Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes

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Toxinotyping and PCR ribotyping are two methods that have been used to type *Clostridium difficile* isolates. Toxinotyping is based on PCR-RFLP analysis of a 19 kb region encompassing the *C. difficile* pathogenicity locus. PCR ribotyping is based on comparison of patterns of PCR products of the 16S–23S rRNA intergenic spacer region. Representative strains (101) from a *C. difficile* PCR ribotype library and 22 strains from previously described toxinotypes were analysed to compare ribotyping with toxinotyping. Within this panel of strains all 11 toxinotypes (0–X) described previously and an additional 5 novel toxinotypes (XI–XV) were observed. PCR ribotyping and toxinotyping correlated well and usually all strains within a given ribotype had similar changes in toxin genes. The new toxinotype XI comprises strains that did not express toxins TcdA or TcdB at detectable levels, but contained part of the *tcdA* gene. Strains of toxinotype XII exhibit changes only in the 5' end of the *tcdB* gene. Toxinotype XIV is composed of strains that have a large insertion at the beginning of the *tcdA* gene. A total of 25 of the 89 tested PCR ribotypes of *C. difficile* contained variant strains. It was estimated that they represent 7.7% of the total number of strains in the Anaerobe Reference Unit collection.

Keywords: *Clostridium difficile*, toxinotyping, PCR ribotypes, *tcdA*⁺*B*[−] strains, variant toxin genes

INTRODUCTION

Clostridium difficile is the aetiological agent of pseudo-membranous colitis (PMC) and a major cause of nosocomial antibiotic-associated diarrhoea (AAD) (Knoop *et al.*, 1993; Riley, 1998). *C. difficile* infections may be sporadic, but outbreaks are common and a number of typing schemes have been developed for epidemiological analysis (Riley, 1994). Phenotypic typing methods have included analysis of bacteriophage, bacteriocin and antibiotic susceptibility, PAGE protein profiling and serotyping (Delmée *et al.*, 1985, 1986; Sell *et al.*, 1983; Burdon *et al.*, 1979). Initial molecular methods involved plasmid analysis and whole-genome restriction analysis (Mulligan *et al.*, 1988; O'Neill *et al.*, 1991). More recently, arbitrarily primed PCR (AP-PCR;

Barbut *et al.*, 1993; van Dijck *et al.*, 1996), PFGE (Collier *et al.*, 1996; van Dijck *et al.*, 1996) and PCR ribotyping of the 16S–23S rRNA intergenic spacer (O'Neill *et al.*, 1996; Stubbs *et al.*, 1999) have become the methods of choice. An international *C. difficile* typing study is currently comparing seven different techniques (Brazier *et al.*, 1997). The application of different typing methods has revealed that *C. difficile* is a heterogeneous species, consisting of several groups, some of which are commonly associated with disease or have caused particular outbreaks (Delmée *et al.*, 1985; van Dijck *et al.*, 1996).

Two toxins, enterotoxin A (TcdA) and cytotoxin B (TcdB), have been recognized as the main virulence factors of *C. difficile*. These toxins belong to the group of large clostridial cytotoxins (LCT) and are characterized by a high molecular mass (> 250 kDa) and the ability to glycosylate small GTPases from the Rho and Ras subfamilies (von Eichel-Streiber *et al.*, 1996). The enterotoxin and cytotoxin genes, *tcdA* and *tcdB*, and three additional genes (*tcdC*, *tcdD* and *tcdE*) are

Abbreviations: UPGMA, unweighted pair group method with arithmetic averages.

arranged within a well-defined chromosomal region termed the toxinogenic element (Hammond & Johnson, 1995) or pathogenicity locus (PaLoc; Braun *et al.*, 1996).

Variant strains of *C. difficile* were first recognized because of a failure to produce TcdA (Borriello *et al.*, 1992; Lyerly *et al.*, 1992; Depitre *et al.*, 1993). Subsequent analysis of toxin genes has revealed that two groups of these TcdA⁻B⁺ strains exist; the first group is represented by an atypical strain (8864 or CCUG 20309) and the second group includes strains from serogroups F and X (Borriello *et al.*, 1992; Lyerly *et al.*, 1992; Depitre *et al.*, 1993; Rupnik *et al.*, 1997; Soehn *et al.*, 1998; von Eichel-Streiber *et al.*, 1999). Recently, a PCR-RFLP method (known as toxinotyping) has been developed to analyse changes in the PaLoc and to type strains of *C. difficile* (Rupnik *et al.*, 1998). This method has indicated that, in addition to TcdA⁻B⁺ strains, certain TcdA⁺B⁺ strains also differ from a representative strain of *C. difficile* (VPI 10463) in the PaLoc. These variant strains have been assigned to 10 toxinotypes (I–X), whilst strains with a PaLoc similar to strain VPI 10463 have been assigned to toxinotype 0 (Rupnik *et al.*, 1998).

The toxinotyping method correlates well, but not entirely, with conventional serotyping (Rupnik *et al.*, 1998), and the aim of the present study was to compare toxinotyping with PCR ribotyping. In addition, a large PCR ribotype library (Stubbs *et al.*, 1999) of *C. difficile* strains was screened in an attempt to obtain further information on the prevalence of variant strains and the emergence of novel toxinotypes.

METHODS

Bacterial strains. One hundred and twenty-three *C. difficile* strains were analysed in the present study. Twenty-two strains from the collection of M. Delmée (UCL, Brussels, Belgium) contained representative strains of the different toxinotypes (Rupnik *et al.*, 1998). Nineteen of these strains were of unknown PCR ribotype, whilst three strains (8864, 1470 and 7701) had been ribotyped previously during the international *C. difficile* typing study (Brazier *et al.*, 1997). An additional 101 strains, belonging to 7 non-toxinogenic and 79 toxinogenic PCR ribotypes, were obtained from the *C. difficile* library (Stubbs *et al.*, 1999) of the Public Health Laboratory Service (PHLS) Anaerobe Reference Unit (ARU). This library consisted of 116 ribotypes at the time of the study, but we omitted the majority of ribotypes including nontoxinogenic strains. Only seven representative nontoxinogenic ribotypes were selected, whose ribotype banding pattern was related to ribotypes with variant strains.

Growth conditions and toxin production. Strains were grown on Fastidious Anaerobe Agar (FAA; Lab M) supplemented with 6% horse blood in an anaerobic atmosphere at 37 °C. Production of TcdA and TcdB was determined by the ToxA TEST immunoassay (TechLab, BioConnections) and by Vero cell cytotoxicity assay (Brazier, 1993).

PCR ribotyping. Crude template nucleic acid was prepared by resuspension of cells harvested from FAA in a 5% (w/v) solution of Chelex-100 (Bio-Rad) and boiling for 12 min. Cell debris was removed by centrifugation (15000 g for 10 min) and PCR ribotyping was performed according to methods

documented previously (O'Neill *et al.*, 1996; Stubbs *et al.*, 1999). Profiles were analysed with GelCompar image analysis software (version 4.0; Applied Maths) and dendrograms were produced with the unweighted pair group method with arithmetic averages (UPGMA) and fine alignment (Stubbs *et al.*, 1999).

Toxinotyping. Initially, strains were screened for variation in the first 3 kb of *tcdB* (PCR fragment B1, Fig. 3) and a 3 kb fragment spanning the repetitive region of *tcdA* (PCR fragment A3) by amplification and subsequent digestion with restriction enzymes *HincII*, *AccI* and *EcoRI*, as described previously (Rupnik *et al.*, 1998). For strains that exhibited changes in these regions, the entire PaLoc was amplified in ten overlapping fragments (Rupnik *et al.*, 1998): B1 (3 kb), B2 (2 kb) and B3 (2 kb) for *tcdB*; A1 (3 kb), A2 (2 kb) and A3 (3 kb) for *tcdA*; PL1 and PL2 for sequences upstream of *tcdB*; PL3 for the intergenic region between *tcdA* and *tcdB*; and PL4 for the region downstream of *tcdA*. Toxinotyping (PCR-RFLP) of these amplification products was performed as described previously (Rupnik *et al.*, 1998).

16S rDNA sequencing. 16S rRNA genes were amplified with the universal primers pA and pH (Hutson *et al.*, 1993). Amplification products were cleaned with QIAquick-spin PCR clean-up columns (Qiagen) and a 500 bp region at the 5' end of the gene was sequenced with primer KK (Hutson *et al.*, 1993) using the ABI-PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer). Sequences were compared to those in the EMBL database with BLASTN (dispatch1@ncbi.nlm.nih.gov) and analysed further using DNASIS (Hitachi Software).

RESULTS

Ribotyping and toxinotyping of isolates

In the present study we investigated two groups of strains. Twenty-two strains from the Brussels collection representing different toxinotypes (Rupnik *et al.*, 1998) were ribotyped, whereas 101 strains of known ribotype from the ARU Cardiff collection were toxinotyped.

Out of the 22 isolates from the Brussels collection, 17 isolates were assigned to 6 known PCR ribotypes (012, 019, 023, 063, 066, 075) and 5 strains from toxinotypes I, II and III were designated new PCR ribotypes 080, 102 and 103 (Table 1).

One hundred and one strains from the ARU PCR ribotype library belonged to 86 PCR ribotypes. Six strains representing 6 nontoxinogenic ribotypes were confirmed not to contain toxin genes, whereas two strains from the seventh nontoxinogenic ribotype (033) were found to contain at least parts of toxin genes. Among 79 toxinogenic ribotypes studied, another 30 variant strains were found in 21 ribotypes (two of them, 003 and 012, also contained strains of toxinotype 0). Strains were of known toxinotypes (I, III, IV, V, VI, VIII, IX) or represented five new toxinotypes, which will be described below. Sixty-three strains from 58 ribotypes were typed to toxinotype 0. Two of those 58 ribotypes (003, 012) also contained variant strains of toxinotype I.

In total, 123 strains were typed by ribotyping and toxinotyping. Finally, strains were grouped into 89 ribotypes, 86 of them described previously and 3 newly

Table 1. Distribution of toxinotypes in 25 ribotypes found to include variant *C. difficile* strains

Sixty-five ribotypes including only toxinotype 0 strains or nontoxigenic strains are not shown.

Toxin production	Ribotype	Number of studied strains*	Toxinotypes found (no. of strains)	
			Cardiff collection	Brussels collection
A ⁺ B ⁺	003	2/3	0 (1), I (1)	
	012	1 + 1/74	0 (1)	I (1)
	019	1 + 3/4	IX (1)	IX (3)
	023	2 + 2/45	IV (2)	IV (2)
	027	1/1	III (1)	
	034	3/9	III (3)	
	045	2/4	VI (2)	
	056	2/19	XII (2)	
	058	2/12	IV (2)	
	059	1/1	IV (1)	
	063	0 + 3/4		IV (1)†, VI (1), VII(1)
	066	1 + 5/9	VI (1)	V (2), VI (3)
	070	1/2	XIII (1)	
	075	1 + 1/2	III (1)	III (1)
	078	2/13	V (2)	
	080‡	0 + 3/3		III (3)
	102‡	0 + 1/1		I (1)
	103‡	0 + 1/1		II (1)
	111	1/1	XIV (1)	
	122	1/1	XV (1)	
A ⁻ B ⁺	036	0 + 1/1		X (1)†
	017	6 + 1/32	VIII (6)	VIII (1)†
	047	1/4	VIII (1)	
	110	1/2	VIII (1)	
A ⁻ B ⁻	033	2/2	XIa (1), XIb (1)	

* Number of studied strains is given as (Cardiff strains + Brussels strains)/all strains included in ribotype.

† Strains from Brussels collection which have been previously ribotyped (see Methods).

‡ New ribotypes.

found. Twenty-five ribotypes were found to include variant *C. difficile* strains from 15 variant toxinotypes; 23 ribotypes had only variant strains, whereas 2 ribotypes included changed strains and ordinary strains.

Production of toxins correlated well with the presence and the type of toxin genes (Table 1). Although the majority of ribotypes with variant strains produced both toxins, strains producing only TcdB (A⁻B⁺ strains) were found in four ribotypes and, interestingly, strains of ribotype 033 were noncytotoxic (A⁻B⁻) but contained at least some parts of the *tcdA* gene.

Correlation between toxinotypes and PCR ribotypes

In most cases both methods correlated well. The occasional exceptions indicated that one method is more discriminatory than the other. Toxinotypes were sub-

divided by PCR ribotyping more readily than PCR ribotypes were subdivided by toxinotyping. Strains in toxinotypes I, III, IV, V, VI and VIII could be differentiated into several PCR ribotypes. PCR ribotypes 003 and 012 contained a strain of toxinotype 0 as well as a variant strain from toxinotype I. PCR ribotypes 063 and 066 also contained strains of more than one toxinotype.

UPGMA analysis of PCR ribotype patterns clustered variant toxinotype strains into several groups (Fig. 1). PCR ribotypes of strains within a single toxinotype were similar (e.g. toxinotype III) but could also be distributed across two or three different groupings (e.g. toxinotypes I, IV and VIII). UPGMA analysis of all PCR ribotypes of the ARU Cardiff collection (results not shown) indicated that variant toxinotypes did not form a single distinct group, but were distributed into smaller distinct groups with similar ribotype patterns, for example ribotypes 033, 045, 066 and 078.

Fig. 1. UPGMA dendrogram showing similarities in PCR ribotype profiles for strains from 25 different ribotypes which contain variant strains (toxintype also shown).

Description of new toxinotypes

Within the strains analysed in the present study, five novel toxinotypes (termed XI–XV) were observed and the characteristic restriction patterns obtained with fragments B1 and A3 are presented in Fig. 2 and Table 2.

Two new toxinotypes, XII and XIII, exhibited minor changes in toxin genes compared to the reference strain of *C. difficile* VPI 10463 (toxinotype 0; Rupnik *et al.*, 1998). Toxinotype XIII produced a novel *EcoRI* restriction pattern in the repeat region of *tcdA* (Fig. 2; fragment A3, restriction pattern 9), but was otherwise similar to the type strain. Toxinotype XII produced a novel *HincII/AccI* pattern for fragment B1 (Fig. 2; fragment B1, type 6) which seems to be an intermediate between restriction types 1 (found in toxinotypes 0, I and II) and 2 (found in toxinotype IV).

Toxinotype XIa/b contained strains that produced amplification products covering part, but not all, of the *tcdA* gene. The middle and the 3' regions (A2 and A3 fragments) of the *tcdA* gene were amplified, but attempts to amplify the 5' A1-PCR fragment or shorter fragments within this region (Rupnik *et al.*, 1997) were unsuccessful. No PCR products were obtained in amplification reactions with primers designed to *tcdB*. Toxinotype XIa/b exhibited identical A2 fragment restriction patterns to toxinotypes IV, V, VI and VII (Fig. 3). Heterogeneity within toxinotype XI was observed when analysing the A3 fragment. Both toxinotype XI strains produced a truncated A3 PCR fragment; strain IS58

produced a fragment containing a deletion similar to toxinotype VI, whilst the other strain (R11402) had a deletion similar to toxinotype VIII (Fig. 2, Table 2). Given the heterogeneity in the A3 region, toxinotype XI strains were assigned to two subtypes, XIa and XIb (Table 2).

Toxinotypes XIV and XV had identical restriction profiles for the B1 and A3 fragments (Table 2). B1 represents a new type of restriction profile (Fig. 2; restriction pattern 7), whereas the A3 region in type XIV and XV was the same as in toxinotypes III, IV and IX. However, toxinotypes XIV and XV differed in the B2, B3, A1 and A2 fragments, as presented in Fig. 3. A prominent characteristic of toxinotype XIV is an insertion in the A1 fragment (Fig. 1), which is absent in toxinotype XV.

16S rDNA sequences

Sequencing the first approximately 500 bp of the 16S rRNA genes from strains R8637 (019/IX), R10456 (058/IV), CD196 (027/III), IS81 (034/III), IS93 (075/III), R6928 (023/IV) and CCUG 20309 (036/X) indicated 100% similarity with the sequence for the toxinotyping reference strain, *C. difficile* VPI 10463, and for *C. difficile* strains ATCC 9689^T and DSM 11209. Four strains, IS51 (066/VI), IS58 (033/XIa), R6786 (045/VI) and R7605 (078/V), exhibited a consistent single base change (transition of T to C) at position 145.

Fig. 2. PCR-RFLP patterns of the B1, A3 and A1 fragments, which were used for toxinotyping. For the B1 fragment, *HincII* (H) and *AccI* (A) restrictions were tested. A comparison of five previously described types (1–5) and two new types (6 and 7) of PCR-RFLP patterns is presented. Restriction pattern 6 is typical for new toxinotype XII and restriction pattern 7 for new toxinotypes XIV and XV. Currently, nine different types of A3 fragment are recognized according to differences in *EcoRI* restriction patterns or the length of the fragment. Five types of A3 fragment (1, 2, 3, 4 and new type 9) show different *EcoRI* restriction profiles, whereas types 5, 6, 7 and 8 have no *EcoRI* restriction sites. Types 5, 6 and 7 are A3 fragments with different lengths of deletions. For the A1 fragment, only representatives of newly described toxinotypes XII–XV are shown (no fragment could be amplified in toxinotype XIa/b). Toxinotype XIV has a characteristic insertion, whereas *PstI* restriction shows that the *PstI* site is conserved. Lanes M contain molecular mass markers.

DISCUSSION

Variation in the toxin genes, *tcdA* and *tcdB*, of *C. difficile* was analysed initially by toxinotyping strains of the large serogroup-based collection in Brussels of M. Delmée, and variant strains were delineated into ten toxinotypes (I–X; Rupnik *et al.*, 1998). In the present study, a collection of *C. difficile* isolates ($n = 2200$), which form the basis of a PCR ribotype library (Stubbs *et al.*, 1999), was screened systematically for changes in toxin genes of representative isolates ($n = 101$). All

toxinotypes (except toxinotype II) that had been reported previously (Rupnik *et al.*, 1998) were observed in this collection, indicating that changes in the PaLoc of *C. difficile* are stable.

The present study also revealed an additional five new toxinotypes, XI–XV. Two of these toxinotypes (XII and XIII) exhibit minor differences in only one of the ten PCR fragments covering the PaLoc. Toxinotypes reported previously (Rupnik *et al.*, 1998) either have changes in all regions of the PaLoc (major toxinotypes III–X) or

Table 2. Comparison of characteristic restriction patterns in some PCR fragments in novel toxinotypes XI–XV

Numbers refer to restriction profiles shown in Fig. 2. —, Not amplified; d, deletion; i, insertion.

Toxinotype	B1 fragment types of <i>HincII/AccI</i> restriction patterns	B3 fragment types of <i>HindIII/RsaI</i> restriction patterns	A3 fragment types of <i>EcoRI</i> restriction patterns	PL2 fragment types of <i>NsiI</i> restriction patterns	Standard strain
Type 0	1	1	1	1	VPI 10463
Type XIa	—	—	5d	—	IS58
Type XIb	—	—	7d	—	R11402
Type XII	6	1	1	1	IS25
Type XIII	1	1	9	1	R9367
Type XIV	7	5	2	4i	R10870
Type XV	7	4	2	4i	R9385

changes only in the 3' repeat regions of the *tcdA* gene (minor toxinotypes I and II) when compared to a *C. difficile* reference strain, VPI 10463 (Rupnik *et al.*, 1998). A novel toxinotype, XII, is unique, having differences in the 5' region of the *tcdB* gene only, whilst all other regions of both toxin genes are identical to the reference strain.

The new toxinotypes XIV and XV are of interest because they are identical in regions which have typically been used as markers for existing toxinotypes (*HincII/AccI* restriction patterns for the B1 region and *EcoRI* restriction patterns for the A3 region), but these toxinotypes differ in other regions of the PaLoc. In addition, toxinotype XIV strain has a large insertion at the beginning of *tcdA* gene, which was shown to be a new type of mobile genetic element (Braun *et al.*, 2000).

Strains constituting the novel toxinotype XI (a/b) do not express detectable levels of TcdA or TcdB, but the current PCR method indicates that at least two-thirds (fragments A2 and A3) of the *tcdA* gene is present. Because no actual enterotoxin is produced, toxinotype XI strains are not true A⁺B⁺ strains, but we designate them *tcdA*⁺B⁺ strains. A similar *tcdA*⁺B⁺ organism was isolated from a recurrent *C. difficile* infection by Cohen *et al.* (1998). As in the current study, the presence of a part of the *tcdA* gene was determined by PCR, but toxin production was not reported. Toxinotype XI strains could have only part of the PaLoc left or they could possess regions of the PaLoc that cannot be detected using the current PCR primers. A nontoxigenic *C. difficile* strain that should have both toxin genes was described by Mathis *et al.* (1999).

Typing results for toxinotyping and PCR ribotyping correlated well and most strains within a PCR ribotype belong to a single toxinotype. Where a PCR ribotype includes two or more different toxinotypes, these have similar toxinotype profiles and it is possible that one toxinotype has evolved from the other. PCR ribotypes 063 and 066 include strains from related toxinotypes (V, VI and VII) and have similar characteristics in most regions of the *tcdA* and *tcdB* genes, but they differ in the

size of the deletion in the repeat region of the *tcdA* gene. As discussed previously, it is possible that strains of toxinotypes VI and VII are descendants of type V strains with different deletions in the repetitive region of *tcdA* (Rupnik *et al.*, 1998). The results of the present study seem to support this suggestion. The same situation as in ribotypes 063 and 066 is observed in ribotype 033. This toxinotype also includes two strains with a very similar PaLoc differing only in the length of the deletion in *tcdA*. These two strains are included not in two separate toxinotypes but rather in subtypes XIa and XIb with the intention of indicating that they are related. Probably the same change from type to subtype level will have to be done for toxinotypes V, VI and VII in the future.

PCR ribotype 063 also contains toxinotype IV, which is dissimilar to types VI and VII in restriction sites. It is unlikely that these toxinotypes have a common ancestor that could explain their identical ribotype. Toxinotype IV is found in a number of PCR ribotypes that cluster into two separate groups (Fig. 1) and it is possible that the toxin genes of toxinotype IV have transferred horizontally via some type of mobile genetic element.

Two PCR ribotypes (003 and 012) contain standard and variant strains. Toxinotype I strains differ from toxinotype 0 at the 3' end of the *tcdA* gene only. This region contains a number of repetitive sequences and is prone to homologous recombination (von Eichel-Streiber & Sauerborn, 1990). Therefore, toxinotype I strains may be the result of relatively frequent mutation events in strains with ordinary toxin genes (toxinotype 0).

In toxinotypes with large numbers of strains (III, IV, V, VI, VIII), PCR ribotyping could be used to subtype toxinotypes. PCR ribotype profiles for strains within a single toxinotype exhibited similar patterns and cluster analysis (Fig. 1) showed that these types may have arisen from a single ancestor. Three toxinotypes (I, IV and VIII) are distributed among unrelated PCR ribotypes and, as discussed previously, this could be due either to independent mutations or to horizontal transfer.

Sequence analysis of the 16S rRNA gene of strains representative of toxinotypes revealed few differences.

Fig. 3. Schematic presentation of toxin genes in reference strain VPI 10463 in comparison to variant genes of five new toxinotypes. (a) Five genes of the PaLoc in a representative strain of toxinotype 0: two toxin genes (*tcdA* and *tcdB*) and three additional genes (*tcdC*, *tcdD*, *tcdE*). Hatched areas represent the repetitive sequences of both toxin genes. (b) Toxin genes of toxinotypes XI–XV. Light grey shading represents the regions that are variant but the same as found in previously described toxinotypes I–X. In the boxes, toxinotypes with identical restriction sites are shown. Dark grey shading shows the regions with new restriction patterns. In toxinotype XI, only part of the *tcdA* could be amplified. Insertions in toxinotypes XIV and XV are schematically presented. Restriction enzymes: A, *Accl*; E, *EcoRI*; Ec, *EcoRV*; H, *HindIII*; Ha, *HaeIII*; Hc, *HincII*; N, *NsiI*; P, *PstI*; R, *RsaI*; S, *SpeI*.

Only four strains from toxinotypes V, VI and XI have a single base change when compared to all other strains, including VPI 10463. These four strains belong to PCR ribotypes and toxinotypes that exhibit similar profiles (in both methods) and would seem to represent a well-defined subpopulation within *C. difficile*.

In a previous study (Rupnik *et al.*, 1998), 21 % of strains analysed were found to possess variant toxin genes. However, almost half of the 47 variant strains described belonged to a single toxinotype (type VIII). The Cardiff

ARU collection includes 116 ribotypes, of which 79 are toxinogenic. Almost one-third of those toxinogenic ribotypes contain variant strains. With the exception of ribotype 017 with 32 isolates and ribotype 023 with 43 isolates, ribotypes with variant strains contain only a few strains (Table 1; Stubbs *et al.*, 1999). All strains analysed in a given PCR ribotype (with the exceptions of ribotypes 003 and 012) had either variant toxin genes or non-variant toxin genes. If the correlation between toxinotypes and ribotypes is assumed to be consistent for all strains within the *C. difficile* library of the Cardiff

ARU ($n = 2200$; Stubbs *et al.*, 1999), the prevalence of variant strains can be estimated to be 7.7% of all strains and 8.8% of toxinogenic strains. Despite this relatively low percentage, variant strains seem to be prevalent as strains described in this study are from various locations (UK, USA, Australia) and were isolated from human hospitalized and community patients, animals and environmental sources. Further studies are required to see if and how the pathogenesis of variant strains differs from standard toxin-producing strains of *C. difficile*.

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