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

Maja Rupnik,1 Jon S. Brazier,2 Brian I. Duerden,2 Miklavz Grabnar1 and Simon L. J. Stubbs2

Author for correspondence: Maja Rupnik. Tel: +386 61 256 5584. Fax: +386 61 257 3390. e-mail: maja.rupnik@mf.uni-lj.si

1 University of Ljubljana, Department of Biology, Vecna pot 111, 1000 Ljubljana, Slovenia
2 Anaerobe Reference Unit, Public Health Laboratory, University Hospital of Wales, Cardiff, UK

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 aetiologic agent of pseudomembranous 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 \( C. \text{ difficile} \) 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 \( C. \text{ difficile} \) (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 \( C. \text{ difficile} \) (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). 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 \( C. \text{ difficile} \) 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 \( C. \text{ difficile} \) strains were analysed in the present study. Twenty-two strains from the collection of M. Delmee (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 \( C. \text{ difficile} \) typing study (Brazier et al., 1997). An additional 101 strains, belonging to 7 non-toxinogenic and 79 toxinogenic PCR ribotypes, were obtained from the \( C. \text{ difficile} \) 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 nontoxigenic strains. Only seven representative nontoxigenic 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. 2) and a 3 kb fragment spanning the repetitive region of tcdA (PCR fragment A3) by amplification and subsequent digestion with restriction enzymes Hinfl, 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 (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 nontoxigenic ribotypes were confirmed not to contain toxin genes, whereas two strains from the seventh nontoxigenic ribotype (033) were found to contain at least parts of toxin genes. Among 79 toxigenic 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

<table>
<thead>
<tr>
<th>Toxin production</th>
<th>Ribotype</th>
<th>Number of studied strains*</th>
<th>Toxinotypes found (no. of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cardiff collection</td>
</tr>
<tr>
<td>A−B+</td>
<td>003</td>
<td>2/3</td>
<td>0 (1), I (1)</td>
</tr>
<tr>
<td></td>
<td>012</td>
<td>1 + 1/74</td>
<td>0 (1)</td>
</tr>
<tr>
<td></td>
<td>019</td>
<td>1 + 3/4</td>
<td>IX (1)</td>
</tr>
<tr>
<td></td>
<td>023</td>
<td>2 + 2/45</td>
<td>IV (2)</td>
</tr>
<tr>
<td></td>
<td>027</td>
<td>1/1</td>
<td>III (1)</td>
</tr>
<tr>
<td></td>
<td>034</td>
<td>3/9</td>
<td>III (3)</td>
</tr>
<tr>
<td></td>
<td>045</td>
<td>2/4</td>
<td>VI (2)</td>
</tr>
<tr>
<td></td>
<td>056</td>
<td>2/19</td>
<td>XII (2)</td>
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<tr>
<td></td>
<td>058</td>
<td>2/12</td>
<td>IV (2)</td>
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<td>059</td>
<td>1/1</td>
<td>IV (1)</td>
</tr>
<tr>
<td></td>
<td>063</td>
<td>0 + 3/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>066</td>
<td>1 + 5/9</td>
<td>VI (1)</td>
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<td></td>
<td>070</td>
<td>1/2</td>
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<td></td>
<td>075</td>
<td>1 + 1/2</td>
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<td>078</td>
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<td>V (2)</td>
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<tr>
<td></td>
<td>080†</td>
<td>0 + 3/3</td>
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<tr>
<td></td>
<td>102†</td>
<td>0 + 1/1</td>
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<td>103†</td>
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<td></td>
<td>111</td>
<td>1/1</td>
<td>XIV (1)</td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>1/1</td>
<td>XV (1)</td>
</tr>
<tr>
<td>A−B−</td>
<td>036</td>
<td>0 + 1/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>017</td>
<td>6 + 1/32</td>
<td>VIII (6)</td>
</tr>
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<td></td>
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</tr>
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<td>110</td>
<td>1/2</td>
<td>VIII (1)</td>
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<tr>
<td>A−B+</td>
<td>033</td>
<td>2/2</td>
<td>X1a (1)</td>
</tr>
</tbody>
</table>

*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 subdivided 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.
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 *Clostridium difficile* VPI 10463 (toxinotype 0; Rupnik et al., 1998). Toxinotype XIII produced a novel *Eco*RI 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 *Hin*ClII/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), R6828 (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 9689T 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.
Novel toxinotypes of C. difficile

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 Xla/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. Delmee, 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...
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 (HindII/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 non-toxigenic 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.
Novel toxinotypes of *C. difficile*.

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, AccI; E, EcoRI; Ec, EcoRV; H, HindIII; Ha, HaellI; 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 toxigenic. Almost one-third of those toxigenic 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 88% 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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