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Isolation and Characterization of toxin A-negative, toxin B-positive Clostridium difficile in Dublin, Ireland

Denise Drudy
*Technological University Dublin*, denise.drudy@dit.ie

N. Harnedy
*Mater Misericordiae Hospital*,

S. Fanning
*University College Dublin*

R. O’Mahony
*University College Dublin*

L. Kyne
*Mater Misericordiae Hospital*

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Isolation and characterisation of toxin A-negative, toxin B-positive Clostridium difficile in Dublin, Ireland

D. Drudy¹, N. Harnedy², S. Fanning¹, R. O’Mahony¹ and L. Kyne²

¹Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, and ²Department of Medicine for the Older Person, Mater Misericordiae Hospital, Dublin, Ireland

ABSTRACT

Clostridium difficile is a major cause of infectious diarrhoea in hospitalised patients. Most pathogenic C. difficile strains produce two toxins, A and B; however, clinically relevant toxin A-negative, toxin B-positive (A⁻B+) strains of C. difficile that cause diarrhoea and colitis in humans have been isolated worldwide. The aims of this study were to isolate and characterise A⁻B+ strains from two university hospitals in Dublin, Ireland. Samples positive for C. difficile were identified daily by review of ELISA results and were cultured on selective media. Following culture, toxin-specific immunoassays, IMR-90 cytotoxicity assays and PCR were used to analyse consecutive C. difficile isolates from 93 patients. Using a toxin A-specific ELISA, 52 samples produced detectable toxin. All isolates were positive using a toxin A/B ELISA. Similarly, all isolates were positive with the cytotoxicity assay, although variant cytopathic effects were observed in 41 cases. PCR amplification of the toxin A and toxin B genes revealed that 41 of the previous A⁻B+ strains had a c. 1.7-kb deletion in the 3'–end of the tcdA gene. Restriction enzyme analysis of these amplicons revealed the loss of polymorphic restriction sites. These 41 A⁻B+ isolates were designated toxino type VIII by comparison with C. difficile strain 1470. PCR ribotyping revealed that all A–B+ isolates belonged to PCR-ribotype 017. A–B+ C. difficile isolates accounted for 44% of the isolates examined in this study, and appeared to be isolated more frequently in Dublin, Ireland, than reported rates for other countries.

Keywords Clostridium difficile, cytotoxicity assay, ELISA, molecular characterisation, PCR, toxins

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INTRODUCTION

Clostridium difficile is a common nosocomial pathogen and a major cause of infectious diarrhoea among hospitalised patients [1,2]. Colonisation with C. difficile is associated with a wide spectrum of clinical presentations, ranging from asymptomatic carriage to fulminant pseudomembranous colitis [3]. Recently, several institutions worldwide have reported an increase in the incidence of severe disease caused by C. difficile [4–7]. This may be related to several factors, including the changing demographics of patients admitted to hospitals, infection control policies, or the emergence of more virulent strains of C. difficile with increased antimicrobial resistance [7,8] (42nd Annual meeting of the Infectious Disease Society of America, 2004, abstract LB-2).

Two structurally similar toxins, denoted A and B, are the main virulence determinants linked with C. difficile-associated disease (CDAD), and most pathogenic strains of C. difficile produce both toxins (A⁺B⁺) [9,10]. The role of these toxins in the pathogenesis of CDAD has been well-described [10]. Both toxin A and toxin B are pro-inflammatory, cytotoxic and enterotoxin in the human colon [11,12]. These toxins are encoded by two genes, tcdA and tcdB, that map to a 19.6-kb pathogenicity locus (PaLoc) containing additional regulatory genes [13]. C. difficile isolates with varying genetic modifications within the PaLoc have been described [14,15]. These include variant C. difficile isolates that produce functional toxin proteins...
TcdA and TcdB, and toxin-variant isolates that fail to produce detectable toxins [14,16–18]. Toxin A-negative, toxin B-positive (A–B+) C. difficile strains were thought originally to be non-pathogenic. However, several recent reports have demonstrated their clinical importance [19–21]. Although outbreaks caused by A–B+ C. difficile are rare, several sporadic cases of infection and cases of pseudomembranous colitis (PMC) have been documented from several countries [20–23], and estimated prevalence rates of A–B+ C. difficile strains vary widely [24].

To date, four A–B+ strain types have been reported. Two toxinotypes, type VIII (strain 1470) and type X (strain 8864) have been well-described [25,26]. Both of these strains are truncated in the 3’-region of the repetitive domain of tcdA. In addition, both strains have alterations in their tcdB genes whereby restriction fragment length polymorphisms (RFLPs) give rise to altered glucosylation of RHO proteins and induce a differential cytopathic effect (CPE) when variant toxin B is compared with wild-type toxin B from A–B+ strains [27]. More recently, two additional A–B+ toxinotypes (type XVI and type XVII) have been described in Asia [15]. The molecular mechanism responsible for the absence of toxin A production in these newer toxinotypes has not yet been elucidated. Of the four A–B+ strain types, toxinotype VIII is considered to be the most clinically significant, and has been associated with the three reported outbreaks involving A–B+ C. difficile [19,20].

The present study reports, for the first time, the isolation of A–B+ C. difficile (PCR ribotype 017, toxinotype VIII) from a number of healthcare settings in Dublin, Ireland.

MATERIALS AND METHODS
C. difficile strains and patients

Between 1 February and 31 July 2004, all C. difficile toxin-positive faecal samples from new cases of C. difficile diarrhoea at two major university-affiliated teaching hospitals in Dublin (St Vincent’s University Hospital and the Mater Misericordiae University Hospital) were investigated. These hospitals have 570 and 490 beds, respectively. Both hospitals test all samples for which a C. difficile toxin assay is requested, in addition to testing non-requested liquid stool specimens from all inpatients and outpatients aged >65 years. The Premier toxin A/B ELISA (Meridian Bioscience Inc., Cincinnati, OH, USA), which detects both toxins A and B, was used in both laboratories for C. difficile toxin detection. Incidence rates at both hospitals were 7/1000 patient admissions for the period of the study. While several repeat samples were collected from patients throughout the investigation, only the first isolate from each patient was included for analysis in this study. In addition, 17 random samples from a third university hospital, collected during the months of February and May, were investigated, as well as six random samples acquired from three general practice surgeries and three nursing homes.

Of 85 new C. difficile cases in the two university hospitals during the study period, 15 samples were unavailable for culture because of insufficient remaining specimen following routine microbiological investigations. The remaining 70 faecal samples were cultured on Cologserine-Cefoxitin-Fructose Agar (CCFA) (LIP, Galway, Ireland). Identification of C. difficile was confirmed by morphology, Gram’s stain, odour and UV fluorescence, and latex agglutination (Microgen Bioproducts, Camberley, UK). C. difficile controls included strains VPI 10463 (A–B+), 630 (A–B+), 57267 (A–B+), 1470 (A–B+) and 8864 (A–B+). A non-toxigenic strain (R10567) served as a negative control in all experiments.

Detection of C. difficile toxins

Toxin-specific immunoassays and cytotoxicity assays were used to determine in-vitro toxin production. C. difficile isolates were inoculated into brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and were cultured anaerobically for 48 h. Broth cultures were centrifuged, after which the supernatants were filtered through 0.2-µM Acrodisc syringe filters (Pall Corp., Portsmouth, UK) and stored at −20°C for up to 3 months before analysis of toxin production. Toxin A was detected using the C. difficile Tox A ELISA (Tech Laboratory, Blacksburg, VA, USA) according to the manufacturer’s instructions. The C. difficile Tox A/B ELISA (Tech Laboratory) was used to detect the presence of toxin A and/or toxin B. Toxin B was detected using a tissue culture cytotoxic assay and the IMR-90 fibroblast cell line (CAMR, Salisbury, UK). In brief, filtered bacterial supernatants were added to IMR-90 monolayers, after which cytotoxicity was determined by examining for cell rounding after 24 and 48 h. The specificity of the CPE was confirmed by neutralisation with Clostridium sordellii antitoxin (Tech Laboratory).

Molecular analysis of tcdA and tcdB

The genes for toxins A and B, tcdA and tcdB, were characterised by PCR as described previously [14,28]. All primers were synthesised commercially by MWG Biotech (Ebersberg, Germany). Genomic DNA was purified from overnight Schaedler broth cultures of C. difficile using the Wizard Genomic DNA kit (Promega, Madison, WI, USA), followed by quantification of template DNA using the PicoGreen ds DNA Quantitation kit (Molecular Probes, Eugene, OR, USA). Primer sequences and restriction enzymes used for PCR and RFLP analysis, respectively, are shown in Table 1. The relative locations of the toxinotyping primers on the C. difficile PaLoc are shown in Fig. 1(A). All PCRs were performed on a Perkin-Elmer 2400 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in final reaction volumes of 50 µL using conditions described previously [28,29]. Amplified products were visualised following electrophoresis on conventional agarose 1.5% w/v gels stained with ethidium bromide 0.5 mg/mL in 1 x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Gels were
visualised and photographed using the Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA). Two PCR products, denoted A3 and B1, were selected for restriction enzyme analysis with EcoRI and HincII (Promega). Digests were visualised as described previously and strain toxinotypes were assigned according to the restriction digest patterns obtained.

**Table 1. Primers and restriction enzymes used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Size (bp) for VPI 10463</th>
<th>Target gene (region)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK9</td>
<td>5’-CCACCAGCTGCAGCCATA-3’</td>
<td>1200</td>
<td>tcdA (A3)</td>
<td></td>
</tr>
<tr>
<td>NK11</td>
<td>5’-TGATGCTAAATGTAATGTTAAATACAATC-3’</td>
<td>3100</td>
<td>tcdA (A1)</td>
<td></td>
</tr>
<tr>
<td>A1C</td>
<td>5’-GGAGGTTTTTATGTAATGTTAAATACAATC-3’</td>
<td>2000</td>
<td>tcdA (A2)</td>
<td>EcoRI</td>
</tr>
<tr>
<td>A2C</td>
<td>5’-TTAATGCTCTACATACAAACGAGG-3’</td>
<td>3100</td>
<td>tcdA (A3)</td>
<td>HincII</td>
</tr>
<tr>
<td>A3N</td>
<td>5’-CTTGTATATAAATCAGGTGCTATCAATC-3’</td>
<td>3100</td>
<td>tcdB (B1)</td>
<td></td>
</tr>
<tr>
<td>A3C</td>
<td>5’-ACGGTGATTATATTGTTAAATACAATC-3’</td>
<td>2000</td>
<td>tcdB (B2)</td>
<td></td>
</tr>
<tr>
<td>A4N</td>
<td>5’-CATGTATATAAATCAGGTGCTATCAATC-3’</td>
<td>2000</td>
<td>tcdB (B3)</td>
<td></td>
</tr>
<tr>
<td>A2N</td>
<td>5’-ATAGACTTACTTCCTATATGACG-3’</td>
<td>3100</td>
<td>tcdB (B3)</td>
<td></td>
</tr>
<tr>
<td>A3N</td>
<td>5’-CTTGTATATAAATCAGGTGCTATCAATC-3’</td>
<td>3100</td>
<td>tcdB (B3)</td>
<td></td>
</tr>
<tr>
<td>A4N</td>
<td>5’-ATAGACTTACTTCCTATATGACG-3’</td>
<td>2000</td>
<td>tcdB (B3)</td>
<td></td>
</tr>
<tr>
<td>A3N</td>
<td>5’-CTTGTATATAAATCAGGTGCTATCAATC-3’</td>
<td>3100</td>
<td>tcdB (B3)</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** (A) Schematic representation of the *Clostridium difficile* pathogenicity locus (PaLoc). The relative locations of the primer sequences (Table 1) used to amplify regions of *tcdA* and *tcdB* are shown. (B) Example of an agarose 1.5% w/v gel showing the amplicons from the A3 PCR assay using primer pair A3C and A4N targeted at the repeated sequences of *tcdA*. The arrows, in descending order, correspond to the A’ B+ amplicon (3100 bp) and the A B+ amplicon (1500 bp) (see Tables 1 and 2). Lanes: M, 1-kb DNA ladder; 1, (A B+VPI 10463; 2–6, A B+ clinical isolates; 7, A B+ control strain F-1470; 8–11, A B+ clinical isolates; 12, A B+ control. (C) Example of an agarose 1.5% w/v gel showing restriction digest patterns of the A3 fragments from (A) following digestion with EcoRI. Lane assignment as indicated for (B). A’ B+ *C. difficile* strains only show the RFLPs indicated by the filled arrow.

**16–23S PCR ribotyping**

PCR ribotyping was performed as described by Stubbs et al. [30] with minor modifications. DNA fingerprints were stored as tagged image file format (TIFF) files and imported into BioNumerics software v.4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Dendograms were created using the DICE coefficient and the unweighted pair group method with arithmetic mean (UPGMA) for cluster correlation using default settings.

**RESULTS**

Between February and August 2004, *C. difficile* isolates were cultured from the faeces of 93 symptomatic adult patients. Initially, these isolates were examined for in-vitro production of toxins A and B. Using the Toxin A ELISA, 52 isolates produced detectable toxin A (Table 2). The remaining 41 isolates tested negative.
However, when the Toxin A/B ELISA was used, toxin production was detected with all isolates. Furthermore, all isolates induced a CPE when investigated using the cell culture cytotoxicity assay (Table 2). The CPE observed for the 41 isolates that were negative in the toxin A ELISA was atypical, demonstrating complete cell rounding of the fibroblast body with no cytoplasmic extensions (data not shown).

All *Clostridium difficile* isolates were investigated by amplifying the *tcdA* gene with primer pair NK 9-NK 11 to detect genetic alteration(s) in the 3’-region (Table 1). Forty-one isolates carried a deletion and yielded a shorter amplicon (700 bp) than that produced from the reference strain VPI 10463 (1200 bp) (Fig. 1(A), Table 2). These unique isolates were classified as toxin A B+ and were evaluated further by toxinotyping (PCR-RFLP), involving six PCRs designed to amplify the complete *tcdA* (A1, A2, A3) and *tcdB* (B1, B2, B3) genes (Fig. 1(A)) [29,31]. In all 41 cases, there was a 1.7-kb deletion in the 3’-region of the *tcdA* gene (denoted by the open bar in Fig. 1(A); see also Fig. 1(B) and Table 2). Amplification of selected domains within the A1 and A2 regions of *tcdA* and the B1, B2 and B3 regions of *tcdB* showed no alterations in amplicon size in comparison with the control strain 10463 (A’B’+) (Table 2).

Restriction enzyme digests of the *tcdA* locus revealed the loss of some restriction sites in these A’B’ strains. Furthermore, the A3 amplicon could not be digested by EcoRI (Fig. 1(C)). Similarly, HincII digestion profiles of the B1 product were consistent with the loss of one or more HincII restriction sites (denoted by the hatched bar in Fig. 1(A); see also Fig. 2). Based on these restriction patterns, all 41 isolates were designated as toxinotype VIII, according to the toxinotyping scheme of Rupnik *et al.* [14]. The *C. difficile* isolates were analysed by 16–23S PCR ribotyping, producing DNA banding patterns of 10–15 bands, ranging in size from c. 200–1500 bp. Analysis of the banding patterns obtained for the 41 A’B’ isolates showed clonality, with identical banding patterns observed (data not shown). Ribotyping analysis revealed nine distinct ribotypes among the 35 A’B’ isolates (Table 3). There were seven different ribotypes among the 23 A’B’ isolates from hospital A, and seven ribotypes among the 12 A’B’ isolates from hospital B (data not shown). There were five ribotypes that were common to both university hospitals, while two distinct ribotyping patterns were found in one hospital only (data not shown).

Toxin A’B’ *C. difficile* strains were isolated from 44% of the patients in this study. The distribution of the A’B’ isolates in the two university hospitals is summarised in Table 3. A’B’ *C. difficile* strains were recovered from patients in these two university hospitals, as well as from patients in a

### Table 2. Summary of results for detection of A’B’ *Clostridium difficile*

<table>
<thead>
<tr>
<th>No. (%) of samples</th>
<th>Toxigenic status</th>
<th>Toxin A ELISA</th>
<th>Toxin A+B ELISA</th>
<th>Cell culture cytotoxicity Assay</th>
<th>Primer pair and amplicon size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NK 9-11  A1  A2  A3  B1  B2  B3</td>
</tr>
<tr>
<td>52 (56)</td>
<td>A’B’</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.2 3.1 2.0 3.1 2.0 2.0</td>
</tr>
<tr>
<td>41 (44)</td>
<td>A’B’</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.7 b 3.1 2.0 1.5 b 2.1 2.0</td>
</tr>
</tbody>
</table>

*Variant cytopathic effect observed.
Shorter amplicon caused by deletion in *tcdA*.

### Table 3. Distribution of A’B’+ and A’B’ *Clostridium difficile* isolates in two university hospitals in Dublin, Ireland

<table>
<thead>
<tr>
<th>Hospital</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of isolates</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>No. A’B’ <em>C. difficile</em></td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>% per institution</td>
<td>(57)</td>
<td>(40)</td>
</tr>
<tr>
<td>No. of ribotypes</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>No. A’B’ <em>C. difficile</em></td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>% per institution</td>
<td>(43)</td>
<td>(60)</td>
</tr>
<tr>
<td>No of ribotypes</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

A, Mater Misericordiae University Hospital.
B, St Vincent’s University Hospital.
third university hospital and from three community specimens. In total, 75% (70/93) of all the isolates examined were cultured from patients attending the two large university hospitals (Table 3), where the prevalence rates for A\(^{-}\)B\(^{+}\) C. difficile isolates were 43% and 60%, respectively. Overall, 35 (50\%) of 70 patients at the two university hospitals were infected with A\(^{-}\)B\(^{+}\) C. difficile (Table 3).

**DISCUSSION**

Toxin A-negative, toxin B-positive (A\(^{-}\)B\(^{+}\)) C. difficile has been isolated from many institutions in several countries on four continents [24]. Varying prevalence rates have been reported: a rate of 2% was reported in a multicentre study in the USA following analysis of C. difficile isolates from six clinical settings [32]; in the UK, a prevalence rate of 3\% was found among 43 isolates from nine of 35 hospitals that submitted strains for typing to the Anaerobic Reference Laboratory in Cardiff [33]; and in France, a rate of 3\% for isolates from 25 different hospitals in Paris was reported [34]. In contrast, A\(^{+}\)B\(^{-}\) prevalence rates as high as 39\% have been described in a Japanese study [35], and a recent study in Israel reported A\(^{+}\)B\(^{-}\) C. difficile rates of 56\% [36].

C. difficile is cultured in 55\% of European laboratories, but culture rates per country vary considerably, from 20\% to 100\% [37]. C. difficile is not cultured routinely in Irish hospitals, giving rise to a knowledge gap concerning the molecular epidemiology of Irish C. difficile strain types and the prevalence of A\(^{+}\)B\(^{-}\) C. difficile. Limited typing of isolates from previous outbreaks at two of the participating institutions in the present study suggested the presence of A\(^{-}\)B\(^{+}\) C. difficile (PCR ribotype 017) in both hospitals. Between March 1999 and January 2000, six (14\%) of 42 isolates from the third university hospital were A\(^{+}\)B\(^{-}\), while nine (60\%) of 15 isolates from the Mater Misericordiae University hospital were A\(^{+}\)B\(^{-}\) between March 2001 and March 2003 (J. Brazier, personal communication).

The overall frequency of A\(^{-}\)B\(^{+}\) C. difficile isolates in this study was higher (44\%) than non-outbreak rates reported for other countries [24]. This may reflect the collection of isolates analysed. A limitation of the present study was that 75\% of the isolates investigated were from two university hospitals, in which the toxin variant strain accounted for 43\% and 60\%, respectively, of the isolates collected. These data may be biased by the local epidemiology of C. difficile in these institutions, e.g., persistence of a clonal strain from an outbreak that is known to have occurred during 2003 in the Mater Misericordiae hospitals, where the incidence rate peaked at 20 cases/1000 patient admissions. However, to our knowledge, no outbreak of A\(^{+}\)B\(^{-}\) C. difficile occurred at St Vincent’s University hospital. Furthermore, analysis of 50 C. difficile isolates collected at this hospital between 1997 and 1998 revealed that 65\% of isolates characterised were clonal A\(^{+}\)B\(^{+}\) strains and that A\(^{+}\)B\(^{-}\) C. difficile were not prevalent in this hospital during that period (personal unpublished results). However, it is possible that A\(^{+}\)B\(^{-}\) C. difficile may have replaced the predominant A\(^{+}\)B\(^{-}\) strain types in recent years. Similarly, an Argentinian hospital reported that A\(^{-}\)B\(^{+}\) strains replaced A\(^{-}\)B\(^{+}\) strains completely over a 4-year period, with no variation in incidence or clinical presentation of CDAD [38].

A further possible limitation of the present study was the random nature with which samples were collected from the third university hospital, GP surgeries and nursing homes. However, these additional data confirmed the presence of these variant isolates in additional healthcare settings. All isolates were collected in a non-epidemic setting, as demonstrated by the stable incidence rates of C. difficile at the two major centres; however, the reasons for clonal spread and persistence of these variant strain types remain to be determined.

A recent survey by the ESCMID Study Group on C. difficile found that 58\% of laboratories surveyed in eight European countries use diagnostic methods for C. difficile that can detect only toxin A [37]. The hospitals collaborating in the present study used diagnostic methods that detect both toxin A and toxin B; it is therefore unlikely that any cases of toxin-variant C. difficile would not have been detected. Failure to use standardised C. difficile diagnostic methods that detect both toxin A and B could lead to significant under-reporting of C. difficile. This is of particular concern in countries in which the prevalence of toxin A\(^{+}\)B\(^{-}\) C. difficile strains is known to be high. Furthermore, outbreaks described previously have documented an increased severity of C. difficile disease associated with these A\(^{-}\)B\(^{+}\) isolates; the ability to clearly discriminate
between variant and non-variant *C. difficile* therefore has direct clinical relevance in terms of infection control management [19,20]. Standardised approaches to the investigation of *C. difficile* are essential in order to increase understanding of the factors leading to institutional outbreaks of *C. difficile*.

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