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

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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 cytoxicity 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 toxinotype 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 enterotoxic 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

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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 in-patients 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 Cyloserine-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 cytotoxin 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 × TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Gels were

Table 1. Primers and restriction enzymes used in this study

Primer	Primer sequence	Size (bp) for VPI 10463	Target gene (region)	Restriction enzyme
NK9	5′-CCACCAGCTGCAGCCATA-3′		*tcdA* (A3)	
NK11	5′-TGATGCTAATAATGAATCTAAAATGGTAAC-3′	1200		
A1C	5′-GGAGGTTTTTATGTCTTTAATATCTAAAGA-3		*tcdA* (A1)	
A2N	5′-CCCTCTGTTATTGTAGGTAGTACATTTA-3′	3100		
A2C	5′-TAAATGTACTACCTACAATAACAGAGGG-3′		*tcdA* (A2)	
A3N	5′-CTTGTATATAAATCAGGTGCTATCAATA-3′	2000		
A3C	5′-TATTGATAGCACCTGATTTATATACAAG-3′		*tcdA* (A3)	*Eco*R1
A4N	5′-TTATCAAACATATATTTTAGCCATATATC-3′	3100		
B1C	5′-AGAAAATTTTATGAGTTTAGTTAATAGAAA-3′		*tcdB* (B1)	*Hin*cII
B2N	5′-CAGATAATGTAGGAAGTAAGTCTATAG-3′	3100		
B2C	5′-ATAGACTTACTTCCTACATTATCTGAA-3′		*tcdB* (B2)	
B3N	5′-CATCTGTATAAATATTTGGTGAAATTAC-3′	2000		
B3C	5′-AATTTCACCAAATATTTATACAGATG-3′		*tcdB* (B3)	
B4N	5′-ATTTAACATATTTTTATCTATTCA-3′	2000		

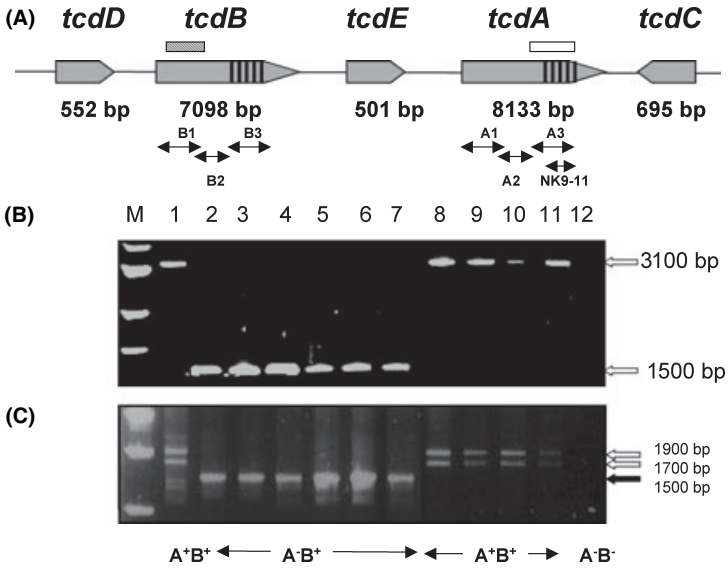

Fig. 1. (A) Schematic representation of the *Clostridium difficile* pathogenicity locus (PaLoc). The relative locations of the primer sequences (Table 1) used to amplify regions of *tcdA* and *tcdB* are shown. (B) Example of an agarose 1.5% w/v gel showing the amplicons from the A3 PCR assay using primer 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 *Eco*RI. Lane assignment as indicated for (B). A^-B^+ *C. difficile* strains only show the RFLPs indicated by the filled arrow.

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 *Eco*RI and *Hin*cII (Promega). Digests were visualised as described previously and strain toxinotypes were assigned according to the restriction digest patterns obtained.

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). Dendrograms 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.

Table 2. Summary of results for detection of A^-B^+ *Clostridium difficile*

No. (%) of samples	Toxigenic status	Toxin A ELISA	Toxin A+B ELISA	Cell culture cytotoxicity Assay	Primer pair and amplicon size (kb) NK 9-11	A1	A2	A3	B1	B2	B3
52 (56)	A^+B^+	+	+	+	1.2	3.1.	2.0	3.1	3.1	2.0	2.0
41 (44)	A^-B^+	-	+	+[a]	0.7 [b]	3.1.	2.0	1.5 [b]	3.1	2.0	2.0

[a]Variant cytopathic effect observed.
[b]Shorter amplicon caused by deletion in *tcdA*.

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 *C. 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 *c.* 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 *Eco*RI (Fig. 1(C)). Similarly, *Hin*cII digestion profiles of the B1 product were consistent with the loss of one or more *Hin*cII 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

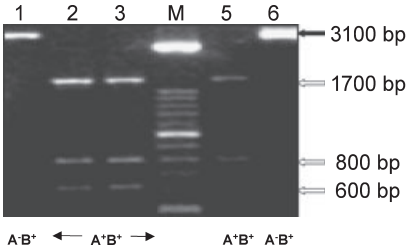

Fig. 2. Amplicons and RFLPs in the *tcdB1* locus. Example of an agarose 1.5% w/v gel showing the restriction patterns of B1 PCR amplicons following restriction digest with *Hin*cII. Lanes: 1 and 6, A^-B^+ *Clostridium difficile*; 2 and 3, A^+B^+ *C. difficile*; 5, *C. difficile* VPI 10463; and M, 100-bp molecular size marker. A^-B^+ *C. difficile* strains show RFLPs indicated by the filled arrow.

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

	Hospital	
Institution Identifier	A	B
Number of isolates	40	30
No. A^+B^+ *C. difficile*	23	12
% per institution	(57)	(40)
No of ribotypes	7	7
No. A^-B^+ *C. difficile*	17	18
% per institution	(43)	(60)
No of ribotypes	1	1

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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