was more frequently found among isolates obtained in the south-east region compared with the central region of Hungary (29.1 versus 10.4 %). Similar differences in the spread of different ribotypes, in particular 027, which were found during earlier studies in Hungary may be due to the existing order for admissions of patients to hospitals. We also summarized the changing pattern of PCR ribotypes of Hungarian *C. difficile* isolates over time, based on earlier published data.

INTRODUCTION

Since the acceptance of *Clostridium difficile* as the main cause of antibiotic-associated diarrhoea and pseudomembranous colitis in 1978 (Bartlet *et al.,* 1978), its importance has increased worldwide. It has been accepted now as the most common aetiological agent of hospital-acquired diarrhoea, but it causes infection in outpatient settings as well. To follow the epidemiology of *C. difficile* infection, initially typing methods were applied mainly based on phenotypic characteristics, such as antibiotic resistance, PAGE of soluble proteins, bacteriophage and bacteriocin patterns, slide agglutination and Western blotting (Wurst *et al.,* 1982). With the development of genotypic methods, such as restriction endonuclease analysis of the total bacterial genome, PFGE and arbitrary primed PCR, the possibility of using typing data to provide increased discrimination between isolates or provide support for a common source has increased (Brazier *et al.,* 1997). With the emergence and spread of the B1/NAP1/027 *C. difficile* strain in North America and Europe, the importance of using typing methods, which give comparable results for local or more general spread of the hypervirulent strain, has increased. Several genotypic methods such as PCR ribotyping, amplified fragment length polymorphism, multilocus sequence typing, multiple locus variable-number tandem repeat analysis and surface layer protein A sequence typing were compared for

Abbreviation: CE, capillary gel-based electrophoresis.

applicability in routine or reference laboratories for discrimination of strains by Killgore *et al.* (2008). Their conclusion was that all these methods can detect outbreak strains within a hospital; however, inter-institutional strain tracking and intra-typic discrimination were only possible with restriction endonuclease analysis and multiple locus variable-number tandem repeat analysis (Killgore *et al.,* 2008). From the above-mentioned methods, PCR ribotyping, using the classical agarose gel-based method, has been adopted in many reference laboratories in Europe to differentiate banding patterns, as the method of choice for *C. difficile* typing and surveillance (Cartwright *et al.,* 1995; Stubbs *et al.,* 1999). Indra *et al.* (2008) published the development of a high-resolution capillary gel-based electrophoresis (CE) PCR ribotyping method to provide comparable data for different laboratories and overcome the problems associated with inter-laboratory comparison of the results of strain typing by the classic agarose gel-based method. With the widespread use of agarose gel-based and CE ribotyping in European reference laboratories, it is now possible to perform Europe-wide surveillance studies to evaluate the spread of the PCR ribotype 027 (and others) in different European countries (Barbut *et al.,* 2007; Freeman *et al.,* 2010, 2015; Bauer *et al.*, 2011). A number of studies have reported on the prevalence of 027 and other ribotypes in Eastern European countries as well (Terhes *et al.*, 2006; Rafila *et al.,* 2014; Drabek *et al.*, 2015; Krutova *et al.,* 2015; Nyc *et al.*, 2015; Pituch *et al.*, 2015).

The aim of the present study was to evaluate the PCR ribotypes of recent toxigenic *C. difficile* strains obtained from diarrhoeal faeces in two geographically different parts of Hungary using CE-based PCR ribotyping.

METHODS

Isolates. A total of 192 toxigenic *C. difficile* strains were analysed in this study. The strains were isolated from diarrhoeal faeces from inpatients (154 isolates) and outpatients (38 isolates) in two large laboratories, Synlab Hungary (Budapest) and the Institute of Clinical Microbiology, University of Szeged, between 1 October and 1 December 2014. Strains collected in Budapest were derived mostly from different hospitals in Budapest (29 isolates); however, 41 further isolates were collected from inpatients of various hospitals situated in the central and the northern parts of Hungary. The inpatient isolates (84) in Szeged were collected from nine different departments of the University Hospital of Szeged. Only toxigenic strains were included in this study, as demonstrated by a positive result using the QUIK CHEK COMPLETE (TechLab) immunochromatography-based method evaluated visually after 10 min incubation time at room temperature. All strains were grown on Schaedler agar with vitamin K1 and 5 % sheep blood (Becton-Dickinson) at 37 °C for 24 h under anaerobic conditions (GasPack Jar; Becton-Dickinson). Limited demographic data were collected, such as age of the patients and whether they were diagnosed with diarrhoea in an inpatient or an outpatient setting.

DNA preparation. A single colony was selected for DNA preparation after 24 h anaerobic subculture of the isolates. DNA preparation for PCR detection of *cdt*A, *cdt*B and the binary toxin genes of selected isolates and for ribotyping of all 192 isolates was carried out as described earlier by Stubbs *et al.* (1999). Briefly, the cells were suspended in a 5 % (w/v) solution of Chelex-100 (Bio-Rad) in molecular grade distilled water in 1.5 ml Eppendorf tubes. The solutions were incubated in boiling water for 12 min and then centrifuged at 15 000 ***g*** for 10 min. The supernatant was removed, placed in a fresh tube and stored at −20 °C until use.

CE ribotyping was carried out at the Austrian Agency for Health and Food Safety (Vienna, Austria) as previously described by Indra *et al.* (2008). Briefly, primers targeting 16S and 23S rDNA (VBC-Biotech) were used as described by Bidet *et al.* (1999), with the modification that the 16S rDNA primer was labelled at the 5′ end with carboxyfluorescein. The PCR ribotyping method exploits the presence of polymorphisms in the 16S–23S rDNA intergenic spacer region. Analysis of the PCR fragments was performed using an ABI 3130 genetic analyser with a 41 cm capillary loaded with POP7 gel and the GeneScan 1200 LIZ Dye Size Standard (Thermo Fisher). The size of each peak was calculated using PEAK SCANNER software 1.0 (Applied Biosystems). Data obtained were analysed according to the web-based database (http://webribo.ages.at) created for CE-based PCR ribotyping results by Indra *et al.* (2008).

Detection of toxin genes by PCR. The method described by Terhes *et al.* (2004) was used for detection of the major toxin genes *tcdA* and *tcdB* and for detection of the binary toxin genes *cdtA* and *cdtB* for two isolates from this study collected in Szeged and two further toxigenic *C. difficile* strains isolated in 2011 also from patients in Szeged for an earlier European study.

RESULTS AND DISCUSSION

In this study, *C. difficile* strains were collected in two large laboratories, representing central and south-East Hungary, to determine the distribution of the ribotypes of *C. difficile* during a limited period of collection time (1 October to 1 December 2014). Among 192 recent toxigenic *C. difficile* strains, altogether 31 different ribotypes were found in the WEBRIBO database at the time of the investigation (first half of 2015) (Table 1). The most frequently found ribotypes were 027, 036, 014 and 176 (33.3, 19.7, 6.7 and 6.7 % of all isolates, respectively). Six isolates were the same as the known Austrian ribotypes designated as AI-3, AI-12, AI-75 and AI-83, and 16 isolates (8.3 %) were considered as 'novel' ribotypes, as their pattern does not fit any known ribotype pattern in the WEBRIBO. No further study was done for designation of these new ribotypes. Most of the isolates, 141 (73.4 %), originated from patients aged >61 years and were dominated by ribotypes 027 and 036 (52 and 33 isolates, respectively).

Table 2 shows the distribution of the four dominant ribotypes among inpatient and outpatient isolates obtained in Budapest and Szeged. The ribotype 027 (45.8 %) was the most frequently found ribotype among the isolates collected in Budapest, followed by ribotypes 176 and 036. In Szeged, the highest number of isolates belonged to ribotype 036 (29.1 %), followed by 027 (20.8 %). Other ribotypes represented with only one to three isolates were much more frequent among isolates obtained in Szeged (30.2 %) compared to those isolated in the laboratory in Budapest (19.8 %).

Since the emergence of hypervirulent, fluoroquinolone-resistant *C. difficile* 027 in the USA and Canada followed by its distribution in different European countries, interest in isolation and typing of *C. difficile* strains has increased tremendously; however, very few data were available for a long

Table 1. Distribution of *C. difficile* ribotypes by age group

Ribotypes	Age groups									
	3–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81 to >90	Total
002	0	0	0	0	0	0	0	1	0	1
003	0	0	0	0	0	0	0	1	0	1
010	1	0	0	0	0	0	0	0	0	1
012	1	0	0	0	0	1	0	1	0	3
014	**1**	**0**	**1**	**2**	**0**	**1**	**4**	**3**	**1**	**13**
018	0	0	0	0	2	0	0	0	0	2
020	0	0	0	0	0	0	0	1	2	3
027	**0**	**1**	**1**	**2**	**4**	**4**	**16**	**19**	**17**	**64**
036	**0**	**0**	**1**	**1**	**2**	**1**	**6**	**14**	**13**	**38**
056	0	0	0	0	0	0	1	0	0	1
066	0	0	0	0	0	1	0	0	0	1
070	0	0	0	0	0	0	0	0	1	1
078	0	0	0	0	0	0	0	1	1	2
087	0	0	2	0	0	0	0	0	0	2
126	0	1	0	0	0	0	0	1	1	3
176	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**6**	**2**	**13**
203	1	0	1	0	1	0	1	2	1	7
209	0	1	0	0	0	0	1	0	0	2
400	0	0	0	0	1	0	0	0	0	1
430	0	0	0	0	0	0	0	0	2	2
449	0	0	0	0	1	0	1	0	1	3
456	0	0	0	0	0	0	0	1	0	1
484	0	0	0	0	0	1	0	0	0	1
541	0	0	0	0	0	0	0	0	1	1
591	1	0	0	0	0	0	0	0	0	1
653	0	0	0	1	0	0	0	0	0	1
698	0	1	0	0	0	0	0	0	0	1
AI*	0	0	1	1	0	0	1	0	3	6
'New'†	**2**	**0**	**0**	**1**	**0**	**1**	**1**	**7**	**4**	**16**
Sum	7	5	7	9	12	11	33	58	50	192

*AI-3, AI-12, AI-75, AI-83.
†Not found in the database WEBRIBO at the time of the study.

time from Eastern European countries including Hungary. Earlier, as in many countries, only classical agarose gel-based ribotyping was used in different studies for differentiating *C. difficile* isolates in our country (Urban *et al.,* 2001; Terhes *et al.,* 2004, 2006, 2009a). The first data on the ribotyping of *C. difficile* isolates from one centre in Hungary were published in 2001 (Urban *et al.,* 2001) (Table 3). According to classical agarose gel-based typing results, the 65 toxigenic strains belonged to 15 ribotypes; besides the 14 known ribotypes dominated by 087, only one, at that time 'new', ribotype was found with no formal recognition later. The second opportunity to test 83 toxigenic isolates collected from four sites in Hungary yielded 17 known and 3 'new' ribotypes not included at that time in the database in Cardiff (Terhes *et al.,* 2006). Compared to the previous study (Urban *et al.,* 2001), the dominating ribotypes were different and two binary toxin gene positive strains were also found, which belonged to ribotypes 023 and 075. The first Hungarian 027 *C. difficile* strain was isolated in 2007 (Terhes *et al.,* 2009b), together with seven other binary toxin gene positive strains, which belonged to ribotypes 078 and 131 (Terhes *et al.,* 2009a). Unfortunately not all the 120 toxigenic isolates were ribotyped at that time, only those which were binary toxin gene positive (Table 3).

In 2010 and 2011 – rather late compared to Western European countries – the hypervirulent *C. difficile* 027 started to spread in different hospitals throughout Hungary. The National Reference Laboratory for Anaerobes, dealing also with the confirmation and testing of *C. difficile* isolates at that time, was located at the Institute of Clinical Microbiology of the University of Szeged. The laboratory was not financially able to carry out full ribotyping for all isolates in those years; only determination of their toxin gene status

Table 2. Distribution of dominant ribotypes of *C. difficile* strains isolated from inpatients and outpatients at two centres in Hungary

Ribotypes	No. of *C. difficile* strains isolated in					
	Budapest			Szeged		
	Inpatients	Outpatients	Total (%)	Inpatients	Outpatients	Total (%)
014	2	0	2 (2.1)	9	2	11 (11.4)
027	36	8	44 (45.8)	18	2	20 (20.8)
036	10	0	10 (10.4)	27	1	28 (29.1)
176	6	7	13 (13.5)	0	0	0
Others*	9	10	19 (19.8)	24	5	29 (30.2)
'New'†	7	1	8 (8.3)	6	2	8 (8.3)
Sum	70	26	96	84	12	96

*002, 003, 010, 012, 018, 020, 056, 066, 070, 078, 087, 126, 203, 209, 400, 430, 449, 456, 484, 541, 591, 653, 698, AI-3, AI-12, AI-75, AI-83.
†Not found in the WEBRIBO database at the time of the study.

was carried out. All the binary toxin gene positive isolates were typed by comparing the agarose gel banding patterns to the PCR ribotype 027 reference strains (Lume 1 and Lume 11) obtained from the laboratory of Ed Kuijper (Table 3). In 2010, of the 601 toxigenic *C. difficile* isolates sent to the reference laboratory from eight different regions of Hungary, 30.4 % of the strains proved to be 027. Four other binary toxin positive isolates were found belonging to other, undetermined, ribotypes. In 2011, 699 isolates were tested in a similar way, obtained from 11 different laboratories, and 50.2 % of the isolates proved to belong to 027. The ribotype of the seven other binary toxin positive isolates was not determined (Nagy, 2014). Interestingly, in both years, there were considerable differences in the prevalence of ribotype 027 isolates among the toxigenic strains obtained from different regions of the country. In Szeged, only 4 % of 106 and 8 % of 139 isolates belonged to ribotype 027 in 2010 and 2011, respectively, whereas in Budapest,

Table 3. PCR ribotypes of Hungarian *C. difficile* isolates over time

Period of strain collection	No. of collection sites	No. of toxigenic isolates	No. of known ribotypes/'new' ribotypes	Dominant ribotypes (%)	No. of binary toxin positive strains (ribotypes)*	Reference
<2000	1	65	14/1	087 (38.4) 012 (20.0) 001 (12.3)	0	Urban *et al.* (2001)
2002–2004	4	83	17/3	014 (24.8) 002 (13.3) 012 (8.4)	1 (023) 1 (075)	Terhes *et al.* (2006)
2006–2007	4	120	NT	NT	1 (027) 4 (078) 3 (131)	Terhes *et al.* (2009a)
	8	601	NT	027 (30.4)	183 (027) 4 (other ribotypes)	Nagy (2014)
	11	699	NT	027 (50.2)	351 (027) 7 (other ribotypes)	Nagy (2014)
2011–2012	3	75	14/0	027 (70.2) 198 (10.6)	53 (027) 8 (198) 2 (078)	Freeman *et al.* (2015)
2012–2013	10	270	Not known	027 (67 %)	181 (027)	Davies *et al.* (2015)
2014	2	192	31/16	027 (33.3) 036 (19.7) 176 (6.7) 014 (6.7)	64 (027) 38 (036) 2 (078) 1 (066)	Present study

NT, Not tested.
*Determined by PCR detection of the binary toxin gene or accepted according to the published toxinotype of the ribotype.

40 % of 282 and 86 % of 252 isolates proved to be this ribotype, showing huge differences in the spread of the hypervirulent strain in the different regions of Hungary during that period (not published).

In 2011–2012, three laboratories from two different regions of Hungary participated with 75 *C. difficile* isolates in a pan-European surveillance study on antibiotic resistance to *C. difficile,* and during this study the distribution of the ribotypes was also determined (Freeman *et al.,* 2015). Fourteen different known ribotypes were found among the Hungarian isolates with a very high dominance of ribotype 027 (70.2 %) based on the CE-based ribotyping method (Table 3). Most of the strains belonging to this ribotype were obtained from the central region of Hungary, 44 of 50 isolates (88 %); however, only 9 of 25 isolates (36 %) collected in Szeged were ribotype 027. The second most frequent ribotype was 198 (8/25; 32 %) among the strains collected in Szeged (personal communication from the central laboratory for this study, in Leeds).

In 2012–2013, during an Europe-wide point prevalence study, data and samples were collected during one day in winter and one day in summer. In this study, 10 hospitals (mostly situated in Budapest) collected 270 *C. difficile* isolates, and Hungary was among the four countries with Germany, Poland and Romania, where the highest number of 027 isolates was found. Of the 270 isolates collected in Hungary, 181 (67.0 %) belonged to this ribotype (Davies *et al.,* 2014).

In the present study, our aim was to collect isolates during a limited time frame (1 October to 1 December 2014) in two large laboratories serving two regions in the country and to evaluate the prevalence of ribotypes among different age groups, inpatients and outpatient isolates; and to determine regional differences in the distribution of ribotypes using CE-based ribotyping. Besides the existing dominance of 027 (33.3 %) among all isolates, similar to previously observed trends, there was a remarkable difference in its presence among *C. difficile* strains isolated in Budapest from patients in several hospitals of the city and the region and among strains isolated from patients in different departments of the University Hospital Szeged (45.8 and 20.8 %, respectively). The ribotype called 036 according to the WEBRIBO was the second most frequent *C. difficile* ribotype (19.8 %) among all isolates; however, again there was a significant difference in the prevalence of this ribotype isolated in Budapest compared with those isolated in Szeged (10.4 versus 29.1 %, respectively). Besides these two dominant ribotypes, a wide range of different other ribotypes was found, which were represented by one to three isolates, including some which were regularly found in Austria (AI-3, AI-12, AI-75 and AI-83). PCR ribotype 014, found in 6.7 % of all *C. difficile* isolates in this study, has frequently been reported to have been recovered also in France (Barbut *et al.,* 2007), whereas ribotype 176, closely related to 027 (Valiente *et al.*, 2012), the second most frequent ribotype among the *C. difficile* isolates from Budapest, but absent in Szeged in this study (Table 2), has been reported to be dominant among *C. difficile* strains isolated from patients in the Czech Republic (Krutova *et al.,* 2015) and to be present also in Poland (Pituch *et al.,* 2015). Ribotype 036, which was the second most prevalent ribotype among *C. difficile* strains in this study and the leading ribotype among the inpatient isolates from Szeged, was originally described as belonging to toxinotype X as a toxin A-negative, toxin B-positive and binary toxin-positive *C. difficile* strain based on the detection of genes by PCR (Rupnik *et al.,* 2001). In a study published recently about the development of an international library, obtained by standardized CE-based ribotyping and validated by four reference centres in the USA, Canada, The Netherlands and UK, Fawley *et al.* (2015) reported about a possible mix-up between ribotypes 036 and 198 in different libraries. Furthermore, Valiente *et al.* (2012) using the classical agarose gel-based PCR ribotyping showed that ribotypes 176 and 198 have only slight variations in banding patterns compared to 027, and presumed that they have evolved recently from PCR ribotype 027. These literature data led us to look more closely for the presence of toxin genes of two representatives of ribotype 036 from this study (isolates that may be different from those described earlier as representatives of the toxin A-negative, toxin B-positive toxinotype) and two ribotype 198 isolates from Szeged included in the Europe-wide surveillance in 2011–2012 and being the second most common isolate at that time in Szeged (personal communication from the central laboratory for this study, in Leeds). All four strains were positive for the binary toxin genes as well as for *tcdA* and *tcdB* genes, suggesting the possible circulation of the same or very closely related ribotypes in the different departments of the University Hospital, Szeged, over time.

The last 5 years has seen increasing acceptance, worldwide of CE-based PCR ribotyping as the method of choice to follow the epidemiology of *C. difficile* infection, its spread in hospitals or in the community, as well as among animals, and to harmonize the nomenclature throughout Europe, preferably globally (Indra *et al.*, 2008; Kentsch *et al.,* 2013; Fawley *et al.,* 2015). While the first library of *C. difficile* ribotypes set up by the classical agarose gel-based analysis of banding patterns differentiated 116 ribotypes (Stubbs *et al.,* 1999), today we acknowledge the existence of >650 ribotypes (Fawley *et al.,* 2015). The great diversity of the PCR ribotypes of *C. difficile* isolates today and the permanent evolution of new ribotypes not present in the internationally accepted WEBRIBO (http://webribo.ages.at), or in the CE-based consensus library (Fawley *et al.,* 2015), which has recently been used in different Europe-wide studies, makes it problematic to follow epidemiologically important changes. Further studies are needed to determine whether the differences in the distribution of the *C. difficile* ribotypes among isolates in central or south Hungary observed in both present and earlier studies are real differences, showing epidemiologically important variations within the country. Furthermore, harmonization of the various *C. difficile* ribotype libraries is warranted.

ACKNOWLEDGEMENTS

We thank G. Terhes and H. Juhász for PCR detection of the toxin genes of the selected *C. difficile* isolates.

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