Immunological detection and cytotoxic properties of toxins from toxin A-positive, toxin B-positive Clostridium difficile variants

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_Clostridium difficile_ is a major nosocomial pathogen and a causative agent of antibiotic-associated diarrhoea and pseudomembranous colitis. PCR analysis of the toxin A and B genes of this bacterium has revealed 20 variant types (toxinotypes I–XX), many of which can cause human disease. Strains comprising the 15 toxin A-positive, toxin B-positive toxinotypes are not usually differentiated from non-variant strains by routine laboratories that do not utilize PCR tests. Consequently, the toxins from these variant strains have not been investigated thoroughly. The present studies revealed that toxin A-positive (A+B+) strains representing 12 variant toxinotypes all express considerably lower levels of toxin A and are less cytotoxic in vitro than non-variant strain VPI 10463. Truncated forms of toxin A were detected by immunoblotting in toxinotype VI and VII strains and these toxins were differentiated from each other and from toxin A of the non-variant strain. A further novel finding was the ability of toxin A-negative (A–B+) strains of toxinotypes IX, XIV and XV to exhibit an alternative _Clostridium sordellii_-like cytopathic effect on Vero cells, characterized by marked cell clumping. A rapid and simple method for toxin A removal from culture filtrates was developed. This enabled confirmation that the abnormal cytotoxicity observed for these strains is due to an altered toxin B, as has been found in toxin A-negative (A–B+) strains. These findings indicate the potential for differentiation of certain toxin A-positive (A+B+) toxinotypes without the need for PCR techniques.

INTRODUCTION

_Clostridium difficile_ is a major nosocomial pathogen and the predominant aetiologcal agent of antibiotic-associated diarrhoea and potentially fatal pseudomembranous colitis (Riley, 1998; Yassin _et al._, 2001). The main virulence factors of the bacterium are two high molecular mass proteins: toxin A, a potent enterotoxin; and toxin B, a potent cytotoxin. It was originally believed that all pathogenic strains produced both toxins and that toxin A caused the initial damage to the intestine (Lyerly _et al._, 1985). In the 1990s, however, two types of strain were discovered that were toxin A-negative in immunoassays (Borriello _et al._, 1992; Depitre _et al._, 1993; Lyerly _et al._, 1992). More recently, worldwide reports have confirmed the ability of strains that produce only toxin B to cause severe human disease (Alfa _et al._, 2000; Barbut _et al._, 2002; Kuijper _et al._, 2001; Limaye _et al._, 2000; Sambol _et al._, 2000; Samra _et al._, 2002).

The complexity of _C. difficile_ toxins was highlighted following the development of a PCR method for analysing the entire pathogenicity locus that is found only in toxigenic strains (Rupnik _et al._, 1998). Examination of large culture collections in Belgium and the United Kingdom revealed a significant proportion of strains with differences in the toxin genes compared to reference strain VPI 10463, defined as toxinotype 0 (Rupnik _et al._, 1998, 2001). The variant strains were divided into 15 toxinotypes (I–XV) according to these genetic changes (Rupnik _et al._, 1998, 2001). In a recent survey of Asian isolates, a high prevalence of variant strains occurred (38 %) and five additional toxinotypes (XV–XX) were identified (Rupnik _et al._, 2003). The majority of variant toxinotypes have restriction site polymorphisms in both toxin A and B genes, but express both toxins (Rupnik _et al._, 1998, 2001, 2003). Deletions occur in the 3' end of the toxin A gene in 12 toxinotypes; however, only five of these toxinotypes (VIII, X, XI, XVI and XVII) are toxin A-negative by immunoassay due to non-production of this toxin.

Although a useful research tool, PCR toxinotyping is unlikely to find a wide application in routine testing for _C. difficile_ infection since it is time consuming, technically demanding and requires isolated bacteria. Routine laboratories that carry out direct toxin testing of stools may identify infection by toxin A-negative (A–B+) strains due to a negative toxin A immunoassay result, combined with a positive toxin A and B immunoassay result or a positive cytotoxicity test. Also, toxin A-negative (A–B+) strains produce variant forms of toxin B

Abbrivation: LT, lethal toxin.
with altered glucosyltransferase activity for small GTPases (Chaves-Olarte et al., 1999; Soehn et al., 1998) that cause atypical cytotoxicity in mammalian cell lines (Chaves-Olarte et al., 1999; Kuijper et al., 2001; von Eichel-Streiber et al., 1995). Due to their ease of detection, toxin A-negative (A−B+) strains have been extensively characterized. In contrast, toxin A-positive, toxin B-positive variant strains are generally not differentiated from non-variant strains by routine testing. This study analyses for the first time the toxins from strains representing 12 toxin A-positive (A+B+) toxinotypes using immunological techniques and cytotoxicity assays. Toxin B of these strains is compared with toxin B of well-characterized toxin A-negative (A+B−) strains from toxinotypes VIII and X. Our findings indicate the potential of well-characterized toxin A-negative (A+B−) strains from toxinotypes VIII and X. Our findings indicate the potential of well-characterized toxin A-negative (A+B−) strains from toxinotypes VIII and X.

**METHODS**

**Bacterial strains and purified toxins.** Table 1 lists the *C. difficile* strains used in this study and their sources. Non-variant reference strain VPI 10463 (toxinotype 0) was obtained from the American Type Culture Collection (strain 43255). Variant strains of toxinotypes I–X and XII were supplied by Professor M. Delme (Microbiology Unit, Catholic University of Louvain, Brussels, Belgium). Variant strains of toxinotypes XIII–XV and a non-toxigenic control strain, 18980, were provided by Dr M. Rupnik (Department of Biology, University of Ljubljana, Ljubljana, Slovenia). An additional strain, 5340, belonging to toxinotype VIII (M. Rupnik, personal communication) was supplied by Dr S. Sambol (Department of Medicine, VA Chicago Health Care System, Chicago, MI, USA).

Purified toxins A and B from *C. difficile* strain VPI 10463 were obtained from List Biological Laboratories (Campbell, CA, USA). Partially purified lethal toxin (LT) from *Clostridium sordellii* strain VPI 9048 was supplied by Dr H. Genth (Department of Toxicology, Medical School of Hannover, Hannover, Germany). The total protein concentration of this preparation was 663 μg ml⁻¹ (two replicates) as determined by the Pierce BCA assay (Perbio Science) with BSA as the standard. SDS-PAGE revealed that LT comprised 40% of the preparation, equating to 265 μg toxin ml⁻¹, and that no contaminating haemorrhagic toxin was present.

**Toxin production in dialysis sacks.** Forty-eight-hour brain–heart infusion (BHI) (Oxoid) broth cultures were harvested at 3300 g for 5 min at 37 °C. Cells were washed in pre-warmed (37 °C) PBS, pH 7.3, and resuspended in pre-warmed PBS to an optical density (OD) of 0.2 at 550 nm, as determined using a 6105 UV/Vis spectrophotometer (Jenway). Five millilitres of these cell suspensions served as inocula for dialysis sacks of 8000 molecular mass cut-off (Biodesign). The sacks were immersed in 250 ml of half-strength BHI broth and incubated anaerobically at 37 °C for 3 days. Cultures were recovered from sacks and the OD₅₅₀ values were measured. Bacteria were removed by centrifugation (3300 g for 15 min at 4 °C) and supernatants were passed through 0.22 μm pore-size syringe filters (Millipore). Supernatants were concentrated fivefold using Centriprep YM-10 concentrators (Millipore) and protein concentrations were determined as described previously. The culture filtrates were aliquoted, stored at −80 °C, and thawed only once prior to further analysis. Pure toxins A and B from reference strain VPI 10463 were found to retain a similar level of immunological activity after storage at either −80 °C or 4 °C over a 45 day period.

**Table 1. C. difficile strains analysed in this study**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Toxinotype</th>
<th>Source of isolate*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toxin A+B+ strain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPI 10463</td>
<td>0</td>
<td>Unknown</td>
<td>Lyerly et al. (1985)</td>
</tr>
<tr>
<td>EX623</td>
<td>I</td>
<td>Patient with AAD, Belgium, 1990</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>AC008</td>
<td>II</td>
<td>Patient with AAD, France, 1994</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>35004</td>
<td>III</td>
<td>Patient with PMC, Belgium, 1989</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>55767</td>
<td>IV</td>
<td>Patient with AAD, Belgium, 1995</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>SE881</td>
<td>V</td>
<td>Patient with AAD, France, 1995</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>51377</td>
<td>VI</td>
<td>Patient with PMC, Belgium, 1994</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>57267</td>
<td>VII</td>
<td>Patient with AAD, Belgium, 1995</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>SE938</td>
<td>IX</td>
<td>Patient with AAD, France, 1996</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>IS25</td>
<td>XII</td>
<td>Details not available</td>
<td>Rupnik et al. (2001)</td>
</tr>
<tr>
<td>R9367</td>
<td>XIII</td>
<td>Details not available</td>
<td>Rupnik et al. (2001)</td>
</tr>
<tr>
<td>R10870</td>
<td>XIV</td>
<td>Patient with AAD, England, 1997†</td>
<td>Rupnik et al. (2001)</td>
</tr>
<tr>
<td>R9385</td>
<td>XV</td>
<td>Details not available</td>
<td>Rupnik et al. (2001)</td>
</tr>
<tr>
<td><strong>Toxin A−B+ strain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1470</td>
<td>VIII</td>
<td>Asymptomatic infant, Belgium, 1981</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td>5340</td>
<td>VIII</td>
<td>Patient with CDAD, USA, 1993</td>
<td>Sambol et al. (2000)</td>
</tr>
<tr>
<td>8864</td>
<td>X</td>
<td>Patient with unknown symptoms, UK</td>
<td>Rupnik et al. (1998)</td>
</tr>
<tr>
<td><strong>Toxin A−B− strain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18980</td>
<td>Non-toxigenic</td>
<td>Details not available</td>
<td>Wozniak et al. (2000)</td>
</tr>
</tbody>
</table>

*AAD, Antibiotic-associated diarrhoea; PMC, pseudomembranous colitis; CDAD, C. difficile-associated disease. †M. Rupnik, personal communication.*
Preparation of anti-toxin A antibody. The toxin A-specific mAb PCG-4 (isotype IgG2a) (Lyerly et al., 1986) was used for analysis of C. difficile culture filtrates. This antibody was obtained from a decline phase culture of hybridoma HB-8712 (American Type Culture Collection), grown in RPMI supplemented with 20 % (v/v) horse serum (Life Technologies). The culture was centrifuged at 3300 g for 15 min at 4 °C and the resulting supernatant was stored in aliquots at −20 °C. The IgG concentration of the preparation was determined by the IMMUNOTEK enzyme immunoassay (Zepto Metrix) as 2.64 ± 0.36 μg ml⁻¹ (five replicates).

SDS-PAGE and immunoblotting. Analysis of culture filtrates for toxin A was performed after separation of proteins by SDS-PAGE in 7.5 % (w/v) acrylamide gels under non-reducing conditions. In some experiments, 5 % (w/v) acrylamide gels were used and the running time was increased from 80 min to 3 h to improve the resolution of proteins. Precision unstained molecular mass markers (Bio-Rad) were included on gels, and silver staining was carried out using standard methods.

For immunoblotting, separated proteins were electrophoretically transferred to PVDF membranes (BDH Laboratory Supplies) at 1–3 mA cm⁻² of gel for 1 h. All subsequent steps were carried out with agitation at room temperature. Membranes were blocked by incubation for 45 min in 20 ml of 50 mM Tris/HCl, pH 7.4/0.9 % (w/v) sodium chloride/3 % (w/v) BSA (buffer TBS). Toxin A was detected by incubating membranes for 1 h with 15 ml of mAb PCG-4, prepared as described earlier, diluted to 1.5 μg ml⁻¹ in buffer TBS. Membranes were washed four times, each for 5 min, in 250 ml of 50 mM Tris/HCl, pH 7.4/0.9 % (w/v) sodium chloride/0.1 % (w/v) Tween 20 (buffer TTBS). Membranes were then incubated for 1 h in 15 ml of goat anti-polyvalent mouse immunoglobulins–alkaline phosphatase (Sigma) at a 1:100 dilution in buffer TBS. Membranes were washed as described earlier, given a final wash for 5 min in ultrapure water, and developed in 20 ml of substrate solution, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). In some experiments, mAb PCG-4 was replaced with a goat anti-toxin A serum (List Biological Laboratories) diluted at 1:50, and protein A/G–alkaline phosphatase (Perbio Science) was used as conjugate at a 1:300 dilution.

Immunoaassay for toxin A. Culture filtrates were also tested using the Oxoid C. difficile toxin A test, a rapid and sensitive lateral flow immunoaassay (Bentley et al., 1998) (Oxoid). Filtrates were diluted 1:5 in the supplied diluent and the assay was carried out according to the manufacturer’s instructions.

Cytotoxicity assays. C. difficile culture filtrates were tested for cytotoxicity using African green monkey kidney (Vero) cells. Vero cells were grown to 60 % confluency in 96-well tissue culture plates (Scientific Laboratory Supplies) in 100 μl volumes of Dulbecco’s medium containing 10 % (v/v) fetal bovine serum (Life Technologies). Control wells were prepared by replacing 50 μl of medium with 50 μl of goat anti-C. difficile toxin B antiserum (Biocoreconnections). Serial 10-fold dilutions of culture filtrates were prepared in duplicate in sterile PBS containing 5 mg phenol red ml⁻¹, and 100 μl volumes were added to the Vero cells. Plates were incubated for 48 h (37 °C and 7 % CO₂) and examined for cytotoxicity. The cytotoxicity titre was defined as the reciprocal of the highest sample dilution that gave cell rounding in 50 % or more of the cell sheet. Pure toxins A and B from strain VPI 10463 were used to determine the detection limits of the cytotoxicity assay. C. sordellii LT was included in some experiments for comparison.

Removal of toxin A from culture filtrates. In order to determine the contribution of toxin A to the cytotoxic effects observed for the culture filtrates, a simple method for toxin A removal from these preparations was developed. Protein A–agarose (250 mg) (Sigma) was washed three times in 5 ml of 200 mM sodium phosphate, pH 7, and resuspended in this buffer to a total volume of 2 ml. To 1 ml of protein A–agarose was added 9 ml of undiluted toxin A-specific mAb PCG-4, prepared as described earlier. The agarose/antibody solution was mixed at room temperature for 15 min, centrifuged at 3300 g for 10 min at 4 °C, and the supernatant discarded. The antibody-coated agarose was washed in 10 ml of 20 mM sodium phosphate, pH 7, re-centrifuged as above and resuspended in the same buffer to a total volume of 1 ml. An uncoated protein A–agarose control reagent was prepared as described above, but using 20 mM sodium phosphate, pH 7, in place of the PCG-4 antibody. The protein A–agarose reagents were stored at 4 °C overnight prior to use.

Immediately before use, appropriate volumes of the protein A–agarose reagents were centrifuged at 7800 g for 5 min at room temperature, and the supernatants were discarded. Two volumes (original volume prior to centrifugation) of antibody-coated or control protein A–agarose were added to 1 vol. of the C. difficile culture filtrates. The solutions were mixed for 30 min at room temperature and centrifuged as described previously. Supernatants were recovered and re-treated with fresh protein A–agarose reagents as described above. Solutions were re-centrifuged and supernatants were carefully removed and tested for the presence of toxin A using the Oxoid toxin A test as described earlier. Culture filtrates were immediately assayed for cytotoxicity as described previously.

RESULTS

Toxin production in dialysis sacks

The C. difficile strains were grown in dialysis sack culture under nutrient limitation since this is known to stimulate toxin production. All strains gave high levels of growth, with cell yields (OD550) ranging from 14.8 to 45.9 (Table 2). The use of washed bacteria in PBS as inocula for the dialysis sacks ensured that the resulting culture filtrates contained only negligible levels of contaminants from the culture medium. The protein concentrations of the culture filtrates were between 4.4 and 17.5 mg ml⁻¹ (Table 2).

Toxin A detection by immunoblotting

Immunoblot analysis of proteins denatured in SDS 7.5 % (w/v) acrylamide gels was performed in order to compare the immunoreactivity of toxin A produced in vitro by strains from different toxin A-positive (A+B+) toxinotypes. The size of the toxin A proteins was also investigated since several toxinotypes are characterized by deletions at the 3' end of the toxin A gene. Immunoblots were probed using a toxin A-specific mAb, PCG-4. The mAb reacted with pure toxin A, but not with pure toxin B, from the toxinotype 0 non-variant strain, VPI 10463, confirming the specificity of this antibody (Fig. 1). A culture filtrate from strain VPI 10463 contained a strong band of the expected molecular mass (308 kDa) for toxin A and, additionally, several minor bands of lower molecular mass (Fig. 1). Since strain VPI 10463 is known to be a high-level toxin producer, the culture filtrates from the other strains were analysed at a fourfold higher total protein loading. As expected, toxin A was not detected in a culture filtrate from the non-toxigenic control strain 18980 (Fig. 1). The intensities of the toxin A bands in filtrates from the variant strains varied greatly between strains and all of these bands were of considerably lower intensity than the toxin A band for non-variant strain VPI 10463 (Fig. 1). The toxin A
Band intensities for all filtrates were consistent upon repetition of the experiment. Replacement of the PCG-4 mAb with a polyclonal anti-toxin A antibody yielded the same pattern of band intensities as shown in Fig. 1 and this result was again reproducible upon repetition. In all immunoblots toxin A was undetectable in filtrates from the toxinotype V and XV strains; however, these filtrates gave weakly positive reactions in the Oxoid toxin A test (duplicate determinations).

Strains from toxinotypes VI and VII consistently expressed toxins that appeared to be of slightly lower molecular mass than toxin A from strain VPI 10463 (indicated by the arrowheads in Fig. 1). In order to achieve better resolution between these high molecular mass toxins, electrophoresis was carried out in a lower percentage acrylamide gel (5 %, w/v) with an extended running time. This enabled the toxins to move considerably further through the gel, albeit with a slight reduction in band sharpness. Immunoblotting of the separated proteins revealed that the toxin A band for the toxinotype 0 strain was positioned well above the 205 kDa band.

Table 2. Cell yields of *C. difficile* strains in dialysis sack culture and cytotoxic activity of culture filtrates in the Vero cell assay

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Toxinotype</th>
<th>Cell yield (OD550)</th>
<th>Total protein concentration (mg ml(^{-1}))(^*)</th>
<th>Cytotoxicity titre†</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPI 10463</td>
<td>0</td>
<td>29.0</td>
<td>7.5</td>
<td>10(^7)</td>
</tr>
<tr>
<td>EX623</td>
<td>I</td>
<td>30.8</td>
<td>10.4</td>
<td>10(^6)</td>
</tr>
<tr>
<td>AC008</td>
<td>II</td>
<td>45.9</td>
<td>5.6</td>
<td>10(^2)</td>
</tr>
<tr>
<td>35004</td>
<td>III</td>
<td>17.6</td>
<td>5.0</td>
<td>10(^4)</td>
</tr>
<tr>
<td>55767</td>
<td>IV</td>
<td>22.6</td>
<td>6.9</td>
<td>10(^4)</td>
</tr>
<tr>
<td>SE881</td>
<td>V</td>
<td>18.1</td>
<td>5.3</td>
<td>2 × 10(^4)</td>
</tr>
<tr>
<td>51377</td>
<td>VI</td>
<td>27.4</td>
<td>11.2</td>
<td>10(^5)</td>
</tr>
<tr>
<td>57267</td>
<td>VII</td>
<td>26.5</td>
<td>17.5</td>
<td>10(^2)</td>
</tr>
<tr>
<td>SE938</td>
<td>IX</td>
<td>22.8</td>
<td>8.4</td>
<td>10(^6)</td>
</tr>
<tr>
<td>IS25</td>
<td>XII</td>
<td>14.8</td>
<td>13.9</td>
<td>10(^5)</td>
</tr>
<tr>
<td>R9367</td>
<td>XIII</td>
<td>39.9</td>
<td>5.5</td>
<td>10(^3)</td>
</tr>
<tr>
<td>R10870</td>
<td>XIV</td>
<td>21.5</td>
<td>10.2</td>
<td>10(^5)</td>
</tr>
<tr>
<td>R9385</td>
<td>XV</td>
<td>19.0</td>
<td>4.4</td>
<td>10(^4)</td>
</tr>
<tr>
<td>18980</td>
<td>Non-toxigenic</td>
<td>31.1</td>
<td>7.0</td>
<td>–</td>
</tr>
</tbody>
</table>

\(*\) Concentration in culture filtrate, obtained from duplicate determinations.

† Reciprocal of the highest dilution of culture filtrate that gave cytotoxicity, obtained from duplicate determinations. –, No cytotoxicity detected.

Fig. 1. Immunodetection of toxin A in culture filtrates from *C. difficile* variant strains. Proteins were separated by SDS-PAGE (7.5 % acrylamide) and toxin A was identified using mAb PCG-4 as described in Methods. Pure toxins A and B from non-variant strain VPI 10463 (toxinotype 0) were loaded at 50 ng. A culture filtrate of the toxinotype 0 strain was loaded at 5 μg total protein; culture filtrates of strains from variant toxinotypes and from a non-toxigenic (NT) strain, 18980, were loaded at 20 μg total protein. Arrowheads indicate toxin A bands that were consistently of lower molecular mass. M, silver-stained molecular mass standards in kDa.
molecular mass standard, whereas toxin A from the toxino-
type VI and VII strains ran just below this standard (Fig. 2).
Increased separation was also achieved between the trunc-
cated forms of toxin A, revealing that the toxino-
type VII strain expressed a slightly smaller toxin than the toxino-
type VI strain (Fig. 2).

Cytotoxicity assays
Mammalian cell assays principally detect toxin B since this
toxin has been reported as 500–1000 times more cytotoxic
than toxin A for a variety of cultured cells (Chaves-Olarte
et al., 1997). In an assay using Vero cells the minimum
cytotoxic concentrations of purified toxins A and B from
strain VPI 10463 were found to be 5.9 ± 2.3 ng ml⁻¹ (four
replicates) and 4.9 ± 0 pg ml⁻¹ (four replicates), respecti-
vely. The difference in potency between the two toxins
was therefore approximately 1000-fold for this cell type.
Culture filtrates from the toxin A-positive (A+B+) strains
were examined for activity in the Vero cell assay. A culture
filtrate from the non-toxigenic strain 18980 served as a
negative control, having no activity in the assay (Table 2).
The cytotoxicity titres for the variant toxin A-positive
(A+B+) strains showed considerable variation, ranging from
2 × 10¹ to 10⁵ (Table 2). The non-variant strain, VPI 10463,
gave a significantly higher cytotoxicity titre, of 10⁷. The
cytotoxicity of all filtrates was readily neutralized by a goat
antiserum raised against toxin B from strain VPI 10463. The
reproducibility of the cytotoxicity data was investigated by
determining cytotoxicity titres for fresh toxin preparations
obtained from a duplicate dialysis sack culture of strains from
toxinotypes 0, IX, XIV and XV. Titres were either identical to
those found previously or differed by a factor of only 10¹,
showing good reproducibility. The strains that gave the
lowest cytotoxicity titres (constituting toxinotypes II, V,
VII and XIII) were also low toxin A producers (Fig. 1).

![Fig. 2](image)

**Fig. 2.** Immunoblot showing increased resolution of truncated forms of toxin A in *C. difficile* variant strains. Proteins were separated by
SDS-PAGE (5% acrylamide) and toxin A was identified using mAb
PCG-4. Culture filtrates of strains from toxinotypes 0, VI and VII were
loaded at 25, 50 and 80 μg total protein, respectively. M, silver-stained
molecular mass standards in kDa.

The qualitative effects on Vero cells of culture filtrates from
strain VPI 10463 and the toxin A-positive (A+B+) variant
strains were also studied. Filtrates from well-characterized
toxin A-negative (A⁻B⁻) strains belonging to toxinotypes
VII (strain 5340 from a case of *C. difficile*-associated disease) and X (the
sole representative strain, 8864) were included for compar-
ison. The toxino-type 0 strain, VPI 10463, gave rounding of
Vero cells that was evenly distributed throughout the
monolayer (Fig. 3b). Pure toxins A and B from this strain
both exhibited an identical cytopathic effect to that of the
culture filtrate (data not shown). As found in previous
reports (Kato et al., 1998; Kuijper et al., 2001) all three toxin
A-negative (A⁻B⁻) strains gave abnormal cytotoxicity,
characterized by marked clumping of Vero cells in addition
to rounding (Fig. 3d and e show the results for toxino-
type VIII and X strains, respectively). The majority of toxin A-
positive (A+B+) variant strains exhibited a cytopathic effect
that was indistinguishable from that of the toxino-type 0
strain (the results for the toxino-type I strain can be seen in
Fig. 3c). Filtrates of strains belonging to toxinotypes IX, XIV
and XV, however, consistently caused a marked clumping of
Vero cells (Fig. 3f and g show the results for toxino-type IX
and XV strains, respectively). These abnormal cytopathic
effects were identical to those observed with filtrates of the
toxin A-negative (A⁻B⁻) strains, and also with partially
purified LT from *C. sordellii* (Fig. 3h).

Removal of toxin A from culture filtrates
Toxin A-negative (A⁻B⁻) strains have been shown to give
abnormal cytotoxicity in mammalian cell assays due to an
altered toxin B. To further investigate the abnormal clump-
ing of Vero cells observed for the three toxin A-positive
(A+B+) variant strains, a method for toxin A removal from
culture filtrates was developed. This utilized protein A–
agarose coated with the toxin A-specific mAb PCG-4. The
procedure was initially developed using a culture filtrate
from the non-variant strain VPI 10463, which contained a
high level of toxin A. Filtrate treated with PCG-4-coated
protein A–agarose gave a negative result in the Oxoid toxin A
test, whereas filtrate treated with control uncoated protein
A–agarose gave a strong positive result in the Oxoid toxin A
test (Table 3). A filtrate giving a negative Oxoid toxin A test
result should show a cytopathic effect due only to toxin B
since the minimal detection limit of the Oxoid toxin A test
(≤ 1 ng ml⁻¹) is lower than the minimal active concentra-
tion of toxin A in the Vero cell assay. Similarly, culture
filtrates of the toxino-type IX, XIV and XV strains were
positive in the Oxoid toxin A test after control protein A–
agarose treatment but were negative after PCG-4-linked
protein A–agarose treatment, confirming the effective re-
moval of toxin A from these samples (Table 3). The
cytotoxicity titres for all filtrates were unchanged after toxin
A removal, presumably because toxin B cytotoxicity masks
that of toxin A when both toxins are present (Table 3).
Marked clumping of Vero cells by the filtrates of the
toxino-type IX, XIV and XV strains occurred irrespective of

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treatment with control protein A–agarose or PCG-4-linked protein A–agarose (Table 3). These results suggest that toxin A is not involved in the abnormal cytotoxicity observed for these strains, thereby providing evidence that an altered toxin B is responsible.

**DISCUSSION**

Over recent years numerous disease-causing toxin A-negative (A−B+) strains have been identified and studied extensively both at the gene and protein level (Barbut *et al.*, 2002; Kuijper *et al.*, 2001; Limaye *et al.*, 2000; Sambol *et al.*, 2000). PCR toxinotyping of clinical strains has also revealed 15 different types of toxin A-positive (A+B+) variants, many of which are isolates from antibiotic-associated diarrhoea or pseudomembranous colitis cases (Rupnik *et al.*, 1998, 2001, 2003). Since toxin A-positive (A+B+) variants are unlikely to be identified as different from non-variant strains by routine laboratories that do not perform PCR, the toxins produced by this group of variant strains are largely uncharacterized. In

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Table 3. Ability of culture filtrates of *C. difficile* variant strains to cause abnormal cytopathic effects on Vero cells after toxin A removal

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Toxinotype</th>
<th>Control protein A–agarose-treated</th>
<th>PCG-4-linked protein A–agarose-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxoid toxin A test result*</td>
<td>Cytotoxicity titre†</td>
<td>Cytotoxicity effect‡</td>
</tr>
<tr>
<td>VPI 10463</td>
<td>0</td>
<td>++++</td>
<td>10⁷</td>
</tr>
<tr>
<td>SE938</td>
<td>IX</td>
<td>+++</td>
<td>10⁵</td>
</tr>
<tr>
<td>8864</td>
<td>X</td>
<td>–</td>
<td>10⁷</td>
</tr>
<tr>
<td>R10870</td>
<td>XIV</td>
<td>++</td>
<td>10⁵</td>
</tr>
<tr>
<td>R9385</td>
<td>XV</td>
<td>+</td>
<td>10⁴</td>
</tr>
</tbody>
</table>

*Strength of reaction compared to that obtained for the positive control supplied in the kit. –, No reaction; +, weak reaction; ++, moderate reaction; ++++, strong reaction (comparable to that for the positive control); ++++, very strong reaction.
†Reciprocal of the highest dilution of culture filtrate that gave cytotoxicity, obtained from duplicate determinations.
‡Effect obtained with duplicate 1 : 100 and 1 : 1000 diluted culture filtrates. Culture filtrates from strain VPI 10463 and the toxin A-negative (A−B+) strain 8864 served as negative and positive controls, respectively, for cell clumping.
the present study we utilized non-molecular techniques to analyse the toxins from strains representing the majority of toxin A-positive (A+B+) variant toxinotypes.

Immunoblotting of a culture filtrate of non-variant strain VPI 10463 with mAb PCG-4 revealed the presence of several minor bands of lower molecular mass in addition to the expected toxin A band of 308 kDa. Lower molecular mass bands have also been reported for highly purified toxin A (Lyerly et al., 1986) and they presumably arise due to protease activity on the single subunit polypeptide. Culture filtrates from all of the variant strains appeared to contain significantly lower amounts of toxin A than the filtrate from strain VPI 10463, as detected by immunoblotting with the PCG-4 antibody. This mAb binds to two epitopes within the receptor-binding domain of non-variant toxin A, located between amino acids 2097–2141 and 2355–2398 (Frey & Wilkins, 1992). The 3' end of the gene that encodes this receptor-binding domain is highly polymorphic (Rupnik et al., 1998, 2001, 2003). Consequently, decreased binding of the PCG-4 antibody to altered sequences in the variant toxins could account for the seemingly lower levels of toxin A produced by these strains. However, immunoblotting using a polyclonal antibody raised against whole toxin A gave the same results as for the mAb, suggesting that the data predominantly reflect the lower levels of toxin A produced in vitro by variant strains. Filtrates of the toxinoype V and XV strains that were negative for toxin A by immunoblotting gave positive Oxoid toxin A test results, presumably reflecting the lower detection limit of the Oxoid test.

Truncated forms of toxin A were consistently detected by immunoblotting in preparations from toxinoype VI and VII strains, both of which have sizeable deletions in the 3' end of the toxin A gene (Rupnik et al., 1998). To our knowledge there has been only one other report of a strain producing a shorter toxin A protein and this strain was not investigated thoroughly at the molecular level (McMillin et al., 1991). Modification of the PAGE-blottng protocol enabled a better separation of the variant toxins from toxin A of the non-variant toxinoype 0 strain, and also achieved differentiation of the two truncated toxinope molecules. The smaller of the truncated toxins, present in the toxinoype VII strain, is presumably due to a slightly larger deletion in its toxin gene compared with the deletion that characterizes toxinoype VI (Rupnik et al., 1998). It may also be feasible using the revised blotting procedure to identify the truncated products of smaller deletions in the 3' end of the toxin A gene in toxinoypes I and II strains (Rupnik et al., 1998). Although Western blotting is too laborious to be considered as a routine screening test it may be possible to develop a more rapid immunoassay that utilizes a panel of mAbs designed to detect toxinoype-specific deleisons in the receptor-binding domain of toxinope proteins.

Mammalian cell cytotoxicity assays are widely accepted as the method of choice for toxin detection in stools in order to confirm a diagnosis of C. difficile infection. The toxin A-positive (A+B+) variant strains all exhibited considerably less cytotoxicity for Vero cells than non-variant strain VPI 10463. These findings may reflect a reduced potency of the variant toxin B molecules and/or a lower level of toxin production by variant strains. Many of the variant toxinoypes possess genes encoding a third toxin (binary toxin; actin-specific ADP-ribosyltransferase) although the role of this toxin in disease has not been firmly established (Stubb et al., 2000). At high concentration (20 μg ml⁻¹) binary toxin exhibits cytotoxicity for Vero cells resulting in cell rounding (Perelle et al., 1997). Binary toxin is unlikely, however, to contribute to the cytotoxicity data reported in the present study since this toxin requires activation by exogenous proteases for in vitro activity (Perelle et al., 1997).

Cytotoxicity titres varied greatly between toxino A-positive (A+B+) variant strains and low titres correlated with low levels of toxin A production, as expected due to the coordinate regulation of toxin expression (Hundsberger et al., 1997). Since only a single strain was examined from each toxinoype it is not possible to conclude whether there are toxinoype-based differences in toxin expression levels. In agreement with our findings, two strains that very closely resemble toxinoype VII reacted only weakly in an immunoassay for toxin A (Barbut et al., 2002). To the contrary, toxinoype V, VI and VII strains have been reported as highly cytotoxic; however, cytotoxicity titres were not given and the mammalian cell type was not specified (Spigaglia & Mastrantonio, 2002). Toxin synthesis in C. difficile appears to be positively and negatively regulated by the respective products of genes tcdD (also known as txtk) and tcdC that lie within the pathogenicity locus (Hundsberger et al., 1997; Mani & Dupuy, 2001). Many of the variant toxinoypes have polymorphisms within one or both of these genes (Rupnik et al., 1998, 2001; Spigaglia & Mastrantonio, 2002) which may result in non-functional products that alter the rate of synthesis of toxins A and B. The toxino A-negative (A−B+) strain 8864 which has an unusually high cytotoxicity has been found to possess mutations within gene tcdC that give rise to a grossly truncated TcdC polypeptide (Lyerly et al., 1992; Soehn et al., 1998).

A novel finding of this study was the ability of toxino A-positive (A+B+) strains representative of toxinoypes IX, XIV and XV to exhibit an alternative C. sordelli-like cytopathic effect on Vero cells. Strains from toxinoype IX have also been reported to exhibit atypical cytopathic effects on other cell types (Rupnik, 2001). The cytopathic effects caused by these toxino A-positive (A+B+) strains appear identical to those observed for toxino A-negative (A−B+) strains from toxinoypes VIII and X. Toxin A-negative (A−B+) strains are known to produce altered forms of toxin B with extensive sequence variations in the N-terminal enzymic domain (Sambol et al., 2000; von Eichel-Streiber et al., 1995). Toxin B of strain VPI 10463 glucosylates GTPases of the Rho subfamily only (Just et al., 1995), whereas toxin B of strains 8864 and 1470, in common with LT, additionally glucosylate Ras subfamily GTPases (Chaves-Olarte et al., 1999; Popoff et al., 1996; Soehn et al., 1998). One of these Ras subfamily GTPases, R-Ras, is known to control...
integrin–extracellular matrix interactions (Chaves-Olarte et al., 1999) and its glucosylation may be responsible for the clumping of Vero cells. A rapid and simple method for toxin A removal from culture filtrates was developed using antibody-coated Protein A–agarose beads. The use of this procedure provided evidence that the abnormal cytotoxicity observed for the toxinotype IX, XIV and XV strains was also due to an altered toxin B. These three toxinotypes are all characterized by pronounced polymorphisms at the 5' end (enzymic domain) of the toxin B gene (Rupnik et al., 1998, 2001). It would be interesting to determine whether the toxin B proteins expressed by these toxinotypes also glucosylate R-Ras.

Our findings indicate the potential for differentiation of strains from certain toxin A-positive (A+B+) toxinotypes using cytotoxicity assays and immunological tests. To date there have been few detailed reports on the role of toxin A-positive (A+B+) variant strains in human disease. In Italy, toxinotype V and VI strains were isolated from sporadic cases of C. difficile-associated disease, and a toxinotype VI strain was also identified from an outbreak (Spigaglia & Mastrantonio, 2002). In France, two strains closely resembling toxinotype VII were isolated from a patient with recurrent pseudomembranous colitis (Barbut et al., 2002). Improved detection of toxin A-positive (A+B+) variant strains should enable their prevalence and ability to cause particular forms of disease to be investigated further and their response to antibiotic therapy to be determined.

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