EPIDEMIOLOGY AND BACTERIAL TYPING

PCR ribotyping of clinically important *Clostridium difficile* strains from Hungary

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Isolates of *Clostridium difficile* from different hospital wards at the University Hospital of Szeged in Hungary were typed by PCR amplification of rRNA intergenic spacer regions (PCR ribotyping). A total of 15 different ribotypes was detected among the 65 isolates tested. The predominant type, PCR ribotype 087, accounted for 39% of all isolates, in contrast with an international typing study where ribotype 001 was the most common. Two non-toxigenic *C. difficile* strains were found to exhibit the same pattern, which was distinct from those of all the ribotypes described previously, suggesting that this is a new type.

Introduction

Clostridium difficile, an obligately anaerobic gram-positive spore-forming rod, is a common nosocomial enteric pathogen world-wide. It causes pseudomembranous colitis (PMC), antibiotic-associated diarrhoea (AAD) or colitis (AAC) [1, 2]. The spectrum of these diseases ranges from uncomplicated mild diarrhoea to lethal toxic megacolon or colon perforation, or both [3, 4]. Since the 1980s, several studies have documented the nosocomial acquisition of *C. difficile*. Some hospital wards have a high rate of colonisation by these micro-organisms. Despite major efforts to control the spread of *C. difficile*-associated diarrhoea (CDAD) in health-care facilities, this pathogen remains a problem world-wide that continues to be responsible for both endemic and epidemic nosocomial diarrhoea [5–8]. *C. difficile* infections have become a considerable problem in most European countries, including Hungary [5–8]. The laboratory isolation and identification of nosocomial *C. difficile* strains have necessitated the development of typing methods to provide a better follow-up of the epidemiology of the disease and to afford a better insight into the pathogenicity of various strains. Typing methods have been developed on the basis of various phenotypic and genotyping markers. The phenotypic markers include toxin production, antimicrobial resistance patterns, bacteriocin and bacterio-

phage susceptibility patterns and whole-cell protein electrophoretic patterns, including ³⁵S methionine-labelled protein patterns [9–11]. Immunological markers have been described for serotyping based on the Western blot method and serogrouping by slide agglutination [12–14]. Although serotyping and phage typing are simple and useful methods, only a few laboratories can perform them because these methods require the maintenance of stocks of sera and specific phages. It has been shown that phage typing may be difficult to use because of the frequent lysogeny of *C. difficile* strains [10]. Isolates have also been fingerprinted by pyrolysis mass spectrometry (PMS), according to the profiles of the volatile products [15, 16]. Although this is a rapid technique, the disadvantage of PMS is that results for isolates that have been processed in different batches cannot be compared directly. Epidemiological markers based on genotypic characteristics are generally more discriminating and specific than phenotypic markers. Plasmid profiles, DNA restriction endonuclease and ribosomal rRNA restriction patterns have been used in the fingerprinting of *C. difficile* [17–19]. O'Neill and colleagues [20] developed a modification of the PCR ribotyping method based on polymorphisms in the 16S–32S rRNA intergenic spacer region for the routine typing of *C. difficile*. This part of the genome has been shown to be very heterogeneous, in contrast to the rRNA genes themselves, which are highly conserved. *C. difficile* was shown to possess up to 10 or more copies of the rRNA genes in its genome, which varied not only between strains, but also between different copies on the same genome. This modified PCR ribotyping

Received 20 Feb. 2001; revised version accepted 11 June 2001.

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method offers several advantages over other methods and appears to hold good promise for the typing of wild isolates of *C. difficile*.

The aim of this study was to determine the most common ribotypes of *C. difficile* in the 1200-bed University Hospital of Szeged, Hungary, and compare them with those found in the PHLS Anaerobe Reference Unit in Cardiff, UK.

Materials and methods

Bacterial isolates

Of the 65 *C. difficile* isolates investigated in this study, 57 were isolated from diarrhoeal faecal samples and 8 from other clinical materials (Table 1). Three reference strains were included as controls: *C. difficile* NCTC 11382 (toxin A-positive, toxin B-positive), *C. difficile* CCUG 20309 (toxin A-negative, toxin B-positive) and *C. difficile* NCTC 11206 (toxin A-negative, toxin B-negative). Isolation and identification of *C. difficile* were performed according to standard methods.

Toxin detection

Single colonies were subcultured on pre-reduced Columbia Agar Base (Oxoid) supplemented with cattle blood 5%, vitamin K₁ and haemin and incubated at 37°C under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, OR, USA) for 24 h for toxin A detection. The production of toxin A by isolates of *C. difficile* was determined by an ELISA method (ToxA TEST Immunoassay; TechLab, BioConnections, Leeds, W. Yorkshire). Toxin B was detected by cytotoxicity assay on Vero cells. The cytopathic effects of filtered chopped-meat glucose broth culture supernates (diluted 1 in 10 – 1 in 10⁴) on Vero cells were used to detect toxin B. All toxin B-positive samples were neutralised with specific *C. difficile* goat antitoxin.

PCR ribotyping method

Isolates were cultured anaerobically overnight on Fastidious Anaerobe Agar (FAA; LabM, Bury) supplemented with horse blood 6% in anaerobic conditions. Approximately 10 colonies were picked and crude template nucleic acid was prepared by resuspending the cells in Chelex-100 (BioRad, Hemel Hempstead) 5% w/v and boiling for 12 min. After the removal of cellular debris by centrifugation (15 000 g for 10 min), 10 µl of supernate were added to 90 µl of PCR mixture containing 50 pmol of each primer, Taq polymerase (Pharmacia) 2 U and 2.25 mM MgCl₂. Reaction mixtures were subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. Amplification products were concentrated to a final volume of 25 µl by heating at 75°C for 90 min before electrophoresis at 100 mA and 200 V in Metaphor agarose (FMC Bioproducts,

Table 1. Origin of isolates from the University Hospital of Szeged and their PCR ribotypes

Origin of strains	Clinical material	Number of isolates	Number of isolates belonging to PCR ribotype														
			001	002	009	010	012	015	032	040	068	070	087	092	114	124	New type
Ward																	
Internal medicine	Faeces	17	2		1		3			1	1		8		1		
Paediatrics	Faeces	17	5		1	1	3		1				4			1	
Surgery	Faeces	6		1	2							1	2				
	Drain	3					1						1				1
	Wound	1											1				
	Bile	1	1														
Haematology	Faeces	5											3			1	
Dermatology	Faeces	1											1				
Ophthalmology	Conjunctiva	1				1											
Neurology	Faeces	1											1				
Cardiac surgery	Faeces	1					1										
ICU	Faeces	9					4						4				
	Wound	1					1							1			
	Intra-abdominal	1						1									

Control strains NCTC 11382, CCUG 20309 and NCTC 11206 belonged to PCR ribotypes 043, 036 and 038, respectively.

Rockland, ME, USA) 3% for 4.5 h at 8°C. Products were visualised by staining the gel for 20 min in ethidium bromide 0.5 µg/ml. To enable normalisation of all gel patterns, a molecular size standard (100 bp; Advanced Biotechnologies, Epsom) was run at five-lane intervals [20].

PCR ribotyping uses specific primers complementary to the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene to amplify the variable-length intergenic spacer region. The oligonucleotide primers used were 5'-CTG GGG TGA AGT CGT AAC AAG G-3' (positions 1445–1466 of the 16S rRNA gene) and 5'-GCG CCC TTT GTA GCT TGA CC-3' (positions 20–1 of the 23S rRNA gene) [20].

Gel images were analysed with GelCompar image analysis software (version 4.0; Applied Maths, Kortrijk, Belgium). The criterion for the proposal of a new library type was the existence of clearly discernible, reproducible differences in PCR ribotype pattern from those of all other existing types.

Results

The sources and PCR ribotypes of the isolates are listed in Table 1. All the isolates originated from different patients in various wards at the University Hospital of Szeged. Most of them were hospitalised in the Internal Medicine, Paediatrics and Surgery Departments and Intensive Care Unit.

In-vitro toxin testing of the isolates revealed that there were both toxigenic and non-toxigenic isolates in the samples. During a period of 3 months, 57 *C. difficile* strains were isolated from 252 faecal samples; 44 of them (77%) were toxin-producing, as detected by an

immunoassay for the detection of *C. difficile* toxin A and cytotoxicity on Vero cells (toxin B). Six (75%) of the eight clinical isolates from samples other than faeces collected during the same period were toxin-producing. Toxin A-negative, toxin B-positive strains were not isolated during this period.

The PCR ribotypes consisted of patterns comprising 3–12 bands, with the size of the bands varying from *c.* 250 to 600 bp (Fig. 1). A total of 15 different ribotypes was detected among the 65 isolates tested (Table 2) [12, 21]. The 50 toxigenic isolates (77%) could be classified into seven visually distinct ribotypes, and the 15 non-toxigenic isolates into eight PCR ribotypes. Of the 50 toxigenic isolates tested, 46 (92%) belonged to three PCR ribotypes (087, 012 and 001); type 087 was the most common ribotype, accounting for 50% (25 of 50) of the toxigenic isolates tested. The remaining four isolates (8%) belonged to four other ribotypes. There was a wider distribution of ribotypes among the non-toxigenic isolates. A total of 15 toxin-negative isolates was investigated and ribotype 009 was isolated most frequently (4 of 15). Two non-toxigenic isolates belonging to ribotype 010 were isolated from conjunctiva and from the faeces of a newborn infant. This PCR ribotype is frequently isolated from the faeces of neonates and small children.

There was no significant correlation between the PCR ribotypes and the origin of the isolates investigated in this study as regards wards or patients' rooms. Some of the *C. difficile* isolates originating from different wards of the University Hospital of Szeged in Hungary belonged to the same ribotype as *C. difficile* isolates collected in the UK by the Anaerobe Reference Unit in Cardiff. All but two of the Hungarian isolates could be typed by this PCR ribotyping method, the exceptions were non-toxigenic isolates (one from faeces and one

Fig. 1. PCR ribotype profiles found most frequently in Hungarian isolates of *C. difficile*. Lane L, 100-bp ladder; 1, 2, ribotype 015; 3, 8, ribotype 087; 4, 5, 7, 9, ribotype 012; 6, ribotype 010; 10–13, ribotype 001.

Table 2. Distribution of PCR ribotypes among Hungarian *C. difficile* isolates

PCR ribotype	Reference strain of the ribotype*	Number (%) of isolates	Production of toxin		Serogroup [†] of the reference strain	Toxino-type [‡]
			A	B		
001	R8366	8 (12.3)	+	+	G	0
002	R8375	1 (1.5)	+	+	A2	0
009	R8269	4 (6.3)	–	–	I	
010	R8270	2 (3.0)	–	–	D,E6	
012	R6187(S51)	13 (20.0)	+	+	C,A,G,K	0
015	R6685(S38)	1 (1.5)	+	+	G	0
032	R6598	2 (3.0)	–	–	UT	
040	R100917	1 (1.5)	–	–	UT	
068	IS56	1 (1.5)	–	–	UT	
070	R9367	1 (1.5)	+	+	K	I
087	R11840	25 (39.0)	+	+	G	0
092	R10630	1 (1.5)	+	+	UT	0
114	R11212	1 (1.5)	–	–	UT	
124	R11919	2 (3.0)	–	–	UT	
New type	–	2 (3.0)	–	–	UT	

UT, untypable.

*Ribotype reference strains maintained in the Anaerobe Reference Unit in Cardiff, UK.

[†]Serogroup of the reference strains has been made to Delmée serotype [12].[‡]Toxino-type of the reference strains was determined according to methods described previously [21].

from intra-abdominal drainage) (Table 2). These two isolates exhibited the same pattern, which was distinct from those of all the ribotypes described previously, suggesting that it is a new type. More investigations are required to classify it with the cluster correlation algorithm. The integrity of the library was tested with control *C. difficile* strains: *C. difficile* NCTC 11382, ribotype 043, was used as a toxigenic control strain. The non-toxigenic control strain was *C. difficile* NCTC 11206, which belonged to PCR ribotype 038, and the control toxin A-negative, toxin B-positive strain, CCUG 20309, belonged to ribotype 036.

Discussion

PCR ribotyping has recently been proposed as an effective means of studying *C. difficile* epidemiologically [22–24]. The PHLS Anaerobe Reference Unit in Cardiff routinely uses this method [20]. It provides a typing service for referred isolates and, from >2000 strains examined, a library consisting of 116 distinct ribotypes has been constructed. In total, 54 different PCR ribotypes have been identified from hospital patients. Type 001 accounts for 68% of the total of all isolates from hospital patients in England and Wales [25]. Type strains within the library have also been analysed by other typing schemes and the PCR ribotyping method correlates with other typing schemes and allows subtyping of many of the types produced by other methods (Table 2) [25].

The International Typing Study (involving seven groups of experts from the UK, Belgium, Australia and the USA) in 1997 organised by Brazier [26], revealed that certain types were more common in each country, indicating the distribution of the same types in hospitals in these countries. There are only a few

publications about the genetic relationship of pathogenic strains of *C. difficile* from various other parts of the world, especially with regard to the Eastern European countries. In Eastern Europe there has been only one study, in a Polish maternity hospital [27], where arbitrarily primed PCR and PCR ribotyping methods were used. All environmental isolates and 11 of 31 neonatal isolates were of a single type, type 1. There have been attempts to establish some form of standardisation in the nomenclatures to describe strains typed by the various study groups. At present these various types are uncoordinated and there is a lack of understanding as to how types relate to one another. The Polish ribotyping results have not been compared to the reference library of the Anaerobe Reference Unit in Cardiff and it could not be determined which PCR ribotype corresponds to their type 1.

The present study compared the ribotypes of 65 *C. difficile* isolates originating from patients in Szeged, Hungary, with the library of *C. difficile* ribotypes in the Anaerobe Reference Unit in the UK. Although this sample size is small, the isolates originating from Hungarian inpatients display a very different distribution of PCR ribotypes from that found by the Anaerobe Reference Unit in the UK. The most predominant ribotype in the Hungarian survey of 65 isolates was PCR ribotype 087, a toxigenic type, which accounted for 39% of all isolates, in contrast with the results of the international typing study where its prevalence was much lower. This type was not common in England and Wales either; only eight isolates of this type were found among several hundred investigated isolates [25]. The present study found two non-toxin-producing isolates, of a previously unrecognised type. No significant correlation was found between the distribution of the PCR ribotypes and the origin of the isolates during this period. Although no data have been published on the

prevalence of *C. difficile* infection or the epidemiology of *C. difficile*-associated diarrhoea in Hungary, the isolation of toxigenic *C. difficile* from hospitalised patients suggests that this pathogen may be responsible for certain cases of diarrhoea of undiagnosed origin and validates our efforts to establish its significance and conduct epidemiological studies in Hungary.

This work was supported by a grant from Hungarian Eötvös Scholarship and by grant no. T032385 from the Hungarian National Research Foundation (OTKA).

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