New types of toxin A-negative, toxin B-positive strains among clinical isolates of *Clostridium difficile* in Australia

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A total of 817 human clinical isolates of *Clostridium difficile* from all Australian states were screened for A[−]B⁺ strains by toxin gene PCR assays. Nine (1.1 %) strains were confirmed to be A[−]B⁺ by enzyme immunoassay for toxin production. Of these, six (66.7 %) were binary toxin-positive by PCR. Using PCR ribotyping and toxinotyping, the A[−]B⁺ strains could be grouped into seven ribotypes and three toxinotypes. Only one of the ribotypes had been reported previously (017). The prevalence of ribotype 017 was low in this study with only two strains detected. Two new A[−]B⁺ toxinotypes were also defined (XXX, XXXI). Toxinotype XXX had a toxin B gene similar to that of toxinotype IV (A⁺B⁺) but with a novel cytopathic region. Toxinotype XXXI was similar to other A[−]B⁺ types (X, XVII), but had a larger deletion to the toxin A gene than in either of those types. The types of A[−]B⁺ strains identified in this study differed markedly from those described in other regions.

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INTRODUCTION

Clostridium difficile is the most frequently diagnosed cause of infectious nosocomial diarrhoea and pseudomembranous colitis. The characteristic diarrhoea and inflammation of *C. difficile* infection is toxin-mediated. Pathogenic strains typically produce two major toxins, toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin), which are recognized as the major virulence factors. The genes for these toxins, together with three regulatory genes, are chromosomally located on the 19.6 kb pathogenicity locus (PaLoc) (Rupnik, 2008). Non-toxicogenic strains lack this element (Cohen *et al.*, 2000).

Strains with variations in the genes for toxin A and B (*tcdA* and *tcdB*, respectively) often produce a third unrelated binary toxin (CDT) (Stubbs *et al.*, 2000). This toxin is encoded on a separate region of the chromosome comprising the two components of CDT (*cdtA*, *cdtB*) and a regulatory gene (*cdtR*) (Carter *et al.*, 2007). The role of binary toxin in disease is not well understood but CDT-positive strains are associated with more severe disease in

humans and are found more commonly in animals (Barbut *et al.*, 2005; Rupnik, 2007).

The majority of *C. difficile* strains produce both toxin A and B but a small number have deletions in *tcdA* and produce only a functional toxin B (Voth & Ballard, 2005). These strains were initially thought to be non-toxicogenic as they were isolated from asymptomatic individuals and did not cause disease in a mouse model of infection (Borriello *et al.*, 1992). Subsequently, clinically relevant toxin A-negative, toxin B-positive (A[−]B⁺) strains have been well described in humans (Alfa *et al.*, 2000; Drudy *et al.*, 2007; Kuijper *et al.*, 2001; Pituch *et al.*, 2001; Sato *et al.*, 2004) and animals (Thakur *et al.*, 2010). There is also evidence that the prevalence of clinically significant A[−]B⁺ strains may be increasing (Goorhuis *et al.*, 2009).

Until recently, nearly all pathogenic A[−]B⁺ strains were thought to possess a 1.8 kb deletion in the 3' repeating region of *tcdA*, and classed as toxinotype VIII (Rupnik *et al.*, 1998; Soehn *et al.*, 1998). Only one other PaLoc variant had been described (a 5.9 kb deletion in the 3' *tcdA* region, toxinotype X), although this was isolated from an asymptomatic patient (Soehn *et al.*, 1998). Recent reports from Asia have described A[−]B⁺CDT⁺ strains with

Abbreviations: ARU, Anaerobe Reference Unit; PaLoc, pathogenicity locus.

different PaLoc arrangements, suggesting that the molecular epidemiology of these strains may be different in this region (Rupnik *et al.*, 2003).

Little is known about the molecular epidemiology of A⁻B⁺ *C. difficile* in Australia. This study examines the frequency and molecular diversity of A⁻B⁺ strains among 817 recent Australian *C. difficile* clinical isolates of human origin. All A⁻B⁺ strains were characterized by PCR toxin gene screening, PCR ribotyping and toxinotyping.

METHODS

Bacterial isolates. A total of 817 *C. difficile* human clinical strains isolated between 2005 and 2010 were included in this study. These were recovered from patients in all of Australia's states and territories, in hospitals, long-term care facilities and in the community. The identity of the isolates was confirmed using standard techniques (Bowman & Riley, 1988; Brazier, 1998).

Detection of toxin genes by PCR. All isolates included in this study were screened for the presence of the toxin A and toxin B genes by PCR (Kato *et al.*, 1991, 1998). Isolates were also screened for changes in the repeating region of *tcdA* with the primer pair NK11–NK9 as described by Kato *et al.* (1998). Isolates that were PCR-positive for *tcdB* and generated an approximately 1200 bp product with NK9–NK11 were designated A⁺B⁺. Isolates that were PCR-positive for *tcdB* but were PCR-negative or generated a truncated product with either primer pair for *tcdA* were tested for TcdA and TcdB production as described below. The presence of both binary toxin genes, *cdtA* and *cdtB*, was detected by PCR assay (Stubbs *et al.*, 2000).

Toxin production. The production of both toxin A and toxin B was determined using the immunoassay Cdiff 2-AB stick (Operon). A cell culture cytotoxin assay using Vero cells was also used to confirm toxin B production and to characterize the cytopathic effect (Bowman & Riley, 1988).

Detection of PaLoc accessory genes. The presence of the *tcdE* gene (Braun *et al.*, 1996) was confirmed by PCR, as was the presence of the *tcdC* gene, which was also sequenced to detect any deletions (Spigaglia & Mastrantonio, 2002).

PCR ribotyping. All A⁻B⁺ isolates were typed by PCR ribotyping (Stubbs *et al.*, 1999). Potentially novel ribotypes were referred to the

Anaerobe Reference Unit (ARU), Cardiff, UK, for confirmation. An AU (Australian) type number was used to identify the ribotype of a strain where a UK number had yet to be issued by the ARU.

Toxinotyping. Each strain was characterized by toxinotyping (Rupnik *et al.*, 1997, 1998). Two fragments were used for toxinotyping, B1 and A3. New types were referred to the Institute for Public Health, Maribor, Slovenia, for analysis of the entire PaLoc by RFLP.

RESULTS AND DISCUSSION

Among the 817 isolates tested, 19 (2.3 %) had an abnormal PCR result for either *tcdA* PCR assay. Nine (47.4 %) of these were confirmed as TcdA-negative by enzyme immunoassay. The remaining strains were identified as variant A⁺B⁺ toxinotypes (V, VI) possessing deletions in *tcdA* that did not abrogate toxin production. Of the nine A⁻B⁺ strains, binary toxin genes were detected in six (66.7 %).

A 700 bp amplification product with the primer set NK11–NK9 characteristic of toxinotype VIII strains was only seen in three Australian isolates (Table 1). Two of these were ribotype 017, while the other belonged to a ribotype not reported previously. Both of the ribotype 017 strains were isolated in Eastern Australia in the state of Victoria. A total of seven A⁻B⁺ ribotype groups were identified, six of which had not been reported to the ARU before (Fig. 1). Most of these ribotype groups contained only one isolate: the exception was AU 13, which contained two isolates.

Two new A⁻B⁺ toxinotypes were identified in this study. The majority of Australian A⁻B⁺ ribotypes belonged to toxinotype XXX. Most of these had very similar ribotyping profiles, differing by one to two bands. The *tcdB* gene of this toxinotype had a new RFLP pattern for the B1 fragment (catalytic region) while the rest of *tcdB* was similar to that of toxinotype IV (A⁺B⁺). None of the PCR fragments covering the *tcdA* gene could be amplified. The PaLoc accessory genes *tcdE* and *tcdC* were also negative in PCR assays. Only one human strain belonging to

Table 1. Characteristics of Australian *C. difficile* A⁻B⁺ strains

Ribotype*	No. of strains	<i>tcdA</i> †		<i>cdtA/B</i>	<i>tcdC</i>	<i>tcdE</i>	Toxinotype
		Non-rep.	Rep.				
UK 017	2	+	700 bp	–	+	+	VIII
AU 219	1	+	700 bp	–	+	+	VIII
UK 291	1	–	–	+	–	–	XXX
AU 13	2	–	–	+	–	–	XXX
UK 280	1	–	–	+	–	–	XXX
UK 281	1	–	–	+	–	–	XXX
UK 237	1	–	–	+	–	–	XXXI

*AU (Australian) type is used where ARU has yet to assign UK ribotyping number.

†Non-rep., non-repeating fragment of *tcdA*; Rep., repeating fragment of *tcdA*.

Fig. 1. Australian *C. difficile* A⁻B⁺ PCR ribotypes.

toxintype XXXI was identified from a 94-year-old woman hospitalized in Western Australia. The same ribotype and toxinotype, however, are common animal isolates (unpublished data) in the same state. Toxinotype XXXI was similar to toxinotypes X and XVI, but lacked the A1 and PL3 fragments, suggesting an even larger deletion in *tcdA* than is present in toxinotype X. Both of the new toxinotypes displayed an abnormal cytopathic effect *in vitro* similar to that of the *Clostridium sordellii* TcsL rather than that of TcdB from non-variant *C. difficile* strains (Torres, 1991).

Previous typing studies of A⁻B⁺ isolates from multiple countries have shown a high degree of similarity among strains (Johnson *et al.*, 2003; van den Berg *et al.*, 2004). One particular clindamycin-resistant strain of apparent clonal origin (toxintype VIII, PCR ribotype 017) has dominated international typing studies of A⁻B⁺ and been the cause of epidemics in Canada, the Netherlands, Ireland and Poland (Alfa *et al.*, 2000; Drudy *et al.*, 2007; Kuijper *et al.*, 2001; Pituch *et al.*, 2007). Although the frequency of A⁻B⁺ strains in our collection was similar to that observed elsewhere (Brazier *et al.*, 1999), they were surprisingly heterogeneous with only two strains belonging to ribotype 017. The majority of Australian A⁻B⁺ isolates were new, belonging to ribotypes and toxinotypes not previously reported. Unlike ribotype 017, all but one of the Australian ribotypes were binary toxin-positive and displayed a variant cytotoxicity due to changes in the cytopathic region of *tcdB* resulting in changes to substrate specificity. These characteristics are also present in most A⁻B⁺ toxinotypes other than VIII.

The epidemiology of *C. difficile* in the Asia-Pacific region differs from elsewhere. While a study of Asian isolates identified two new A⁻B⁺ toxinotypes, the majority of A⁻B⁺ strains were still ribotype 017 (Rupnik *et al.*, 2003). A recent study of 75 isolates from Shanghai, however, identified 25 A⁻B⁺ isolates of which only 14 belonged to ribotype 017, with the rest spread among three other ribotypes (Huang *et al.*, 2009).

In the present study, we have described the prevalence and type of A⁻B⁺ strains among *C. difficile* clinical isolates in Australia. We have also described two new A⁻B⁺

toxinotypes. Further studies are required to show whether these strains are found in other parts of the world, or are peculiar to the Australasian region.

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