

# The role of toxin A and toxin B in *Clostridium difficile* infection

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***Clostridium difficile* infection is the leading cause of healthcare associated diarrhoea in Europe and North America<sup>1,2</sup>. During infection, *C. difficile* produces two key virulence determinants, toxin A and toxin B. Experiments with purified toxins have suggested that toxin A alone is able to evoke the symptoms of *C. difficile* infection, but toxin B is unable to do so unless it is mixed with toxin A, or there is prior damage to the gut mucosa<sup>3</sup>. However, a recent study suggested that toxin B is essential for *C. difficile* virulence and that a strain producing toxin A alone was avirulent<sup>4</sup>. This creates a paradox over the individual importance of toxin A and toxin B. Here we show that isogenic mutants of *C. difficile* producing either toxin A or toxin B alone can cause fulminant disease in the hamster model of infection. By using a gene knock-out system<sup>5,6</sup> to permanently inactivate the toxin genes, we found that *C. difficile* producing either one or both toxins displayed cytotoxic activity *in vitro*, which translated directly into virulence *in vivo*. Furthermore, by constructing the first ever double mutant strain of *C. difficile*, in which both toxin genes were inactivated, we were able to completely attenuate virulence. Our findings re-establish the importance of both toxin A and toxin B and highlight the need to continue considering both toxins in the development of diagnostic tests and effective counter-measures against *C. difficile*.**

Toxin A and toxin B both catalyse the glucosylation, and hence inactivation, of Rho-GTPases; small regulatory proteins of the eukaryotic actin cell cytoskeleton. This leads to disorganisation of the cell cytoskeleton and cell death<sup>7</sup>. The toxin genes, *tcdA* and *tcdB*, are situated on the *C. difficile* chromosome in a 19.6 kilobase pathogenicity locus (PaLoc), along with the three accessory genes, *tcdC*, *tcdR* and *tcdE* (Fig. 1a). To address the individual importance of toxin A and toxin B, we used the ClosTron gene knock-out system<sup>6</sup> to inactivate the toxin genes of *C. difficile*. This system inactivates genes by inserting an intron into the protein-encoding DNA sequence of a gene, thus resulting in a truncated and non-functional protein. The intron sequence itself encompasses an erythromycin resistance determinant which permits selective isolation of mutants. Furthermore, it has been shown experimentally that the insertions are completely stable, meaning that inactivation of a gene is permanent<sup>5</sup>.

Using the ClosTron system, we targeted insertions to *tcdA* and *tcdB* at nucleotide positions 1584 and 1511, respectively (Fig. 1a). In both cases, this placed the intron within DNA sequence encoding the toxin catalytic domain. Three separate isogenic mutants of the toxin A-positive, toxin B-positive ( $A^+B^+$ ) *C. difficile* strain 630 $\Delta$ *erm*<sup>8</sup> were constructed; two 'single-mutants', with toxin profiles  $A^-B^+$  and  $A^+B^-$ , respectively, and a 'double-mutant' with toxin profile  $A^-B^-$ . The  $A^-B^-$  double-mutant was made from the  $A^+B^-$  single-mutant by targeting *tcdA* with a second intron which carried the chloramphenicol / thiamphenicol resistance gene *catP* instead of the usual erythromycin resistance determinant.

The genotype of each toxin mutant was characterised by PCR and DNA sequence analysis to confirm the exact location of each intron insertion made (data not shown). Southern blot analysis of EcoRV-digested genomic DNA samples, using an intron-

specific probe, confirmed that the  $A^-B^+$  and  $A^+B^-$  mutants each had a single insertion, while the  $A^-B^-$  mutant had a double insertion (Fig. 1b). It is noteworthy that three bands were expected for the  $A^-B^-$  double-mutant strain because the *catP* gene harbours an EcoRV site. The phenotype of each strain was confirmed by Western blot analysis. Use of a toxin A-specific antibody-probe confirmed that the  $A^-B^+$  and  $A^-B^-$  mutants no longer produced toxin A (Fig. 1c). Likewise, use of a toxin B-specific antibody-probe confirmed that the  $A^+B^-$  and  $A^-B^-$  mutants no longer produced toxin B (Fig. 1d).

Subsequently, *in vitro* cell cytotoxicity assays were carried out using HT29 (human colon carcinoma) cells and Vero (African green monkey kidney) cells. Each of these cell lines is susceptible to both toxin A and toxin B, although HT29 cells are more sensitive to toxin A and Vero cells are more sensitive to toxin B<sup>9</sup>. The action of toxin A and toxin B causes the cells to 'round' (that is, lose morphology) and die; a phenomenon which is clearly visible by light microscopy. We incubated cultured cells for 24 h with 4-fold dilution series of *C. difficile* culture supernatants. To obtain the most objective data set possible, we determined the endpoint titre of each dilution series, rather than implementing a subjective cell scoring system. Endpoint titre was defined as the first dilution in a series for which HT29 or Vero cell morphology was indistinguishable from the negative controls (that is, cells which had been incubated with uninoculated *C. difficile* culture medium).

As expected, the  $A^-B^-$  double toxin mutant did not display any cytotoxic activity towards either HT29 or Vero cells (Fig. 2a,b). Compared to the  $A^+B^+$  parental strain, the  $A^-B^+$  mutant displayed reduced toxicity towards HT29 cells, although the difference was not statistically significant, and a similar degree of toxicity towards Vero cells (Fig. 2a,b). These findings were anticipated given the respective sensitivities of HT29 cells and Vero cells to toxin A and toxin B<sup>9</sup>. However, unexpectedly, when compared to the  $A^+B^+$  parental strain, the  $A^+B^-$  mutant displayed increased toxicity towards HT29 cells,

although the difference was not statistically significant, and a similar degree of toxicity towards Vero cells (Fig. 2a,b). We reasoned that this may occur due to increased expression of toxin A by the  $A^+B^-$  mutant; a phenomenon which has been reported previously<sup>4</sup>. Indeed, qRT-PCR analysis confirmed that expression of toxin A was an average of 3.3-fold greater in the  $A^+B^-$  mutant than the  $A^+B^+$  parental strain (data not shown). We do not know the reason for this. However, considering the respective sensitivities of HT29 and Vero cells to toxin A<sup>9</sup>, this finding explains our unexpected cytotoxicity results, even accounting for the fact that the  $A^+B^-$  mutant does not produce any toxin B.

To confirm that the cytotoxic activity we had observed on HT29 cells and Vero cells were indeed attributable to the respective actions of toxin A and toxin B, we carried out toxin neutralisation assays. Culture supernatants of each *C. difficile* toxin mutant and the  $A^+B^+$  parental strain were incubated with either toxin A-specific or toxin B-specific neutralising antibodies, prior to inoculation onto HT29 and Vero cell monolayers. Importantly, culture supernatants were diluted equivalently such that, for the  $A^+B^+$  parental strain, only toxin A activity was detected on HT29 cells and only toxin B activity was detected on Vero cells (that is, toxin B activity towards HT29 cells was diluted-out completely and toxin A activity towards Vero cells was diluted-out completely) (Fig. 2c,d). As expected, the toxin A-specific antibody neutralised all toxic activity produced by the  $A^+B^-$  mutant and the toxin B-specific antibody neutralized all toxic activity produced by the  $A^-B^+$  mutant (Fig. 2c,d). Interestingly, the increased production of toxin A by the  $A^+B^-$  mutant was clearly visible in this assay, as the cytotoxic activity of this strain towards Vero cells was not diluted-out completely as it was for the  $A^+B^+$  parental strain (Fig. 2d).

Having fully characterised our *C. difficile* toxin mutants *in vitro*, we tested the virulence of each in the hamster model of infection. Hamsters were each challenged

with 100 spores of a single *C. difficile* strain, 5 days after an oral dose of clindamycin (30 mg/kg). Each toxin mutant and the A<sup>+</sup>B<sup>+</sup> parental strain were administered to eight hamsters in total. All hamsters became colonised by the *C. difficile* strain administered between 1 and 3 days post-challenge, with the exception of one which received the A<sup>+</sup>B<sup>-</sup> single toxin mutant (Fig. 3a). Following colonisation, hamsters which received the A<sup>+</sup>B<sup>+</sup> parental strain, the A<sup>-</sup>B<sup>+</sup> mutant or the A<sup>+</sup>B<sup>-</sup> mutant all developed symptoms of *C. difficile* infection, which resulted in a mean time to death of 1.0 day, 1.3 days and 4.0 days, respectively (Fig. 3b). In contrast, none of the hamsters colonised by the toxin null A<sup>-</sup>B<sup>-</sup> double mutant developed any symptoms of disease during the 14-day experimental period, indicating that this strain is completely attenuated for virulence. Bacteriological and PCR analysis of caecum samples taken from each hamster *post mortem* confirmed that the only infecting strain of *C. difficile* was, indeed, the strain administered in every case, thus ruling out any possibility of cross-contamination between cages or contamination from the environment (Supplementary Fig. 1).

In conclusion, it is clear that both toxin A and toxin B play an important role in *C. difficile* infection as we have shown here that a strain which produces either toxin on its own or both together is virulent. It is pertinent to question why we found that an A<sup>+</sup>B<sup>-</sup> strain of *C. difficile* is virulent; a result which is in direct contrast with a similar study published recently<sup>4</sup>. This discrepancy may arise due to inherent differences between the hamsters used in each study. However, perhaps more likely is that there is one or more key differences between the strains of *C. difficile* studied. Although both strains are erythromycin-sensitive derivatives of strain 630<sup>10, 11</sup>, they were isolated independently through serial sub-culture<sup>8, 12</sup>. Therefore, either strain could have acquired one or more secondary mutations, which may affect the action of either one or both of the toxins. However, it is notable that our findings align with those of previous studies which have suggested a role for both toxin A and toxin B in *C. difficile* infection<sup>3, 13-15</sup>. Moreover, given that the human colon is the principle site of pathology in patients infected with *C.*

*difficile*, it stands to reason that an A<sup>+</sup>B<sup>-</sup> strain of *C. difficile* is virulent *in vivo*, as it is toxin A which displays the greatest cytotoxicity towards laboratory cultured human colon cells (that is, HT29 cells).

It is important to note that inherent variability exists between the toxins of some *C. difficile* strains; particularly in the case of toxin B<sup>16-19</sup>. In practical terms, this means that the toxins from different strains can vary in enzymatic activity (that is, different GTPase substrates may be glucosylated) and/or host-cell specificity. Consequently, it is not appropriate to over-interpret our findings and make general conclusions about the toxins produced by all toxigenic strains of *C. difficile*. Nonetheless, our results clearly demonstrate that a strain of *C. difficile* producing either toxin A or toxin B alone may be virulent and thus we have re-established the importance of both toxins in *C. difficile* infection.

It is interesting to note that a number of clinical cases of *C. difficile* infection have been attributed to naturally occurring A<sup>-</sup>B<sup>+</sup> strains<sup>20, 21</sup>, but there have been no reports of naturally occurring A<sup>+</sup>B<sup>-</sup> isolates to date. This would suggest that A<sup>+</sup>B<sup>-</sup> strains do not exist, but it may also be an artefact of routine diagnostic testing practices. Either way, our results show that A<sup>+</sup>B<sup>-</sup> strains may be virulent and even if they do not exist in nature already, they may yet evolve. Consequently, it is imperative that both toxin A and toxin B continue to be considered in routine diagnostic settings and in the development of effective countermeasures against *C. difficile*.

## Methods Summary

Mutants were constructed from the parental strain *C. difficile* 630Δerm<sup>8</sup> using the ClosTron system<sup>5, 6</sup>. The retargeted plasmids pMTL007C-E2::*Cdi-tcdA*-1584s,

pMTL007C-E2::Cdi-*tcdB*-1511a and for the double pMTL007S-C7::Cdi-*tcdA*-1584s were transferred into *C. difficile* via conjugation. The single ClosTron mutants were isolated on erythromycin plates. The double mutant was isolated on thiamphenicol plates.

For cytotoxicity assays, the four strains were grown overnight in 5 ml TY under anaerobic conditions as previously described<sup>22</sup>. The cell densities were standardised before centrifugation and filtration. Supernatants were diluted in a 4-fold series and 20µl of dilutions were added onto monolayers of Vero and HT29 cells preincubated in 96 well plates for 48 h (at 37°C, 5% CO<sub>2</sub>). Cytotoxicity was recorded after 24 h. Statistical analysis was performed using one way ANOVA tests. For the neutralization assay appropriate dilutions of supernatants were pre-incubated with a suitable concentration of anti-TcdA or anti-TcdB serum (polyclonal, tgcBIOMICS) for 1 h at 37°C and then added as previously described to Vero and HT29 cells and evaluated after 24 h. Golden Syrian hamsters were dosed with clindamycin (30 mg/kg) 5 days prior to being infected orally with 100 spores each. Hamsters were monitored for signs of infection (including weight-loss, behavioural changes and wet-tail) and sacrificed when the endpoint was met. Faecal pellets were collected daily and plated to confirm the presence or absence of *C. difficile*. Caecum samples were homogenized, plated and *C. difficile* counts obtained. PCR was performed to determine the *C. difficile* genotype isolated from all samples. Supplementary Information, showing PCR results, is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Author Contributions** The study was conceived by N.M. and designed by S.K., S.C. and J.H. Construction of mutants and *in vitro* characterisation was carried out by S.K. *In vivo* work was carried out by S.C., M.K. and A.C. Analysis of data was carried out by S.K. and M.K. with assistance from S.C. and J.H. The manuscript was written by S.K. and S.C. with critical input from all other authors. Funding for the study was sourced by N.M. and A.C.

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**Figure 1. Characterization of *C. difficile* toxin mutants.** **a**, The pathogenicity locus PaLoc of *C. difficile* 630 showing the intron insertion sites for the toxin mutants. **b**, Southern blot using an intron specific probe. The control plasmid (pMTL007C-E2) and the genomic DNA of the four strains was digested with EcoRV, which resulted in a band of ca. 9 kb for the plasmid, 10 kb for the *tcdB*-mutation (in  $A^+B^-$  and  $A^-B^-$ ), just over 3 kb for the *tcdA*-single mutant ( $A^-B^+$ ) and 1.8 and 1.2 kb for the *tcdA*-mutation in the double mutant, due to an additional EcoRV site in the *catP* gene. **c**, Western blot probing culture supernatants with anti-TcdA-antibody (tgcBIOMICS). **d**, Western blot probing culture supernatants with anti-TcdB-antibody (tgcBIOMICS).

**Figure 2. In vitro cytotoxicity.** **a** and **b**, Supernatants of the parental strain  $A^+B^+$  and the three mutants  $A^-B^+$ ,  $A^+B^-$  and  $A^-B^-$  were used in cell culture assays to measure cytotoxicity. HT29 cells (a) and Vero cells (b) were cultured to a flat monolayer before adding *C. difficile* supernatants in 4-fold dilutions series. After a 24 h incubation toxin endpoint titres were determined. Data represent the mean  $\pm$  s.d.; n = 3. **c** and **d**, Toxin neutralization assays. Appropriate dilutions of supernatants were pre-incubated with a suitable concentration of anti-TcdA or anti-TcdB serum for 1 h at 37°C and then added to HT29 cells (c) and Vero cells (d) which were evaluated after 24 h. Scale bar represents 2 mm.

**Figure 3. Virulence of *C. difficile* strains in hamsters.** Groups of 8 hamsters were challenged with *C. difficile* 630  $\Delta erm$  ( $A^+B^+$ ), or one of the toxin mutant strains,  $A^+B^-$ ,  $A^-B^+$  or  $A^-B^-$ . **a**, Colonisation of Golden Syrian hamsters by each

strain is presented as time from inoculation to colonisation in days. n = 8. **b**, Time from colonisation to death. The duration of the experiment was set at 14 days. n = 8.

## Methods

**Strains and growth conditions.** Strains used in this study were *E. coli* TOP10 (Invitrogen) as a cloning host, *E. coli* CA434<sup>23</sup> as a conjugal donor and *C. difficile* 630 $\Delta$ erm<sup>8</sup> and mutants. All strains were stored at -80°C stocks upon arrival and maintained as frozen stocks ever since. *E. coli* cultures were grown on Luria Bertani medium, aerobically, 37°C and shaking if liquid unless stated otherwise. *C. difficile* cultures were grown in BHIS<sup>24</sup> or TY<sup>25</sup>, anaerobically, 37°C in an anaerobic workstation (Don Whitley, UK). Antibiotics were used at the following concentrations where appropriate: Chloramphenicol (25  $\mu$ g/ml or 12.5  $\mu$ g/ml), thiamphenicol (15  $\mu$ g/ml), spectinomycin (250  $\mu$ g/ml or 750  $\mu$ g/ml), erythromycin (2.5  $\mu$ g/ml), D-cycloserine (250  $\mu$ g/ml) and ceftioxin (8  $\mu$ g/ml).

**Molecular biology techniques.** Qiagen mini prep kits were used to purify plasmids. Genomic DNA was obtained by phenol-chloroform extraction. Digests, PCRs and DNA purification were all done according to general protocols<sup>26</sup>. DNA sequencing was performed by Geneservice, UK.

**Construction and characterization of mutants.** The *C. difficile* single mutant strains A<sup>-</sup>B<sup>+</sup> and A<sup>+</sup>B<sup>-</sup> were made using ClosTron technology as described previously<sup>5, 6</sup>. The A<sup>-</sup>B<sup>-</sup> double mutant strain was made from the A<sup>+</sup>B<sup>-</sup> mutant using a *catP*-based ClosTron using the 'pseudo-suicide' vector principle as described elsewhere<sup>25</sup>. The following

retargeted plasmids pMTL007C-E2::*Cdi-tcdA*-1584s, pMTL007C-E2::*Cdi-tcdB*-1511a and for the double pMTL007S-C7::*Cdi-tcdA*-1584s were used. To verify the correct insertions, primers used for *tcdA* were: *Cdi-tcdA*-F2 (5'-TCAATTGACAGAACAAGAAATAAATAGTCTATGGAGC-3') and EBS universal<sup>5</sup>, and *Cdi-tcdA*-R2 (5'-TACCCCATTTGTCTTCAGAAAGAGATCCACC-3') and *ErmRAM*-R (5'-ACGCGTGCGACTCATAGAATTATTTCTCCCG-3'); and for *tcdB* were: *Cdi-tcdB*-F1 (5'-TGATAGTATAATGGCTGAAGCTAATGCAGATAATGG-3') and *ErmRAM*-R, and *Cdi-tcdB*-R1 (5'-CTTGCATCGTCAAATGACCATAAGCTAGCC-3') and EBS universal.

**Southern blotting.** Mutants were verified by Southern blot using an intron specific probe. 2 µg genomic DNA were digested with *EcoRV* (NEB) overnight. The blot was carried out using a DIG high prime labelling and detection kit (Roche) according to the manufacturer's instructions.

**Western blotting.** Supernatants from 96 h cultures, grown anaerobically in TY, were concentrated 8-fold by chloroform-methanol-precipitation. Proteins were standardized and run on Tricine gels 10-20% (Invitrogen) and transferred onto nitrocellulose membrane. The membranes were blocked with milk powder and then incubated with mouse monoclonal anti-TcdA antibody TTC8 and mouse monoclonal anti-TcdB antibody 2CV (tgcBIOMICS) respectively, followed by protein A-HRP (Sigma). The ECL Western blot detection kit from Amersham was used according to the manufacturer's instructions.

**Cell toxicity assays.** The four strains were grown overnight in 5 ml TY under anaerobic conditions as previously described<sup>22</sup>, then the cell density was standardised, the cells centrifuged and supernatants filtered. Supernatants were diluted in a 4-fold series and 20 µl of dilutions were added onto monolayers of Vero and HT29 cells preincubated in 96 well plates for 48 h (at 37°C, 5% CO<sub>2</sub>). Cytotoxicity was recorded after 24 h. For the

neutralization assay appropriate dilutions of supernatants were pre-incubated with a suitable concentration of anti-TcdA or anti-TcdB serum (polyclonal, tgcBIOMICS) for 1 h at 37°C. These were then added to Vero and HT29 cells which were evaluated after 24 h.

Vero and HT29 cells were grown in DMEM or McCoy's 5A, respectively, with 10% v/v foetal calf serum and 1% v/v penicillin-streptomycin at 37°C, 5% CO<sub>2</sub> until confluent. Cells were detached using trypsin, and seeded into 96 well plates at a density of ca.  $2 \times 10^5$  cells/ml. All assays were carried out in triplicate. GraphPad Prism was used for statistical analysis. Significant differences were assessed using one way ANOVA tests.

**qRT-PCR.** The qRT-PCR was carried out as described elsewhere<sup>4</sup>.

**Hamster infection model.** We used a block design with final group sizes of 8 animals. Female Golden Syrian hamsters (100 – 130 g) were housed singly in individually ventilated cages. Each hamster was dosed with clindamycin (30 mg/kg) 5 days prior to being infected orally with 100 spores each. Hamsters were monitored for signs of infection and sacrificed when the endpoint was met. The hamsters were handled individually in a microbiological safety cabinet. In line with UK Home Office requirements to reduce animal suffering, an alternative to death was used as the endpoint. Animals were monitored 3-4 times per day following infection and were assessed for several parameters including presence and severity of diarrhoea, weight-loss, level of activity, starey coat, sunken eyes, hunched posture and response to stimulus. A scoring system based on severity of changes observed (ranging from 0-3 for each parameter) was used to quantify changes in the condition of the animals which were euthanised when a pre-determined cumulative value was reached.

Faecal pellets were collected daily and plated to determine the presence of *C. difficile*. Caecum samples from each hamster were homogenized, plated and *C. difficile* counts

obtained. PCR was performed to determine the genotype of each strain recovered from hamsters. Faecal and caecum samples were plated on Fructose agar (*Clostridium difficile* agar base, Oxoid) with cycloserine cefoxitin, taurocholate, tetracycline and amphotericin to select for *Clostridium difficile*. The following primer sets were used to authenticate the various strain genotypes: oligonucleotides 3800 and 10050<sup>8</sup> to confirm the cells were derived from *C. difficile* 630 $\Delta$ erm, oligonucleotide primers Cdi-tcdA-F2 (5'-TCAATTGACAGAACAAGAAATAAATAGTCTATGGAGC-3') and Cdi-tcdA-R2 (5'-TACCCCATTTGTCTTCAGAAAGAGATCCACC-3') to distinguish between the toxin A insertional mutants and wildtype, and the primers Cdi-tcdB-F1 (5'-TGATAGTATAATGGCTGAAGCTAATGCAGATAATGG-3') and Cdi-tcdB-R1 (5'-CTTGCATCGTCAAATGACCATAAGCTAGCC-3') to distinguish between the toxin B insertional mutation and wildtype. A figure showing annealing of the primers and gel pictures can be found in supplementary information, which is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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