INTRODUCTION

Toxigenic strains of Clostridium difficile commonly produce two large clostridial toxins, toxins A (TcdA) and B (TcdB), to which disease symptoms are attributed. They are encoded by genes tcdA and tcdB. Together with three additional genes (tcdC, D and E), they form a 19-kb chromosomal pathogenicity locus (PaLoc) (Fig. 1) (Braun et al., 1996; Hammond & Johnson, 1995). However, a number of different genetic variants of C. difficile have been described.

Strains with variations in PaLoc sequence have been classified into toxinotypes; 20 variant toxinotypes (I–XX) have been described (Rupnik, 2001; Rupnik et al., 2003a). Strains in which the PaLoc is identical to the reference strain VPI 10463 are referred to as toxinotype 0. Not all variations of toxin genes affect toxin production. Strains of toxinotypes I–VII, IX, XII–XV and XVIII–XX produce both toxins (A+B strains) despite changes in their toxin genes (Rupnik, 2001; Rupnik et al., 2003a). Strains of toxinotype XI have only the 3’ portion of tcdA present and do not produce either TcdA or TcdB (A+B) (Rupnik, 2001), whereas strains of toxinotypes VIII, X, XVI and XVII produce a functional TcdB but no TcdA (A+B) strains.

Abbreviations: CDAD, C. difficile-associated diarrhoea; PaLoc, pathogenicity locus; PMC, pseudomembranous colitis; TcdA, toxin A; TcdB, toxin B.

Some C. difficile strains produce an additional toxin, called binary toxin CDT (Rupnik et al., 2003b). Genes cdtA and cdtB, located on the CDT locus of the chromosome outside of the PaLoc, encode the enzymic (CDTa) and binding (CDTb) components of the binary toxin (Popoff et al., 1988; Perelle et al., 1997). Binary toxin is an actin-specific ADP-ribosyl-transferase and is cytotoxic for eukaryotic cells in culture (Popoff et al., 1988). The role of binary toxin in human
disease is not yet defined but it is suggested that CDT could contribute to the pathogenesis of *C. difficile* disease (Carman et al., 2003; Geric et al., 2003a).

The role of different toxinotypes and the production of binary toxin in the outcome of human disease are not well defined. Studies have described the frequency of binary toxin-positive strains in isolate collections from various sources, but information on such strains within a single hospital is missing. In this study the distribution of different toxinotypes and strains possessing binary toxin genes in clinical *C. difficile* isolates from a Veterans Affairs hospital in the USA was examined.

**METHODS**

**Clinical isolates.** From May 1996 to April 2001, 162 clinical *C. difficile* isolates from 125 patients at the Lakeside division of the Veterans Affairs Chicago Healthcare System in Chicago, IL, USA were saved for subsequent analysis. All isolates were included in the study except for the period of time from February to December 1998 when the laboratory relied primarily on toxin testing, and isolates were not available.

In 1995 the Lakeside VA was a 250-bed tertiary University-affiliated hospital (Mekonen et al., 2002). As a consequence of hospital consolidation within the VA system, the inpatient service at this hospital declined during the study period. There were no major *C. difficile*-associated diarrhoea (CDAD) outbreaks during the study period. In 1996, at the start of this study, there were 556 patient admissions/month with a mean length of stay of 10.7 days. By the end of the study in 2001, there were 341 patient admissions/month with a mean length of stay of 7.8 days. The rate of nosocomial CDAD also decreased during this time period from 8.1/1000 admissions (0.75/1000 patient days) in 1996 to 5.9/1000 admissions (0.67/1000 patient days) in 2001.

Faecal samples collected from patients suspected to be infected with *C. difficile* were cultured anaerobically on selective cycloserine, cefoxitin, fructose and egg yolk agar (CCFA) as described previously (Mekonen et al., 2002). In addition, a cell culture-based stool cytotoxicity assay (Bartels cytotoxicity assay for *Clostridium difficile* toxins; Bartels) was performed on all submitted stool specimens. Bacterial isolates were identified as *C. difficile* in the clinical laboratory according to colony morphology on CCFA, Gram’s stain and biochemical assay with the RapID ANA II System (Innovative Diagnostic Systems). Colonies of *C. difficile* were then subcultured in chopped meat media (CMM; BBL Microbiology Systems) and stored at room temperature. Nine isolates could not be recovered from CMM, leaving 153 isolates from 118 patients for analysis.

**DNA preparation.** Strains were recovered from CMM by subculture onto anaerobic sheep blood agar plates (BD) and a crude DNA extract was obtained from growth on the agar plate after 24 h with 5 % Chelex 100 Resin (Bio-Rad) as described by O’Neill et al. (1996).

**Detection of toxin genes cdtB, tcdA and tcdB and toxinotyping.** The presence of binary toxin gene *cdtB* was detected by PCR as described previously (Stubbbs et al., 2000).

Genes for the large clostridial toxins A and B (*tcdA* and *tcdB*) were detected by PCR and characterized by RFLP and the toxinotypes of all tested isolates were determined as described previously (Rupnik et al., 1997, 1998, 2001). Briefly, toxinotype characterization was performed by PCR amplification of two PaLoc fragments from the 5’ end of *tcdB* (fragment B1) and the 3’ end of *tcdA* (fragment A3) with subsequent restriction enzyme digestion. A detailed description of the toxinotyping method can be found on the website: http://rcul.uni-lj.si/~bircediff.

Strains that showed toxin gene variations not described previously in the toxinotyping scheme were characterized with additional PCR reactions for 11 fragments covering the whole PaLoc region. Fragments PL1–4, B1, B2s, B3s and A1–3 are shown above the schematics. Restriction sites are indicated by vertical lines below each schematic: A, Accl; E, EcoRI; Ec, EcoRV; H, HindIII; Ha, HaeIII; Hc, HincII; N, NsiI; Nc, NcoI; P, PstI; R, RsaI; S, SpeI. Toxinotypes with identical PCR fragment restriction patterns are included within boxes above each schematic. White areas within the schematic indicate regions with restriction sites identical to toxinotype 0, grey for previously described restriction types and dark grey for new restriction types. Insertion of a novel mobile genetic element in toxinotypes XIV and XXII is indicated by hatched symbols above the schematics (Braun et al., 2000). The restriction patterns for the B1 and A3 PCR fragments from the newly described toxinotypes have been designated as follows. Toxinotype XXI: B1 PCR fragment type (HincII/ Accl) 4; A3 PCR fragment type (EcoRI) 1. Toxinotype XXII: B1 PCR fragment type 5; A3 PCR fragment type 1.
Struppi2 (Braun et al., 1996) (Fig. 1). Modified primers were used for amplification of B2 and B3 PCR fragments. The putative translocation region (fragment B2a) was amplified with primers LJP4 (5’-GGCGTGCACTTCTTCAGTTGACATAAAGAT-3’) and LJP7 (5’-GGCGTGGACCAAGATGATTGATAGTATCGAAG-3’) and the binding region (fragment B3x) was amplified with primers LJP9 (5’-GGCGTGCACTTATATGAGTCTTATAGCAAA-3’) and LJPI6 (5’-GGCGTGCACTTCTTCAGTTGACATCACTTAATGAGC-3’). The re-action mixture and cycling conditions were the same as described previously for fragments B1 and A3 (Rupnik et al., 1998), Fragment PL1 was restricted with restriction enzyme HindIII, PL2 with NsiI, PL3 with EcoRV, PL4 with HinHI and SpeI, PLC with Ncol, A1 with PstI and NsiI, A2 with EcoRV and HaeIII, A3 with EcoRI, B1 with HindIII and AccI, B2s with HindIII and RsaI and B3s with HindIII and RsaI and restriction patterns were compared to other known toxino-types.

Strains in which no PaLoc-specific fragments could be amplified, were confirmed as non-toxigenic with Lok1Lok3-PCR (Braun et al., 1996), which gives an approximately 700 bp product only in strains lacking the PaLoc (Fig. 1). In toxigenic strains the expected PCR product by this reaction would be 20 kb and is therefore not amplified under the conditions selected for this amplification protocol.

Detection of toxin production. In vitro production of TcdA and TcdB was determined on all variant isolates. Production of cytotoxin TcdB was determined on 5-day cultures in brain heart infusion broth (Difco Becton Dickinson) by the same cell-culture-based cytotoxin assay used for the stool cytotoxin determination or in in-house-grown McCoy cells. TcdA testing was performed on the same culture supernatant with a toxin A immunoassay (Tox-A Test; TechLab or C. difficile Triage micro; Biosite).

RESULTS AND DISCUSSION

Molecular characterization of the pathogenicity locus (PaLoc) in clinical C. difficile isolates

The 153 isolates were characterized by toxinotyping; 123 (80.4 %) were toxino-type 0 with a PaLoc identical to reference strain VPI 10463 (Table 1). Thirteen strains (8.5 %) were non-toxigenic and lacked the entire PaLoc as confirmed with Lok1Lok3-PCR. Seventeen strains (11.1 %) were variant toxino-types with changes in composition of the PaLoc compared to the reference strain VPI 10463 (Table 1). Fifteen of these strains belonged to eight previously characterized variant toxino-types (I, III, IV, V, VIII, IX, XII and XIV), and two strains showed new RFLPs and were, therefore, designated new toxino-types.

One of the new toxino-types, isolate 6223, designated toxino-type XXI, was from a patient seen in the clinic whose stool cytotoxin test was positive and whose clinical syndrome was consistent with CDAD. This isolate produced TcdA and TcdB with a cytopathic effect in the cell culture cytotoxin assay that was typical for standard TcdB from reference strain VPI 10463.

The other new toxino-type, isolate 6143, designated toxino-type XXII, was also from a patient with a positive stool cytotoxin test and CDAD. This patient developed CDAD after prolonged hospitalization for rhabdomyolysis and renal failure. His isolate produced TcdA and TcdB with a typical cytopathic effect in vitro as well.

<table>
<thead>
<tr>
<th>Category</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. strains</td>
<td>153</td>
<td>100</td>
</tr>
<tr>
<td>Non-toxigenic</td>
<td>13</td>
<td>8.5</td>
</tr>
<tr>
<td>Toxino-type 0 (non-variant)</td>
<td>123</td>
<td>80.4</td>
</tr>
<tr>
<td>Variant toxino-types</td>
<td>17</td>
<td>11.1</td>
</tr>
<tr>
<td>Toxino-type I</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Toxino-type III</td>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>Toxino-type IV</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>Toxino-type V</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Toxino-type VIII</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>Toxino-type IX</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Toxino-type XII</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td>Toxino-type XIV</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Toxino-type XXI (new)</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Toxino-type XXII (new)</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Binary toxin positive*</td>
<td>9</td>
<td>5.8</td>
</tr>
</tbody>
</table>

*Toxino-types III (3 different patient strains), IV (2 strains recovered from same patient), V, IX, XIV and XXII.

Both new toxino-types were initially characterized by new combinations of previously described B1 and A3 PCR fragment restriction patterns (Fig. 1 legend). Further molecular characterization revealed additional PaLoc changes in each of the new toxino-types (Fig. 1): (i) a ~2 kb large insertion within the A1 fragment of toxino-type XXI. This insertion was identical to that found in toxino-type XIV and was shown to be a new type of mobile genetic element (Rupnik et al., 2001; Braun et al., 2000) (Fig. 2a); (ii) a new type of A2 restriction pattern in toxino-type XXI (Fig. 2b) and (iii) a new type of B2s restriction pattern in toxino-type XXII (Fig. 3, restriction pattern 6).

All A+/B- variant strains from this study (except for the toxino-type IX strain) produced characteristic barbarizing effects on cultured cells, where long protrusions were retained, similar to the cytopathic effect of reference strain VPI 10463. Isolates 6124 and 6134 of toxino-type VIII (A- B+), produced an atypical cell rounding effect described previously, in which protrusions disappeared and cells completely round up (Chaves-Olarte et al., 2003).

Frequency of variant toxino-types

Of all the clinical isolates obtained over a 5-year period at one tertiary care hospital, 11 % were variant by analysis of the pathogenicity region on the C. difficile chromosome encoding TcdA and TcdB (PaLoc). The frequency of toxino-types observed in this systematic survey was similar to that observed in surveys on isolate collections from Europe and elsewhere (Rupnik et al., 1998, 2001, 2003a; Kato et al., 1998; Spigaglia & Mastrantonio, 2002). The proportion and the types of variant strains found in these previous studies may have been influenced by the manner in which these strain collections were obtained. Strains of toxino-types III, IV and...
VIII were the most frequent variant toxinotypes in two European collections (Rupnik et al., 1998, 2001). Variant strains from Asia showed a slightly different distribution among toxinotypes (Rupnik et al., 2003a). Only six of 15 variant toxinotypes found in European collections were detected among Asian strains, and five new variants were found as well (XVI–XX).

As expected, strains of toxinotype 0 were the most prevalent among our clinical isolates. Among variant toxinotypes, more than one isolate of toxinotypes III, IV, VIII and XII (Table 1) were found, which were shown to be most numerous in previous studies of strains from collections in Europe and Asia (Rupnik et al., 1998, 2001, 2003a; M. Rupnik, unpublished data). Toxinotypes VI and VII were also frequently noted in European collections (Rupnik et al., 2001; Spigaglia & Mastrantonio, 2002) but they were not seen in this survey and they were also absent from a large collection of strains from Asia.

Fifteen variant strains were of toxinotypes that commonly produce both toxins (A\(^+\)B\(^+\)) and two (1.3 %) were toxino-type VIII, which produces only toxin TcdB (A\(^+\)/B\(^+\)). The low prevalence of A\(^+\)/B\(^+\) strains found in this study is similar to that reported in a previous smaller survey from the same hospital (Mekonen et al., 2002) and other surveys (Lyerly et al., 1998; Brazier et al., 1999; Barbut et al., 2002), whereas some other reports have noted higher rates (Kato et al., 1998; Kader et al., 1998; Pituch et al., 2001). One of the toxinotype VIII isolates was from a patient with a clinical syndrome typical for CDAD evidenced by a positive stool cytotoxin assay, a clinical response to metronidazole, and followed by a symptomatic relapse. The second toxinotype VIII isolate was recovered from a patient with transient diarrhoea that resolved without specific treatment. Although toxinotype VIII isolates were originally isolated from asymptomatic patients (Depitre et al., 1993; Stubbs et al., 2000) and were not thought to be pathogenic, several recent reports have documented isolation of A\(^+\)/B\(^+\) strains from patients with the full range of clinical manifestations seen with CDAD (Kato et al., 1998; Sambol et al., 2000; al-Barrak et al., 1999). Moreover, infections caused by toxinotype VIII strains are not detected by immunoassays for toxin A, which are the only tests for C. difficile disease used by many clinical laboratories (Johnson et al., 2001).

Clinical characteristics of patients infected with variant toxinotypes

The presence of variant strains was compared with the clinical patient histories. The 17 variant toxinotypes were from 16 male patients aged 43–84 (mean age, 67.5 yrs), reflecting the patient demographics at this VA hospital (Table 2). The stool cytotoxin assay was positive in ten patients (62.5 %). Overall, the clinical syndromes associated with these variant strains were similar to what has been reported for CDAD in general. Symptoms ranged from uncomplicated to severe diarrhoea and PMC. CDAD was
documented in twelve patients (75%) and PMC in one patient (6.3%). The strain isolated from the patient with PMC was of toxinotype III, which also possesses binary toxin genes. This toxinotype has been isolated from patients with PMC in a previous report (Rupnik et al., 1998). Three of the four unique patient-cases with toxinotype XII infections occurred within a 3-month period, but only one was nosocomial. A half of the episodes were nosocomial and involved infection by six different toxinotype strains.

Two or more consecutive isolates were obtained from 27 patients. The toxinotype of subsequent *C. difficile* isolates from the same patient was identical in 85% of the cases (21 cases with toxinotype 0, one case with toxinotype IV and one case with a non-toxigenic isolate). In four instances a different strain was isolated. From one patient two samples were taken approximately 1 year apart; the first isolate was non-toxigenic and the second was toxinotype 0. Similarly, from a second patient, two strains were isolated more than 1 year apart and were characterized as toxinotype III and 0. From the third patient, isolates of toxinotypes 0 and III were isolated approximately 2 months apart. Isolates of toxinotype 0 and a non-toxigenic strain were isolated just 3 days apart from a fourth patient. A third strain (toxinotype XII) was isolated from this same patient 2 months later. Therefore, in three cases where different strains were isolated, the interval between isolation of the strains was greater than 1 month. Recurrent episodes of CDAD within 1 month of the original episode are more likely to represent relapses of the original strain (potentially due to persistence of spores in the gut or reacquisition of spores from the contaminated environment).

**Fig. 3.** Restriction patterns of PCR fragments B2s and B3s of all known toxinotypes. Currently, nine B2s PCR fragment restriction pattern types and seven B3s PCR fragment restriction pattern types have been described. Toxinotype III strains may have one of three different restriction patterns for each of these PCR fragments and this toxinotype is now subdivided into subtypes IIIa, IIIb and IIIc. The toxinotypes associated with each particular restriction pattern are shown above each restriction type. H, *Hind*III; R, *Rsa*I; M, 100 bp DNA ladder (Invitrogen).
Although simultaneous carriage of two different strains were isolated 3 days apart from the same patient. In one case, two different strains were isolated 3 days apart from the same patient. Although simultaneous carriage of two different strains (Delmé et al., 1988; Camorlinga-Ponce et al., 1987) may explain this finding, sequential recovery of different strains from the same patient is not unexpected in the setting of a susceptible host (patient receiving antibiotics) living in an environment contaminated with multiple different strains (Johnson & Gerding, 1998; Clabots et al., 1992; Cohen et al., 1998; Johnson et al., 1989).

### Frequency of strains with binary toxin genes

Binary toxin genes were not detected in any of the toxintype 0 isolates or in the non-toxigenic isolates. Nine isolates (5.8%) of the variant toxintypes III, IV, V, IX, XIV and XXII amplified binary toxin gene cdB (Tables 1 and 2). This percentage is lower than shown previously for selected strains isolated from multiple hospitals (13.7%; Spigaglia & Mastrantonio, 2002), but similar to an estimated frequency of 5.5% of all C. difficile isolates in the Anaerobe Reference Unit collection as determined by the percentage of binary toxin-positive ribotypes (Stubbs et al., 2000). As previously observed, strains with genes for binary toxin also have altered genes for TcdA and/or TcdB (toxintypes III–VII, IX–XI and XIV–XVII). The reason for this correlation is unknown, but it is possible that binary toxin confers an evolutionary advantage to strains that produce variant large clostridial toxins (TcdA and TcdB) by the combined action of these toxins.

Thirteen strains (8.5%) lacked the entire PaLoc and had no binary toxin gene present. There is no evidence that these non-toxigenic strains cause disease and it is likely that these patients had symptoms due to enteric diseases or conditions unrelated to C. difficile infection. We have found, in a separate study, C. difficile strains lacking the entire PaLoc that do produce binary toxin and studies are being conducted to determine the pathogenetic potential of these strains (Geric et al., 2003a, b).

In summary, this study of clinical C. difficile patient isolates from a single hospital suggests that variant strains of C. difficile are frequently encountered in the clinical setting. Although many of these variants express both of the large clostridial toxins and are associated with typical CDAD syndromes, characterization of these variants is important in considering potential diagnostic strategies such as PCR or immunoassays that target genes or gene products of the PaLoc. It was recently reported that some prevalent toxintypes might not be detected with real-time PCR if only tcdB is amplified (Bélanger et al., 2003). The epidemiology of variant toxintypes in this survey from the USA shows similarities and differences between variants isolated in Europe and Asia. One particular variant, toxintype VIII, is widely disseminated on at least three different continents and is capable of causing severe disease as well as hospital outbreaks, despite lack of toxin A production. Further surveillance for these clinically significant A-“B” strains is particularly important as they are not detected by current tests used in many clinical laboratories.

### Table 2. Clinical summary of the 16 patients from whom toxin-gene-variant C. difficile strains were isolated

<table>
<thead>
<tr>
<th>Toxintype</th>
<th>BT</th>
<th>Nosocomial</th>
<th>Stool cytotoxin</th>
<th>Syndrome</th>
<th>Treatment</th>
<th>Recurrent diarrhoea</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>+</td>
<td>No</td>
<td>+</td>
<td>CDAD</td>
<td>M</td>
<td>No</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>Yes</td>
<td>+</td>
<td>CDAD</td>
<td>M, V</td>
<td>No</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>No</td>
<td>+</td>
<td>CDAD</td>
<td>M</td>
<td>No</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>Yes</td>
<td>+</td>
<td>CDAD</td>
<td>M, M, V</td>
<td>Yes</td>
</tr>
<tr>
<td>IV</td>
<td>+</td>
<td>No</td>
<td>+</td>
<td>CDAD</td>
<td>M</td>
<td>No</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>Yes</td>
<td>I</td>
<td>CDAD</td>
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<td>No</td>
</tr>
<tr>
<td>VII</td>
<td>+</td>
<td>Yes</td>
<td>+</td>
<td>CDAD</td>
<td>M, M</td>
<td>Yes</td>
</tr>
<tr>
<td>VIII</td>
<td>+</td>
<td>No</td>
<td>-</td>
<td>Diarrhoea</td>
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<td>No</td>
</tr>
<tr>
<td>IX</td>
<td>+</td>
<td>No</td>
<td>-</td>
<td>Diarrhoea</td>
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</tr>
<tr>
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<td>+</td>
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<tr>
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<td>-</td>
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<td>Diarrhoea</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Yes</td>
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<td>CDAD</td>
<td>M</td>
<td>No</td>
</tr>
<tr>
<td>XXI</td>
<td>+</td>
<td>No</td>
<td>+</td>
<td>CDAD</td>
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<td>No</td>
</tr>
<tr>
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<td>Yes</td>
<td>+</td>
<td>CDAD</td>
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ACKNOWLEDGEMENTS

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