**Clostridium difficile** 027/BI/NAP1 Encodes a Hypertoxic and Antigenically Variable Form of TcdB

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**Abstract**

The *Clostridium difficile* exotoxin, TcdB, which is a major virulence factor, varies between strains of this pathogen. Herein, we show that TcdB from the epidemic BI/NAP1/027 strain of *C. difficile* is more lethal, causes more extensive brain hemorrhage, and is antigenically variable from TcdB produced by previously studied strains of this pathogen (TcdB003). In mouse intoxication assays, TcdB from a ribotype 027 strain (TcdB027) was at least four fold more lethal than TcdB003. TcdB027 caused a previously undescribed brain hemorrhage in mice and this correlated with a heightened sensitivity of brain microvascular endothelial cells to the toxin. TcdB003 and TcdB027 also differed in their antigenic profiles and did not share cross-neutralizing epitopes in a major immunogenic region of the protein. Solid phase humoral mapping of epitopes in the carboxy-terminal domains (CTD) of TcdB027 and TcdB003 identified 11 reactive epitopes that varied between the two forms of TcdB, and 13 epitopes that were shared or overlapping. Despite the epitope differences and absence of neutralizing epitopes in the CTD of TcdB027, a toxoid form of this toxin primed a strong protective response. These findings indicate TcdB027 is a more potent toxin than TcdB003 as measured by lethality assays and pathology, moreover the sequence differences between the two forms of TcdB alter antigenic epitopes and reduce cross-neutralization by antibodies targeting the CTD.

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**Introduction**

*Clostridium difficile* is the leading cause of hospital-acquired diarrhea in developed countries [1,2,3,4]. This spore-forming anaerobic bacterium contaminates hospital environments and infects patients undergoing antibiotic therapy within healthcare facilities [2,5,6]. Despite these problems, historically, treatment with antibiotics such as metronidazole and vancomycin has been challenging due to the presence of spores in hospital environments and the ability of the bacterium to contaminate hospital environments and infect patients undergoing antibiotic therapy within healthcare facilities [2,5,6]. Despite these problems, historically, treatment with antibiotics such as metronidazole and vancomycin has been challenging due to the presence of spores in hospital environments and the ability of the bacterium to contaminate hospital environments and infect patients undergoing antibiotic therapy within healthcare facilities [2,5,6].

*C. difficile* produces two large clostridial toxins, TcdA and TcdB, which cause extensive tissue damage and are major virulence factors in human disease [32,33,34]. Our work has focused on understanding how variations in the toxins produced by historical and epidemic strains change the extent of *C. difficile* virulence [35,36]. Of particular interest are the differences in the sequence and activities of TcdB, which has been implicated as a critical *C. difficile* virulence factor [37,38]. We hypothesize that variation between TcdB from previously predominant ribotypes and BI/NAP1/027 strains, is a major contributing factor to the increased virulence of the recently emerged forms of *C. difficile*.

TcdB (∼270 kDa; 2366 amino acids; YP_001087133.1) is a single chain polypeptide toxin where the glucosyltransferase domain is located at the N-terminus (GTD: 1–543), followed by an autoprocessing site between amino acid 543 and 544 which is subject to intramolecular cleavage by the cysteine protease domain (CPD: 544–807), a hydrophobic transmembrane domain (TMD: 956–1128), and a putative receptor binding domain at the C-terminus (CTD: 1651–2366) [39,40,41,42,43,44,45]. The gene encoding TcdB is located within a pathogenicity locus on the chromosome of *C. difficile* along with genes encoding TcdA (enterotoxin; YP_001087137.1), TcdE (YP_001081361.1), and regulators of toxin gene expression (TcdC, YP_001087138.1 and...
Variable Toxicity and Epitope Diversity of TcdB

Author Summary

During the past decade, the *C. difficile* BI/NAP1/027 strain has emerged and in some settings predominated as the cause of *C. difficile* infection. Moreover, in some reports *C. difficile* BI/NAP1/027 has been associated with more severe disease. The reasons for association of this strain with more severe disease and relapse are poorly understood. We compared the toxicity and antigenic profiles of the major *C. difficile* virulence factor, TcdB, from a previously studied reference strain and a BI/NAP1/027 strain. The results indicate TcdB027, the toxin from the BI/NAP1/027 strain, is more lethal and causes more extensive brain hemorrhaging than TcdB003, the toxin produced by a reference strain of *C. difficile*. Furthermore, the results show that the antigenic carboxy-terminal domain (CTD) encodes at least 11 epitopes that differ between the two forms of TcdB. In line with this, experiments demonstrate that antisera against the CTD does not cross-neutralize TcdB003 and TcdB027. The results also suggest that the CTD may not occupy the same role in TcdB027 as TcdB003, and identifying these key differences is a critical step toward understanding the virulence and systemic effects of *C. difficile* associated disease.

Results

TcdB027 Exhibits a Lower Lethal Dose Than TcdB003

In previous work we found that that TcdB027 is more cytotoxic and causes broader tissue damage in a zebrafish embryo model than TcdB003 [35]. To determine how this difference in activity might impact systemic damage and lethality between the two forms of the toxin, in the first set of experiments in this study we determined and compared the lethal doses of TcdB003 and TcdB027 in a murine systemic intoxication model. The previously published lethal dose of 220 μg/kg (i.p.) for TcdB03 [32] was used to establish a range of toxin concentrations for these treatments, but the lethality we observed via i.v. injection was much higher than previously reported. As a result, the initial doses of 100 μg/kg (data not shown), 50 μg/kg, and 25 μg/kg of TcdB003 were much more potent than anticipated, and resulted in a very rapid time to death (Fig. 1A). Therefore, the remaining mice were subjected to much lower doses of 5 μg/kg and 2.5 μg/kg of TcdB003. Based on the results of the TcdB003 treated mice, the TcdB027 group started with a dose of 10 μg/kg and was continued with 1:2 dilutions down to 625 ng/kg of TcdB027. After the mice were injected with TcdB003 or TcdB027, they were followed for up to 7 days and the survival curves of the data from these experiments are shown in Fig. 1B.

The data shown in Fig. 1 indicate mice injected with TcdB027 succumb to the toxin at a lower dose than that observed in mice injected with TcdB003. Within 26 h of treatment all of the mice administered 5 μg/kg of TcdB027 died or reached a moribund condition. In comparison, mice administered the same dose of TcdB003 did not succumb to the toxin until after 40 h and as long as 57 h with a median survival of 48 hr (Fig. 1C). At the next lower dose (2.5 μg/kg), no mice survived TcdB027 treatment, while all of the mice treated with TcdB003 survived (Fig. 1D). Based on these outcomes we estimated the LD50 of TcdB027 to be between 625 ng/kg and 1.25 μg/kg of body weight. In comparison, a higher range for TcdB003 was estimated and fell between 2.5 μg/kg and 5 μg/kg of body weight. Thus, in line with previous studies demonstrating more potent effects on cultured cells and zebrafish embryos, TcdB027 also appears to be more toxic than TcdB003 in a rodent model of intoxication.

TcdB027, but not TcdB003 Causes Extensive Brain Hemorrhaging

The results shown in Fig. 1, combined with our earlier findings in the zebrafish model [35], all point to the fact that TcdB027 is more toxic than TcdB003. Recent work by Steele and colleagues detected TcdA and TcdB circulating in the bloodstream of piglets infected by *C. difficile*, and this correlated with systemic effects that could be blocked by passive administration of antibodies against the toxins [52]. This led us to question whether TcdB027 might also cause more extensive systemic damage than TcdB003 due to its higher potency. To assess this, mice were administered TcdB003 (2.5 μg/kg to 50 μg/kg) or TcdB027 (625 ng/kg to 10 μg/kg) and tissue pathologies were examined. Tissues and organs from mice administered sublethal doses of the toxins did not reveal pathologies that differed from that of control (Fig. 2A). In contrast, abnormal tissue histologies were found in several of the major organs examined from mice intoxicated with lethal doses of TcdB.
Mice treated with either TcdB003 or TcdB027 showed pronounced liver damage with extensive blood-pooling, parenchymal cell loss, and evidence of hemorrhage, which can be visualized by the appearance and expansion of the dark red patches as the survival time progresses (Fig. 2A). To a lesser extent, acute hepatocellular coagulative necrosis and hemorrhage in the spleen along with follicular necrosis and possible apoptotic cells was also detected (data not shown). The severity of the observed pathologies was more related to the length of time of toxin exposure rather than toxin concentration. Figure 2A shows representative liver sections from TcdB003 and TcdB027 treated mice, illustrating that the damage is the more extensive in mice receiving the minimum lethal dose and surviving for the longest period of time.

Despite the difference in lethality, the majority of the in vivo effects of TcdB003 and TcdB027 were identical, with the exception of moderate to severe hemorrhage detected in the brain of TcdB027 treated mice. Indeed, brain hemorrhage was the most obvious difference between mice exposed to the two forms of TcdB. The brains of mice treated with TcdB003 displayed only small lesions while the brain hemorrhage of TcdB027-treated mice was profuse with large multi-focal areas of blood accumulation within the cerebellum and cerebrum (Fig. 2B). These data suggest there may be a loss of endothelial integrity in mice challenged with TcdB, as well as a significant difference in the in vivo targeting and tropism of TcdB003 versus TcdB027.

**TcdB027 Is More Toxic Than TcdB003 to Brain Microvascular Endothelial Cells**

Experiments were next performed to determine the toxicity of the two forms of the TcdB on endothelial cell lines as a possible correlation with the differences in the amount of brain hemorrhage. We first wanted to determine whether endothelial cells displayed increased sensitivity to TcdB compared to the epithelial-like cells (e.g., CHO cells) that are normally used in cytotoxicity assays. Rat Aortic Endothelial Cells (RAEC) exposed to TcdB003 and TcdB027 displayed very similar cytotoxic doses (Fig. 3A). The concentration needed to cause toxicity in 50% of culture cells (TCD50) for TcdB003 was 6.07\( \times \)10\(^{-2}\) M and 2.74\( \times \)10\(^{-2}\) M for TcdB027. Since the major differences in pathology between TcdB003 and TcdB027 occurred in the brain, we next tested rat brain microvascular endothelial cells (RBMVEC) for differences in sensitivity to the two forms of TcdB. Interestingly, there was a 10-fold difference in the cytotoxicity of TcdB027 on the RBMVECs, with the TCD50 being 6.32\( \times \)10\(^{-2}\) M compared to the TCD50 of 8.46\( \times \)10\(^{-2}\) M for TcdB003 (Fig. 3B). These data indicated that TcdB was highly cytotoxic on endothelial cells, as the previous published observations of TcdB003 and TcdB027 toxicity on CHO cells is 2.53\( \times \)10\(^{-1}\) M and 2.37\( \times \)10\(^{-1}\) M respectively. Additionally, the RBMVECs had a greater susceptibility to TcdB027, which correlates with the brain pathologies in Fig. 2B.

**The Carboxy-Terminal Domains of TcdB003 and TcdB027 (CTD003 and CTD027) Differ in Cell Interactions and Their Susceptibility to Antibody Neutralizations**

To further study the differences in the cell and organ targeting between TcdB003 and TcdB027, we focused on the CTD, which is thought to be important in facilitating cell interactions [39,53]. We hypothesized that if this region is indeed important in cell targeting, then the sequence differences between TcdB003 and TcdB027 in this region could be an important factor in the distinct cell tropism and animal pathologies between the toxins. We also predicted that these differences could change the profile of antigenic epitopes, and perhaps neutralizing epitopes, in the CTD. We designed a set of experiments to address both of these possibilities.
In order to evaluate differences in the CTD of TcdB003 and TcdB027 we expressed and purified protein fragments representing this region of each toxin. These fragments consisted of the final 721 amino acids of the TcdB protein, including the CROP region along with approximately 206 residues amino terminal to the CROP region. Based on previous sequence comparisons, there are 89 residues that differ between CTD003 and CTD027.[35]

Initially, each CTD was used as an antigen to immunize rabbits for the collection of CTD antisera, which were then used in TcdB neutralization assays to further determine the impact of the CTD on the activity of both TcdB003 and TcdB027. We first investigated the impact of a CTD003 on the cytotoxicity of both TcdB003 and TcdB027 and found that treatment with a CTD003 neutralized the cytotoxic and cytopathic effects of TcdB003 (Fig. 4A). However, a CTD003 caused no detectible reduction in the cytotoxicity of TcdB027 (Fig. 4A). ELISA analysis confirmed that while a CTD003 was only able to neutralize TcdB003 in cell culture, the polyclonal serum could recognize both TcdB003 and TcdB027 in vitro (Fig. 4B). When the a CTD027 antibody was used in the neutralization assay, we found no protection against either TcdB003 or TcdB027, although the serum strongly reacted with both forms of the toxin as determined by ELISA (Fig. 4A and 4B).

The data shown in Fig. 4 suggested that CTD027 and CTD003 differ in their profile of neutralizing epitopes (i.e. sequences where antibody binding blocks intoxication). It was also possible that TcdB027 shared the same sequences of TcdB003 neutralizing epitopes, but, unlike TcdB003, TcdB027 did not depend on these regions for cellular intoxication. To address this alternative explanation, serum against CTD003 was incubated with a 100-fold excess of CTD003 or CTD027, and the mixture was tested for its ability to neutralize cytotoxicity of TcdB003. We reasoned that if CTD027 contains sequences that are targets for antibody-mediated neutralization of TcdB003 then the preincubation with CTD027 should prevent the antiserum from neutralizing TcdB003. As expected, the addition of CTD003 in the neutralization assay resulted in the inhibition of antibody activity and a return to full cytotoxicity of TcdB003 (Fig. 4C and 4D). In line with the possibility that TcdB027 contains sequences that are neutralizing epitopes in TcdB003, preincubation with CTD027 also blocked the neutralizing effects antisera against TcdB003 (Fig. 4C and 4D).

**Fine Specificity Mapping of Antibody Responses Reveals Unique Epitope Differences between TcdB003 and TcdB027.**

The data from the analysis of antisera against the two forms of TcdB suggested there is likely to be shared epitopes between the two proteins, but the extent of shared and unique epitopes was...
difficult to predict. In order to begin to identify shared and unique epitopes between TcdB003 and TcdB027 we used solid phase peptide based ELISAs to map antibody reactive sequences in the CTD of TcdB. In all, 358 decamer peptides, overlapping by 8 residues and covering the entire CTD003 sequence, were synthesized and tested for reactivity to CTD003 or CTD027 sera. Sera was collected from rabbits immunized with CTD003 or CTD027 (n = 2), and when we compared the peptides recognized by αCTD003 to those recognized by αCTD027 we found an overall difference in the pattern of peptides recognized by antisera from the 2 groups (Fig. 5). Each serum sample was analyzed individually, and the average response of αCTD003 and αCTD027 to the CTD003 peptides is shown in Fig. 5. The analysis identified identical epitopes, overlapping epitopes, and epitopes unique to each form of the toxin. The analysis identified approximately 7 regions that were recognized only by αCTD003 (Fig. 5). The analysis also found 4 regions recognized by only αCTD027 and 13 regions where there was overlap or exact matches in the epitopes recognized by both sera (Fig. 5). The majority of the peptides identified are localized in the CROP domain, and many of the epitopes that differ in recognition between αCTD003 and αCTD027 are located sequentially, within the first seven repeats of the CTD. As summarized in Fig. 5, peptides recognized by only the αCTD003 serum were variable regions between the two toxins, with as many as 6 amino acid differences as in the case of peptide 21. In contrast, the peptides recognized by only αCTD027 were highly conserved between the two forms of TcdB, with only one peptide (#7), with a single amino acid change. These data suggest that sequence variation of TcdB027 impacts antibody recognition of conformational epitopes and may contribute to differences in conformational epitopes as well.

Mouse Antiserum against ToxoidB027 Is Cross-Protective In Vitro and In Vivo

The observation that the CTD of TcdB027 is a poor target for the production of antibodies that prevent toxicity on CHO cells, raised concerns about the overall antigenicity of TcdB027. The majority of the amino acid sequence variation between TcdB003 and TcdB027 occurs in the CTD, so we reasoned that producing antibodies using the holotoxin as an antigen could have better potential to be broadly neutralizing. Both TcdB003 and TcdB027 were inactivated using formaldehyde to create ToxoidB003 and ToxoidB027. These toxoids were used as antigen to immunize mice and test for protective antibodies against TcdB. After two subsequent boosts, serum was collected from the mice, and the neutralizing effects were tested in vitro. The data in Fig. 6A shows that the mouse antiserum toward ToxoidB027 protected against the cytotoxic effects of both TcdB003 and TcdB027, while anti-ToxoidB003 was not cross-neutralizing and only maintained the cell viability of the CHO cells treated with TcdB003. The immunized mice were next tested for protection from TcdB in vivo, using a 2-fold minimum lethal dose of TcdB003 or TcdB027. Consistent with the in vitro neutralization data, all mice immunized with ToxoidB027 were completely protected from i.v. challenge of both TcdB003 and TcdB027 (Fig. 6B and 6C). Immunization with ToxoidB003 provided only a slight, yet significant protective effect, increasing the median survival from 15 h to 24 h in mice injected with TcdB003, but only from 9 h to 13 h in mice challenged with TcdB027 (Fig. 6B and 6C). Eventually, all of the ToxoidB003 mice succumbed to the effects of TcdB027, and only two ToxoidB003 mice were fully protected from TcdB003 (Fig. 6B and 6C). Whereas the antisera to the CTD of TcdB027 showed no effect, antibodies to the toxoid form of TcdB027 successfully inhibited toxicity, suggesting that the protective effect against TcdB027 is better conferred by the full-length toxin rather than the CTD in this system.

Discussion

C. difficile infection is a complex illness commonly involving colitis and, in more severe cases, systemic complications [54,55,56]. In the current study we sought to determine how systemic complications vary between two forms of TcdB. To focus on the systemic events mediated by the different forms of TcdB, we bypassed the intestinal stage of this illness by directly administering toxin intravenously. This analysis found that TcdB027 was more lethal and caused more pronounced systemic damage than TcdB003. Further studies revealed this effect correlated with differences in the extent of specific cellular tropisms between the variants of TcdB. Assessing the CTD of TcdB found that this region may contribute to not only differences in tropism, but also accounts for a variability in the antigenic make-up of this domain. Collectively, the data support the notion that TcdB027 is not only more potent than TcdB003, but may have sequence alterations that prevent cross neutralization.

Several recent observations led us to predict that the increased virulence of C. difficile BI/NAP1/027 is due to altered TcdB activity. First, the sequence of TcdB, but not TcdA, varies between
the two strains [35,57]. Second, in cell culture systems, TcdB027 is more potent on a broad range of cell types [35,47,57]. Thus, we hypothesized that TcdB027 could have a lower lethal dose and cause more extensive tissue damage in vivo. Our findings support this hypothesis. When experiments compared the lethal doses of TcdB027 and TcdB003 the BI/NAP1/027 toxin was found to be 4 times more lethal than the ribotype 003 toxin (Fig. 1). More importantly, TcdB027-treated mice died much more quickly and, in some cases, in less than half the time than TcdB003-treated mice. In regards to the pathologies, TcdB027 clearly caused brain damage that was less prominent in mice treated with TcdB003 (Fig. 2). These findings provide insight into the differences in the in vivo effects of TcdB027 and TcdB003, and this variation in toxicity could contribute to more severe disease caused by recently emerged strains of C. difficile.

Very little is known about the underlying mechanisms of C. difficile-induced systemic damage and complications. The extent to which the pathologies observed in toxin-treated mice reflect systemic complications in humans is not known and there is clearly a need for more studies in this area. However, several reports make it reasonable to suspect the toxins contribute to the systemic complications in this disease [54,55,56]. The idea that toxin enters the bloodstream during disease is supported by recent work using a piglet model of C. difficile infection where TcdA and TcdB were detected in the bloodstream of the infected animals [52]. Other work has demonstrated that serum IgG, and not mucosal IgA, against the toxins correspond with protection against illness and relapse [58,59,60] further supporting the notion of systemic effects of these toxins. Thus, the more extensive systemic damage caused by TcdB027 may explain in part why C. difficile NAP1/BI/027 is associated with more severe disease.

Our previous studies found that TcdB003 is cardiotoxic and targets cardiomyocytes with an equal efficiency to TcdB027 [35,61]. In vivo and in vitro data support the notion that the

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**Figure 4. Neutralization of TcdB with αCTD antiserum.** (A) Percent viability of CHO cells treated for 24 hrs with 37 pM of TcdB003 (black) or TcdB027 (gray) alone or after preincubation for 1 h with αCTD003 antiserum or αCTD027 antiserum. Cell viability was determined by WST-8 staining and the error bars represent the standard deviation from the mean of three biological replicates containing three technical replicates. *** p < 0.001 (B) ELISA data showing the specificity of the αCTD antibodies to TcdB003 (black) and TcdB027 (gray) as measured by the optical density at 405 nm. The error bars represent the standard deviation from the mean of three samples. (C) Percent viability of CHO cells treated for 24 hrs with 37 pM TcdB003 alone (black) or combined with αCTD003 antiserum (dark gray), or with αCTD003 antiserum plus 3.7 nM of the CTD003 (white) or CTD027 (light gray) protein fragments. Cell viability was determined by WST-8 staining and the error bars represent the standard deviation of three biological replicates containing three technical replicates. *** p < 0.001 (D) Representative phase contrast photographs of CHO cells after 6 h exposure to (a) 370 pM of TcdB003 alone or 370 pM TcdB027 with (b) 1:100 CTD003 antiserum or 1:100 CTD003 antiserum plus (c) excess CTD003 or (d) CTD027 (e) untreated control.
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two forms are TcdB are very similar in their cardiotoxic effects, but the sequence differences in TcdB027 allow the toxin to target other tissues and cell types more effectively than TcdB003. Consistent with this idea, the TCD050 for TcdB027 and TcdB003 was found to be very similar on aortic endothelial cells, but substantially lower for TcdB027 on brain microvascular endothelial cells. Thus, the evidence to date supports a model where both forms of TcdB are cardiotoxic, but TcdB027 is more potent on other tissue and cell types.

The fact that TcdB027 is a more potent toxin than TcdB003 is now well established by several in vivo and in vitro analyses [35,47], including the ones used in this study. Yet, the sequence changes accounting for these differences in activity have not been defined. There are 198 residue differences between TcdB027 and TcdB003 and each of the residues known to be critical for TcdB activities are conserved between the two forms of this toxin. In previous work we found that TcdB027 undergoes more complete autocleavage because it is able to engage intramolecular substrate more effectively than TcdB003 [36]. This implies the conformation of TcdB027 may be different than that of TcdB003. We have also shown that TcdB027 undergoes dramatic pH-dependent conformational changes more extensively and at a higher pH than TcdB003 [35]. Again, this is unlikely to be related to a single residue change and could be the result of the collective sequence differences.

The finding that antibodies against the CTD neutralized TcdB003 but not TcdB027 on CHO cells could be the result of TcdB027 using an alternative means of cell recognition. Interestingly, Olling et al. have reported that the CROP domain of TcdA is involved in cellular uptake of the toxin, but it is not entirely responsible for cell recognition and binding [49]. In a like manner, it is plausible that the role of the CTD has become less significant in TcdB027 and variations have little effect on the toxin. If so, TcdB027 could bind cells by an alternative manner, which helps
explain the current data that TcdB027 has a broad effect in mice, as well as previous data that shows extensive necrosis in a zebrafish model of intoxication.

The data from the peptide arrays showed CTD003 reactivity with many epitopes in which the sequence varied in TcdB027. Whether these sequence variations evolved as a way of allowing TcdB027 to avoid immune recognition or if this is a means of TcdB027 altering its activity, is not yet clear. If the former is true, it could be possible that a change to one single epitope could be responsible for the lack of neutralization of TcdB027. However, work by Torres and Monath suggests that while the CTD is quite antigenic, antibodies to a single peptide epitope fail to prevent cytotoxicity of TcdB [50]. Finally, in further support of the idea that the two toxins are not identical in their overall structure, three of the epitopes recognized by serum against TcdB027 were not recognized by serum against TcdB003 despite the fact that these sequences were the same (Fig. 5). The conformational differences in the two forms of TcdB could determine whether identical sequences are antigenic.

It is also important to consider this variation in the context of virulence of C. difficile, as well as vaccination. Our previous work suggests that TcdB027 enters cells more rapidly and efficiently than TcdB003 [35]. Given that the CTD is believed to facilitate interactions with the cell surface, it is possible that antigen recognition occurs, but the toxin overcomes this by utilizing a more effective mechanism of cell entry. Arguing against this possibility is the fact that we did not detect even a minor change in the rates of TcdB027-induced cell rounding or the overall level of cell killing. It’s also important to note that our experiments involved preincubating TcdB027 with the antisera. Therefore, if the toxin overcame the neutralizing effect by more efficient cell entry, we would expect to see at least a nominal change in toxicity, but this doesn’t appear to be the case. We believe the reasonable explanation is that the neutralizing epitopes of TcdB027 are sufficiently altered to avoid toxin neutralization or that the toxin has a different mechanism of interacting with and entering the cell. These data also suggest successful vaccines targeting TcdB will need to include antigens from multiple forms of this toxin or, alternatively, be designed to target highly conserved neutralizing epitopes shared among variants of TcdB.

Although further studies are needed, the toxoid of TcdB027 could provide a vaccine that generates a broadly neutralizing response. Given that the CTD027 did not generate an antibody response that protected CHO cells from TcdB027, and past studies have found that TcdB toxoid is not a highly effective vaccine [62,63], we were surprised to find the toxoid of TcdB027 stimulated a potent neutralizing response in mice. It has been known for many years that anti-serum does not cross neutralize TcdA and TcdB, making it reasonable to consider the possibility that anti-serum to the variant forms of TcdB also do not cross neutralize. This does not appear to be the case. As shown in Fig. 6, mice vaccinated with the toxoid form of TcdB027 were completely protected against both TcdB003 and TcdB027. In line with a prior study by Wang et al. [64], the toxoid of TcdB003 evoked only marginal immunoprotection against TcdB, and we found this to be true for mice challenged with either the historical or ribotype 027 form of the toxin. This raises the possibility that converting TcdB003 into a toxoid alters the protein in a way that reduces immunogenicity, but sequence differences in TcdB027 make this form of the toxin more effective as a toxoid.

Overall, these findings demonstrate critical differences between TcdB produced by ribotype 003 and ribotype 027 strains of C. difficile. The sequence variations in TcdB027 impact the toxin’s cytotoxicity, lethality, and antigenic make-up, and likely contribute...
to the overall heightened virulence of *C. difficile* BI/NAP1/027 strains.

**Materials and Methods**

**Ethics Statement**

The animal immunization and toxin challenge studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal procedures reported herein were approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee at OUHSC (IACUC protocol # 09-092-I and 11-016-I). The procedures precluded the use of anesthesia for in vivo lethal challenge assays. To minimize pain and distress, the mice were monitored at least twice daily and any animals with signs of distress such as labored breathing, lethargy, inability to eat or drink, ruffled fur, disorientation, or loss of 20% body weight were euthanized immediately. This method was approved by the IACUC and monitored by a qualified veterinarian.

**Animals, Bacterial Strains, and Cell Culture**

*C. difficile* VPI 10463, a ribotype 003 strain [produces TcdB with identical sequence to the 630/ribotype 012 strain], and *C. difficile* BI17 6493, a ribotype 027 strain (a gift from Dr. Dale Gerding), were used as sources of to purify TcdB003 and TcdB027 respectively.

Female BALB/cJ and C57B/6j mice (Jackson Laboratories), aged 8 weeks, were purchased from The Jackson Laboratories (Bar Harbor, ME) and handled in accordance with IACUC guidelines at University of Oklahoma Health Science Center.

Rat Brain Microvascular Endothelial Cells (RBMEVEC) and Rat Aortic Endothelial Cells were a generous gift from the laboratory of Dr. Eric Howard (University of Oklahoma Health Sciences Center) and have been described previously [65,66]. CHO-K1 cells were purchased from American Type Culture Collection (ATCC). RBMEVEC and RAEC were grown in DMEM containing 10% FBS while CHO cells were grown in F12-K with 10% FBS. All cell types were used between passage 15–30, and were maintained in tissue culture treated T-75 flasks (Corning) at 37°C in the presence of 6% CO2.

**Production of Native Toxin, Toxoid Preparation, and Purification of Recombinant TcdB Fragments**

*C. difficile* was cultured using the dialysis method as previously described [35] and TcdB was isolated using anion-exchange (Q-Sepharose) chromatography in 20 mM Tris-HCl, 20 mM CaCl2, pH 8.0, following a thyroglobulin affinity chromatography protocol to first remove TcdA [67]. Purification of TcdB was confirmed by visualization of a single 270 kDa band by SDS-PAGE, and LC/MS/MS analysis (University of Oklahoma Health Science Center).

Toxoid versions of TcdB003 and TcdB027 were prepared by mixing 500 µl of TcdB (0.4 µg/µl) into 500 µl of 8% formaldehyde with 8.5 mg of lysine to help prevent precipitation and aggregation of the formalized protein [68,69], and incubating at 37°C overnight. The volume was then brought up to 10 ml with PBS, yielding 20 µg/ml of ToxoidB in 0.4% formaldehyde with 0.425 mg/ml lysine. Both toxoid preparations lacked toxic activity as confirmed by the absence of cytopathic effects on CHO cells.

The CTD-encoding region of tcd gene (YP_001087135.1: nucleotides 4961–7111) from the strain VPI 10463 was codon optimized and cloned into pET15b (Genscript). The CTD of the tcd gene (YP_003217086.1: nucleotides 4961–7111) from the NAP1 strain was cloned from a pET15b plasmid containing full-length *tab* that had been codon optimized by Genscript. The CTD gene was amplified using primers 5'-GATCATATGCTG-TATGTTGGTTAACC-3' and 5'-AACGGATCTTTATGCT-TAATACAGA-3' containing BamHI and NdeI sites for cloning into pET15b. The CTDs were expressed using *Escherichia coli* BL21 star DE3 (Invitrogen) at 16°C overnight and then purified by Ni²⁺ affinity chromatography (HisTrap, GE Life Sciences) resulting in proteins representing TcdB1651–2366 from both TcdB003 and TcdB027.

**Lethal Dose Determination and Organ Pathologies**

To determine the differences in the minimum lethal dose of TcdB003 and TcdB027, 100 µl of TcdB003 or TcdB027 dilutions in phosphate-buffered saline was injected intravenously into the tails of BALB/cJ mice using a 27-gauge needle. Twenty mice were given TcdB003 in groups of 4, receiving doses of 2 µg, 1 µg, 500 ng, 100 ng, and 50 ng. Twenty additional mice were injected with doses of 200 ng, 100 ng, 50 ng, 25 ng, and 12.5 ng of TcdB027 (n = 4). The animals were monitored for up to 7 days post challenge for toxin effects and mortality, and mice were euthanized if they became significantly distressed or moribund. Survival was graphed using Kaplan-Meier analyses on GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Immediately after death, the mice were dissected and major organs and tissues were submerged in formalin fixative overnight. Tissue sectioning, slide preparation, H&E staining, and pathology analysis was performed by the Department of Comparative Medicine at OUHSC.

**Animal Immunizations and TcdB Challenges**

Two rabbits per group were immunized with 0.1 mg of the CTD fragment of TcdB003 or TcdB027 in complete Freund’s adjuvant on day 1 and boosted with 0.1 mg in incomplete Freund’s adjuvant on days 14, 21, and 49. Blood samples were collected on days 0, 35, and 56. These experiments were carried out by Cocalico Biologicals Inc. (Reamstown, PA). BALB/cJ mice (20 mice each for ToxoidB003 and ToxoidB027) were injected in equal portions subcutaneously and intraperitoneally with 2 µg of toxoid in PBS emulsified 1:1 in 100 µl of complete Freund’s adjuvant on day 1 and boosted with 2 µg in incomplete Freund’s adjuvant on day 10. Control mice were similarly immunized and boosted using an unrelated peptide. Blood samples were collected via tail bleeds on day 0 and 24, and each bleed was tested by ELISA to evaluate toxoid response.

After completion of the immunizations, the mice were subjected to i.v. challenges of TcdB003 and TcdB027. Each immunization group (ToxoidB003, ToxoidB027, control) contained 20 mice, and 9 from each group were injected via the tail vein with a 2-fold lethal dose of either TcdB003 or TcdB027. The previously established minimum lethal dose was used to set the 2LD100 at 200 ng per mouse for TcdB003 and 50 ng per mouse for TcdB027. The remaining 2 mice from each group were euthanized and exsanguinated for serum collection. The animals were monitored for up to 7 days post challenge for toxic effects and mortality, and mice were euthanized if they became significantly distressed or moribund. Survival was graphed using Kaplan-Meier analyses and compared with the Log-rank test on GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

**Characterization of Antibody Responses**

Direct antigen ELISAs were used to measure the antibody reactivity in animal sera. 1 µg of purified TcdB or CTD fragment was coated per well in polystyrene plates at 4°C overnight. The
plates were washed and blocked with 0.1% BSA in PBS for 1 h at room temperature. Then, the rabbit sera diluted at 1:100 and 1:1000 in PBS-Tween with 0.1% BSA was added in triplicate and incubated for 2–3 h at room temperature. Plates were washed with PBS-Tween and incubated with anti-rabbit IgG conjugated to alkaline phosphatase [Jackson ImmunoResearch Laboratories, Inc] at a dilution of 1:5,000 for 3 h at room temperature, then washed and developed with p-Nitrophenyl Phosphate substrate (Sigma). Plates were read at 405 nm using a Tecan-infinite plate reader (Tecan Group, Ltd.). Plates were read when the positive control reached an OD of 1.0 and the assay was considered invalid if the negative control was over OD 0.2.

**Cytotoxicity and TcdB Neutralization Assays**

Cells were seeded in 96 well plates at a density of 1–2×10⁴ cells per well in DMEM or F12-K (ATCC) containing 10% FBS (ATCC). For TcdB sensitivity measurements on endothelial cells, using a CCK-8 assay according to manufacturer’s instructions with the toxin/antiserum mixture or toxin alone and incubated at 37°C for 1 h at 37°C. The absorbance was read at 405 nm using a Tecan-infinite plate reader (Tecan Group, Ltd.). Next, washes were performed as previous and the peptide ELISAs was developed using 100 µl/well of a 1 mg/ml solution of p-nitrophenyl phosphate dissolved in 150 mM carbonate buffer pH 10.4 containing 100 mM glycerol, 1 mM MgCl₂ and 1 mM ZnCl₂. The absorbance was read at 405 nm using a Tecan-infinite plate reader (Tecan Group, Ltd.), and the results were normalized to the standard positive control peptide having an OD of 1.0. Positive epitopes were defined as at least two consecutive peptides with an OD greater than 2 standard deviations above the mean of pre-blend serum.

**Accession Numbers**

Relevant SwissProt accession numbers are P18177 (TcdB003/ CTD003), P16154 (TcdA003), C9YJ35 (TcdB027/CTD027), C9YJ37 (TcdA027).

**Author Contributions**

Conceived and designed the experiments: JML LDH JAJ JDB. Performed the experiments: JML LDH. Analyzed the data: JML LDH [JDB]. Contributed reagents/materials/analysis tools: JAJ JDB. Wrote the paper: JML JDB.

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