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Distribution of *Clostridium difficile* PCR ribotypes in regions of Hungary

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The objective of this survey was to determine the distribution of *Clostridium difficile* PCR ribotypes present across three Hungarian geographical regions. A total of 105 isolates of *C. difficile* from diarrhoeal faeces of both inpatients and outpatients were examined. The toxigenic status of the strains was determined by PCR for the *tcdA*, *tcdB*, *cdtA* and *cdtB* genes in Szeged (Hungary), while strains were subjected to PCR ribotyping in Cardiff (UK). A total of 31 ribotypes were detected among the 105 *C. difficile* isolates tested. Five PCR ribotypes were distinct from all previously described types, suggesting that they are new. The most common types in Hungary, during the period examined, were PCR ribotype 014 (24·8 %) and PCR ribotype 002 (13·3 %). The distribution of PCR ribotypes differed in the various Hungarian regions: PCR ribotype 012 was frequent (20·7 %) in South Hungary, whereas this type was rare in the Budapest region and was not common to West Hungary. In West Hungary and the Budapest region, PCR ribotype 014 was most frequent (28·9 and 29 %, respectively).

INTRODUCTION

Numerous surveys have revealed that the most frequent nosocomial enteritic pathogen is toxin-producing *Clostridium difficile* (Barbut *et al.*, 1995; McFarland, 1995; Spencer, 1998). The wide application and, in many cases, the misuse of different antibiotics has led to an increasing number of cases of *C. difficile*-associated disease, primarily in the hospital environment (Bartlett, 1992; Brown *et al.*, 1990; Spencer, 1998). At the same time, community-acquired infections are probably underdiagnosed, and few reports deal with this problem (Barbut *et al.*, 2005; Noren *et al.*, 2004; Riley *et al.*, 1995). Because of the emergence of new toxin variant strains, and the spread of toxin A-positive and toxin B-positive strains in hospitals and the community, further investigations of these strains may be important for elucidating the exact role of toxin variant strains in the pathogenesis of disease and for controlling the spread of these bacteria.

Numerous molecular typing methods, such as arbitrarily primed-PCR, RFLP, PFGE and PCR ribotyping, have been used internationally to study *C. difficile* strains of different origins (Brazier, 2001). Most of these typing methods are suitable for following outbreaks, determining recurrences, characterizing endemic strains and tracing the spread of *C. difficile*. PCR ribotyping is a method that is widely used (Rotimi *et al.*, 2003; Stubbs *et al.*, 1999; Urbán *et al.*, 2001) to determine the intraspecies genetic variation of *C. difficile*, and in which the 16S and 23S rRNA intergenic spacer region can be amplified by using specific primers under stringent amplification conditions. The sizes of the amplification products can range from 250 to 600 bp (Brazier, 2001).

In an earlier study in Hungary in collaboration with the Anaerobe Reference Laboratory (Cardiff), 65 *C. difficile* isolates were typed using PCR ribotyping (Urbán *et al.*, 2001). The aim of the present study was to extend this investigation and to determine the distribution of PCR ribotypes in three Hungarian regions.

METHODS

C. difficile **strains and detection of toxin genes.** A total of 105 *C. difficile* isolates from diarrhoeal faeces at Hungarian laboratories in the towns of Szeged, Szolnok (South Hungary), Budapest (Budapest region) and Veszprém (West Hungary), collected between 2002 and 2004, were included in the study. Of the 105 isolates, 65 were obtained from inpatients, and 40 from outpatients. A total of 99 isolates were selected from our previous study (Terhes *et al.*, 2004), in which the main toxin genes and binary toxin genes were analysed, and 6 other toxigenic strains were chosen from our strain collection. The toxin A and B genes (*tcdA* and *tcdB*, respectively), and the binary toxin genes (*cdtA* and *cdtB*), were detected by PCR. PCR was also used to determine the deletion at the 3′ end of the *tcdA* gene (Kato *et al.*, 1991, 1998; Stubbs *et al.*, 2000). These analyses were performed at the Hungarian Anaerobe Reference Laboratory, Szeged. In these cases, the *tcdA/tcdB*-positive control strain was the reference strain VPI 10463 (toxinotype 0), which was obtained from the American Type Culture Collection (Rockville,

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Maryland, USA), while the binary toxin-producing control strains R5989 (ribotype 023), R8637 (ribotype 019) and R10456 (ribotype 058) were part of the collection of the Anaerobe Reference Laboratory, Cardiff, UK.

Culturing conditions and extraction of DNA. For PCR ribotyping, strains were grown on Fastidious Anaerobe Agar (LabM); the plates were incubated at 37 °C for 24 h under anaerobic conditions. After checking the colony morphology, fluorescence under UV light and the purity of the cultures, the cells were resuspended in a 5 % (w/v) solution of Chelex-100 (Bio-Rad). 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 4 °C for 12–24 h.

PCR ribotyping. Amplification reactions were performed in a 100 μl volume containing 50 pmol each primer (16S rRNA gene primer p3 5′-CTG GGG TGA AGT CGT AAC AAG G-3′, position 1445–1466, and 23S rRNA gene primer p5 5′-GCG CCC TTT GTA GCT TGA CC-3′, position 1–20), 200 μM dNTP (Pharmacia), 10 mM Tris/HCl (pH 8·8), 50 mM KCl, 1·5 mM $MgCl_2$, 1 U *Taq* polymerase (Pharmacia), and 10 μl template DNA. PCR was carried out in a thermal cycler, for 1 cycle of 120 s at 95 °C, for starting denaturation, and 30 cycles of denaturation at 92 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 90 s. After the last denaturation step, annealing was carried out at 55 °C for 45 s, and extension at 72 °C for 5 min (Stubbs *et al.*, 1999).

Detection of the PCR products. Amplification products were concentrated to a final volume of 25–30 μl in a heat-block set at 75 °C for 1·5 h. PCR products were detected by electrophoresis in 3 % (w/v) metaphor agarose gels (BMA-BioWhittaker Molecular Applications) for 3 h, at 200 V and 60 mA in TAE buffer [40 mM Tris-acetate, 1 mM EDTA (pH 8·0)]. The DNA banding pattern was visualized under UV light after staining for 20 min in ethidium bromide (0·5 μg ml^{-1}). A molecular mass ladder (Superladder-Low; ABgene) was included every six wells on the gels for normalization of the gel patterns. PCR ribotype profiles were analysed with GelCompar image analysis software (version 4.0; Applied Maths).

RESULTS AND DISCUSSION

Of the 105 *C. difficile* isolates originating from diarrhoeal faeces examined, 83 carried *tcdA* and *tcdB* genes, while 22 were *tcdA/B*-negative. These 22 non-toxigenic strains were isolated from adults aged between 29 and 79 years, except for 5 isolates that originated from children aged less than 3 years. In these cases, diarrhoeal faeces were tested for the presence of other common enteritic pathogens, such as enteropathogenic *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, adenoviruses and rotaviruses. All of these screening tests were negative. No toxin A-negative and toxin B-positive isolates were recovered. A total of 23 different ribotypes were detected, and a comparison with the patterns recorded in the *C. difficile* library of PCR ribotypes in the Cardiff type culture collection revealed 5 new ribotypes, termed PCR ribotypes 161, 162, 164, 165 and 166 (Fig. 1). During the period examined, the most prevalent types among the Hungarian isolates overall were PCR ribotypes 014 (24·8 %), 002 (13·3 %) and 085 (8·6 %). Most of the toxin-negative strains belonged to PCR ribotype 085 (9/22 isolates). The distribution of the ribotypes differed in the three geographical regions: in the Budapest region the dominant types were ribotypes 014, 002 and 018

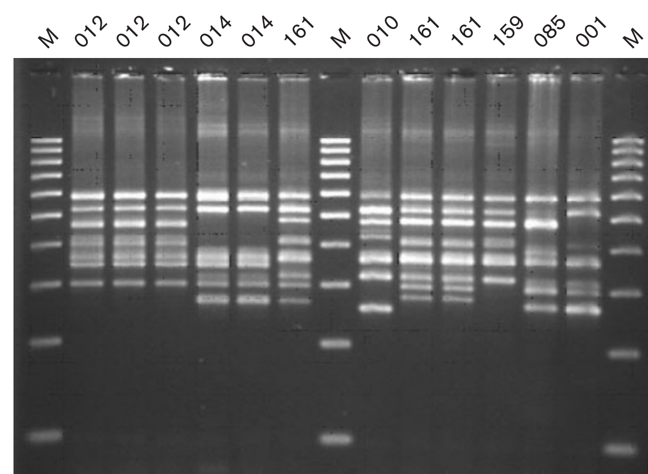

Fig. 1. A representative example of PCR ribotyping patterns of *C. difficile*. M, molecular size marker (Superladder-Low; ABgene). Ribotypes 012 and 014 were characteristic ribotypes during the isolation period, ribotype 161 is a new type, ribotypes 010 and 085 are toxin-negative isolates, ribotype 001 is a toxin-positive isolate and ribotype 159 is a control from the Cardiff strain collection.

(29, 19·4 and 12·9 %, respectively), whereas 014 (28·9 %), 049 (11·1 %), 085 (8·9 %) and 002 (8·9 %) were most frequent in West Hungary, and ribotypes 012 (20·7 %), 002 (13·8 %) and 014 (13·8 %) were characteristic of South Hungary. Type 049 (11·1 %) was present only in West Hungary, while ribotype 005 (9·7 %) was detected only in the Budapest region, and ribotype 035 (6·9 %) was observed only in South Hungary (Table 1). The presence of binary toxin-producing strains was demonstrated in West Hungary and the Budapest region, but not in South Hungary. The frequencies of the various ribotypes were compared between inpatients and outpatients. When the two-sided Fisher's exact test was used, the differences were not significant except in one case (ribotype 012), which was more prevalent among isolates obtained from inpatients.

In 2001, 65 *C. difficile* isolates from the University Hospital of Szeged were typed in cooperation with the Anaerobe Reference Laboratory (Cardiff) by PCR ribotyping. In that survey, 57 strains were isolated from diarrhoeal faeces and 8 from different clinical specimens, e. g. wound, bile and intra-abdominal samples. The strains examined belonged to 15 different ribotypes; the most frequent type, ribotype 087, accounted for 39 % of all the strains (Urbán *et al.*, 2001). During the present study this ribotype was found less frequently (one isolate in South Hungary and one in West Hungary). The results from the local university hospital in 2001 and the results of the present survey indicate changes in the prevalence and distribution of the various *C. difficile* ribotypes.

These data suggest that the distribution of *C. difficile* may change over time and geographically. We have shown that

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Table 1. Distribution of PCR ribotypes in three Hungarian regions

Ribotype	Toxin A/B	Binary toxin	Budapest region	West Hungary	South Hungary	Total
001	+/+	−	2	1	0	3
002	+/+	−	6	4	4	14
005	+/+	−	3	0	0	3
009	−/−	−	0	1	1	2
010	−/−	−	0	3	2	5
011	+/+	−	0	2	0	2
012	+/+	−	1	0	6	7
013	+/+	−	0	1	0	1
014	+/+	−	9	13	4	26
018	+/+	−	4	1	0	5
020	+/+	−	0	0	1	1
023	+/+	+	1	1	0	2
035	−/−	−	0	0	2	2
039	−/−	−	0	1	0	1
046	+/+	−	0	1	0	1
049	+/+	−	0	5	0	5
072	+/+	−	0	1	0	1
075	+/+	+	1	0	0	1
078	+/+	−	0	0	1	1
085	−/−	−	2	4	3	9
087	+/+	−	0	1	1	2
128	−/−	−	0	1	0	1
150	+/+	−	1	1	1	3
161	+/+	−	1	0	2	3
162	+/+	−	0	1	0	1
164	−/−	−	0	1	0	1
165	+/+	−	0	1	0	1
166	−/−	−	0	0	1	1

there can be regional differences within a country. A number of ribotyping surveys in various countries have also demonstrated different distributions of ribotypes. For example, type 001 was detected in the majority of UK hospital infections (55 %), while only comprising 7·5 % of strains causing community-acquired infections in England and Wales (Stubbs *et al.*, 1999). In Kuwait, PCR ribotypes 097 and 078 accounted for ~40 % of all isolates in intensive-therapy units (Rotimi *et al.*, 2003).

In 2001, binary toxin-producing isolates were not observed among strains originating from Szeged University Hospital, whereas binary toxin genes were detected in three isolates between 2002 and 2004. The presence of toxin was also proven using Western blotting. Two of the three binary toxin-positive strains were isolated and identified from severe community-acquired diarrhoea after antibiotic therapy in West Hungary and the Budapest region (Terhes *et al.*, 2004), and PaLoc analysis showed that the strains belonged to toxinotypes III and IV, which produce toxins A and B. These isolates caused severe diarrhoea, but the exact role of the binary toxin in pathogenesis has not yet been clarified. Some clinical studies suggest that the binary toxin can be an additional virulence factor that may enhance the severity of infection (Barbut *et al.*, 2005).

During the past 10 years, numerous molecular typing methods have been developed to differentiate nosocomial *C. difficile* strains. Some of these methods can provide useful information, especially during hospital outbreaks. In each case, the most important expectations are that the methods should be discriminative, reproducible, simple and rapid. PFGE is accepted as the gold-standard technique for the typing of bacterial strains, but the majority of *C. difficile* isolates cannot be typed because of the degradation of the DNA. Moreover, this method is rather labour-intensive and time-consuming (Hyett *et al.*, 1997; Kato *et al.*, 1996). PCR ribotyping is one of the most frequently used methods for the epidemiological investigation of *C. difficile*. The present study compared the ribotypes of *C. difficile* strains from three parts of Hungary with the results of earlier international and national ribotyping studies. Both regional and international differences were observed in relation to the distribution of various ribotypes, and changes in the prevalence of the different ribotypes over time were also apparent. Because of the increasing numbers of hospital infections and outbreaks caused by *C. difficile* the introduction of typing methods in more countries is essential for establishing a database relating to the spread of the most frequent epidemic *C. difficile* strains. Such a database would be of great help in the future demonstration of

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outbreaks, and in the distinction between recurrences and reinfections.

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