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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