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.

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-toxigenic 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-toxigenic 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; Kuipper 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...
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 cytotoxic 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**

<table>
<thead>
<tr>
<th>Ribotype*</th>
<th>No. of strains</th>
<th>tcdA†</th>
<th>tcdA/B</th>
<th>tcdC</th>
<th>tcdE</th>
<th>Toxinotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK 017</td>
<td>2</td>
<td></td>
<td>700 bp</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AU 219</td>
<td>1</td>
<td></td>
<td>700 bp</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>UK 291</td>
<td>1</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>AU 13</td>
<td>2</td>
<td></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
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<td>UK 237</td>
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<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*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.
toxinotype 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 (toxinotype 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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**REFERENCES**


