CLOSTRIDIUM DIFFICILE TOXIN-BASED VACCINE

TECHNICAL FIELD

This invention is in the field of toxin-based vaccines against *Clostridium difficile*.

BACKGROUND ART

C. difficile is a Gram-negative, spore forming anaerobic bacterium that can reside asymptomatically in the intestinal tract of humans. Depletion of other intestinal flora, for example by antibiotic and chemotherapeutic treatment, creates an ecological niche which allows *C. difficile* spores to germinate in the colon, resulting in serious intestinal disease [1]. Antibiotic treatment can therefore transform this normally harmless micro-organism into the causative agent of a spectrum of intestinal diseases, an outcome that is particularly prevalent in hospitalised patients.

C. difficile is the predominant pathogen of nosocomial intestinal infections [2,3] and causes approximately 20% of the cases of antibiotic-associated diarrhoea, up to 75% of the cases of antibiotic-associated colitis, and nearly all cases of pseudomembranous colitis [4]. Host factors such as advancing age, pre-existing severe illness and weakened immune defences predispose individuals to symptomatic infection [1]. Such *C. difficile*-associated disease (CDAD) usually occurs in intensive care units, particularly affecting patients over 60 years of age.

Treatment of CDAD typically involves the cessation of the offending antibiotic, initiation of oral metronidazole or vancomycin therapy and fluid replacement. However, the emergence of antibiotic-resistant enteropathogens has led to concerns over the use of antibiotics to treat CDAD. Moreover, up to 20% of patients relapse within 1-2 weeks of completing a course of antibiotics and the risk of relapse increases markedly with each additional relapse [5,6]. It is also reported that over 50% of the relapse incidents are due to a re-infection with a different *C. difficile* strain, rather than recurrence of the primary infection [7]. Preventive measures are based on patient isolation, implementation of hand hygiene and contact precaution, which have had variable and often limited success.

There is at present, no effective vaccine against CDAD. It is an object of the invention to provide compositions which are effective in raising immune responses against *C. difficile* for use in the development of vaccines for preventing and/or treating *C. difficile* associated diseases.

DISCLOSURE OF THE INVENTION

The invention thus provides an immunogenic composition comprising a combination of *Clostridium difficile* antigens, said combination comprising:

a) a ToxB-GT antigen and a TcdA antigen; or

b) a ToxA-GT antigen and a TcdB antigen.

Thus, the invention provides an immunogenic composition comprising a combination of *Clostridium difficile* antigens, said combination comprising a) a ToxB-GT antigen and a TcdA antigen; or b) a

ToxA-GT antigen and a TcdB antigen. Preferably, the ToxB-GT antigen and/or the ToxA-GT antigen are detoxified.

In one embodiment, the ToxB-GT antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:18 or SEQ ID NO: 60; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:18 or SEQ ID NO: 60, or of a polypeptide having 80% or more identity to SEQ ID NO:18 or SEQ ID NO: 60 and that comprises an epitope of SEQ ID NO:18 or SEQ ID NO: 60; the ToxA-GT antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:4 or SEQ ID NO: 56; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:4 or SEQ ID NO: 56, or of a polypeptide having 80% or more identity to SEQ ID NO:18 or SEQ ID NO:56 and that comprises an epitope of SEQ ID NO:4 or SEQ ID NO:56; the TcdA antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:1; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:1 , or of a polypeptide having 80% or more identity to SEQ ID NO:1 and that comprises an epitope of SEQ ID NO:1; and the TcdB antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:2; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:2, or of a polypeptide having 80% or more identity to SEQ ID NO:2 and that comprises an epitope of SEQ ID NO:2.

In one embodiment, the immunogenic composition comprises a ToxB-GT antigen and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more TcdA antigens, optionally selected from (1) a ToxA-ED antigen (SEQ ID NO: 3), (2) a ToxA-GT antigen (SEQ ID NO: 4), (3) a ToxA-CP antigen (SEQ ID NO:5), (4) a ToxA-T antigen (SEQ ID NO: 6), (5) a ToxA-T4 antigen (SEQ ID NO: 7), (6) a ToxA-B antigen (SEQ ID NO: 8), (7) a ToxA-PTA2 antigen (SEQ ID NO: 9), (8) a ToxA-P5-7 antigen (SEQ ID NO: 10), (9) a ToxA-P5-6 antigen (SEQ ID NO: 11), (10) a ToxA-P9-10 antigen (SEQ ID NO: 12), (11) a ToxA-B2 antigen (SEQ ID NO: 13), (12) a ToxA-B3 antigen (SEQ ID NO: 14), (13) a ToxA-B5 antigen (SEQ ID NO: 15), (14) a ToxA-B6 antigen (SEQ ID NO: 16) or a full-length TcdA antigen (SEQ ID NO:1). The immunogenic composition optionally further comprises 1, 2, 3, 4, 5, 6, 7, 8 or more additional TcdB antigens, optionally selected from (1) a ToxB-ED antigen (SEQ ID NO: 17), (2) a ToxB-GT antigen (SEQ ID NO: 18), (3) a ToxB-CP antigen (SEQ ID NO:19) (4) a ToxB-T antigen (SEQ ID NO: 20), (5) a ToxB-B antigen (SEQ ID NO: 21), (6) a ToxB-B2 antigen (SEQ ID NO: 22) (7) ToxB-B7 (SEQ ID NO: 23) or (8) a full-length TcdB antigen (SEQ ID NO:2).

In one embodiment, the immunogenic composition comprises a ToxA-GT antigen and 1, 2, 3, 4, 5, 6, 7, 8, 9 or more TcdB antigens, optionally selected from (1) a ToxB-ED antigen (SEQ ID NO: 17), (2) a ToxB-GT antigen (SEQ ID NO: 18), (3) a ToxB-CP antigen (SEQ ID NO:19) (4) a ToxB-T antigen (SEQ ID NO: 20), (5) a ToxB-B antigen (SEQ ID NO: 21), (6) a ToxB-B2 antigen (SEQ ID NO: 22) (7) ToxB-B7 (SEQ ID NO: 23) or (8) a full-length TcdB antigen (SEQ ID NO:2). The immunogenic composition optionally further comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15

or more additional TcdA antigens, optionally selected from (1) a ToxA-ED antigen (SEQ ID NO: 3), (2) a ToxA-GT antigen (SEQ ID NO: 4), (3) a ToxA-CP antigen (SEQ ID NO:5), (4) a ToxA-T antigen (SEQ ID NO: 6), (5) a ToxA-T4 antigen (SEQ ID NO: 7), (6) a ToxA-B antigen (SEQ ID NO: 8), (7) a ToxA-PTA2 antigen (SEQ ID NO: 9), (8) a ToxA-P5-7 antigen (SEQ ID NO: 10), (9) a ToxA-P5-6 antigen (SEQ ID NO: 11), (10) a ToxA-P9-10 antigen (SEQ ID NO: 12), (11) a ToxA-B2 antigen (SEQ ID NO: 13), (12) a ToxA-B3 antigen (SEQ ID NO: 14), (13) a ToxA-B5 antigen (SEQ ID NO: 15), (14) a ToxA-B6 antigen (SEQ ID NO: 16) or a full-length TcdA antigen (SEQ ID NO:1).

In one embodiment, the immunogenic composition comprises i) a ToxA-GT antigen or a ToxB-GT antigen; and ii) at least one TcdA antigen selected from ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6; and at least one TcdB antigen selected from ToxB-B, ToxB-B2 antigen, and/or ToxA-B7.

In one embodiment, the immunogenic composition comprises i) a ToxA-GT antigen and a ToxB-GT antigen; and ii) at least one TcdA antigen selected from ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6; and at least one TcdB antigen selected from ToxB-B, ToxB-B2 antigen, and/or ToxA-B7.

In one embodiment, the immunogenic composition comprises a ToxB-GT antigen, a TcdA antigen and a further TcdB antigen, optionally wherein said composition comprises (a) ToxB-GT + ToxA-B2 + ToxB-B, or (b) ToxB-GT + ToxB-B + ToxA-P5-6. In one embodiment, the composition comprises a ToxB-GT antigen, a ToxA-GT antigen, a further TcdA antigen and a further TcdB antigen, optionally wherein said combination comprises ToxB-GT + ToxA-GT + ToxA-B2 + ToxB-B.

In one embodiment, at least two of the antigens in the composition are in the form of a hybrid polypeptide. In another embodiment, none of the antigens are in the form of a hybrid polypeptide.

In some embodiments, the immunogenic composition induces neutralisation titers against *C. difficile* toxin A and toxin B.

In some embodiments, the immunogenic composition comprises at least one further *C. difficile* antigen, optionally wherein said further *C. difficile* antigen is a saccharide antigen.

In some embodiments, the immunogenic composition is a vaccine composition. In some embodiments, the vaccine composition further comprises an adjuvant. In some embodiments, the vaccine composition is use as a pharmaceutical. In some embodiments, the vaccine composition is for use in raising an immune response in a mammal, preferably a human. In some embodiments, the vaccine composition is for use in treating or preventing *C. difficile* associated disease.

In one embodiment, the invention provides a method for raising an immune response in a mammal comprising the step of administering to the mammal an effective amount of the immunogenic composition or vaccine described herein.

All pathogenic strains of C. *difficile* express one or two large exo-toxins (TcdA and TcdB, also referred to herein as ToxA and ToxB, and Toxin A and Toxin B). TcdA and TcdB belong to the large clostridial cytotoxin (LCD) family and exhibit 49% amino acid identity. They are single-polypeptide chain, high molecular weight exo-toxins (308 and 270kDa, respectively) which are organised into multi-domain structures [8,9]. The genes encoding TcdA and TcdB, *tcdA* and *tcdB,* are located in the 19.6kb *C. difficile* pathogenicity locus [10]. Like other members of the LCD family, TcdA and TcdB are organised as modular domains with each domain performing a distinct function [11]. The domain structures of TcdA and TcdB are illustrated in Figure 1.

An overview of the mechanism of action of TcdA/B is provided in reference 11. Briefly, the C-terminus of TcdA/B (denoted "B" in Figure 1) is responsible for toxin binding to the surface of epithelial cells. The C-terminal region of both toxins is composed of residue repeats known as the clostridial repetitive oligopeptides or cell wall binding domains due to their homology to the repeats of *Streptococcus pneumoniae* LytA, and is responsible for cell surface recognition and endocytosis [12]. Recently, the crystal structure of a C-terminal fragment of TcdA has been solved, revealing a solenoid-like structure, which consists of 32 short repeats with 15–21 residues and seven long repeats with 30 residues (reference 13). The C-terminal repeat regions of TcdA and TcdB are similar and may be identified routinely.

Binding of TcdA/B to epithelial cells induces receptor-mediated endocytosis, facilitating entry into the cytoplasm. Once internalised, the toxins require an acidic endosome for transport to the cytosol. A decrease in endosomal pH is thought to induce a conformational change which results in exposure of the hydrophobic translocation domain (denoted "T" in Figure 1) and insertion of the enzymatic N-terminus (comprising an glycosyl-transferase domain and a cysteine protease domain, denoted "GT" and "CP" in Figure 1, respectively), allowing entry into the endosome via pore formation [13]. Recently, references 14 and 15 demonstrated that inositol hexakisphosphate from the host cell induces the autocatalytic cleavage of the N-terminal region at the cysteine protease ("CP") site, thus releasing the N-terminal glucosyltransferase ("GT") domain into the cytosol (the remainder of the toxin is thought to remain in the endosome). Upon cleavage, the GT domain is thought to be capable of transferring glucose residues from UDP-glucose to Rho-GTPases, thus inactivating cell signalling [16]. Inhibition of Rho-GTPases causes a series of cascading effects, including dysregulation of actin cytoskeleton and tight junction integrity which collectively lead to increased membrane permeability and loss of barrier function [17], diarrhoea, inflammation, and an influx of neutrophils and other members of the innate immune response [18].

The TcdA and TcdB exo-toxins are thus the proteins primarily responsible for clinical symptoms caused by *C. difficile* [19, 20, 21] and have been the focus of attempts to develop vaccines to treat and prevent CDAD. Reference 19 found that antibodies against recombinant TcdA are sufficient to prevent diarrhoea if administered prior to challenge. Immune responses to TcdB may also play a role in disease expression and/or immunity, as highlighted by numerous reports of diarrhoea and

pseudomembranous colitis associated with TcdA negative, TcdB positive strains of *C. difficile* [22, 23, 24,25].

Pre-clinical studies using a mixture of formaldehyde-inactivated TcdA and TcdB have suggested that both TcdA and TcdB may be involved in the pathogenesis of *C. difficile*- associated diarrhoea and in generating protective immunity [26]. TcdA and TcdB may be purified from cell cultures, but the inactivation processes represents a major limitation in the preparation of toxoid-based vaccines. Toxin inactivation is typically achieved by formaldehyde treatment, which cross-links amino acids in the toxin polypeptide. The problem with formaldehyde inactivation is that the toxins are potentially subjected to unknown chemical modification and/or partial inactivation. Indeed, formalin-inactivated molecules have been shown to have impaired binding capabilities and reduced immunogenicity [27]. There are also a number of safety issues regarding use of toxoids derived from *C. difficile* toxins purified from cell culture in vaccines.

As discussed in reference 28, TcdA is considered to be primarily responsible for the clinical symptoms of CDAD. Experiments with purified toxoids have indicated that TcdA alone is able to evoke the symptoms of CDAD, but TcdB is unable to do so unless it is mixed with TcdA, or there is prior damage to the gut mucosa [29]. Clinical evidence obtained from animal models indicates that binding domain of TcdA can elicit serum antibodies that neutralize the cytotoxic and lethal effects of TcdA (30, 31, 32, 33). Also, a recombinant non-toxic peptide containing these repeating units has been shown to elicit neutralizing antibodies that can protect laboratory animals against challenge with both TcdA and *C. difficile* (34, 35, 36, 33). Interestingly, however, a recent study showed that toxin B is essential for *C. difficile* virulence and that a strain producing TcdA alone was avirulent (29, 37), and so the current model of *C. difficile* virulence remains unsettled. Thus, it is currently unclear what components of TcdA and TcdB may be used to induce an immune response to treat or prevent CDAD. The current consensus, however, is that effective immunisation against CDAD is likely to require peptides comprising the binding domains of TcdA and TcdB (38, 39) and that antibodies directed against the binding domains confer protection against toxin pathology.

Reference 40 discloses chimeric proteins retaining all of the functional domains present in the wild-type toxins (*i.e.* GT, CP, T and B domains), but in which the binding domain of ToxA has been replaced by the binding domain of ToxB and *vice versa*. In line with the current consensus, it was suggested that the binding domain is the key domain for immunogenicity. In addition, the authors indicated that the chimeric nature of their holotoxin constructs which retained all of the functional domains of the native toxins, was essential.

Surprisingly, however, the inventors have found that native toxin structure is not necessary for immunogenicity and that fragments comprising the GT domain of TcdA or TcdB are particularly suitable for generating an immune response provided that they are combined with TcdB fragments when the GT domain of TcdA is used or TcdA fragments when the GT domain of TcdA is used. The GT domains employed in such combinations are typically detoxified. Such combinations generate

the production of neutralisation titers against both TcdA and TcdB, and are more effective at providing a protective response against CDAD in animal models than combinations comprising binding domain fragments. These combinations thus provide an improved vaccine against CDAD. Furthermore, the use of recombinant polypeptide fragments also avoids safety issues related to the use of toxoids derived from *C. difficile* toxins purified from cell culture in vaccines.

ToxB-GT antigens

The full-length TcdB antigen (also referred to herein as ToxB and ToxinB) comprises the amino acid sequence of SEQ ID NO: 2 (encoded by the nucleic acid sequence of SEQ ID NO: 31). Detoxified TcdB antigen is referred to herein as Toxoid B.

The abbreviation "ToxB-GT" refers to the glucosyl transferase domain of TcdB, which is located within the N-terminal region of the enzymatic domain (ED). The ToxB-GT domain (SEQ ID NO: 18, encoded by the nucleic acid sequence of SEQ ID NO: 47) is a fragment of TcdB that corresponds to amino acids 1-543 of SEQ ID NO: 2.

The ToxB-GT antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 18; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 18, or of a polypeptide having 50% or more identity to SEQ ID NO:18, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 540, or more). Preferred fragments comprise an epitope of SEQ ID NO: 18. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 18 while retaining at least one epitope of SEQ ID NO:18. Amino acid fragments of ToxB-GT may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, or up to 540, consecutive amino acid residues of SEQ ID NO: 18.

The ToxB-GT antigen included in the compositions of the invention may be detoxified. Detoxification may be achieved by mutating the amino acid sequence or the encoding nucleic acid sequence of the wild-type ToxB-GT antigen using any appropriate method known in the art *e.g.* site-directed mutagenesis. Preferably, the ToxB-GT antigen comprises one or more amino acid substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,15, 16, 17, 18, 19, 20, 25, 30, or more mutations), relative to the wild-type ToxB-GT antigen sequence of SEQ ID NO:18. For example, the ToxB-GT antigen comprises one or more amino acid substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more mutations), *e.g.* at amino acid positions 17, 102, 139, 269, 270, 273, 284, 286, 288, 384, 449, 444, 445, 448, 449, 450, 451, 452, 455, 461,

463, 472, 515, 518, and/or 520, relative to the wild-type ToxB-GT antigen sequence of SEQ ID NO:18. For example, the ToxB-GT antigen may comprise substitutions at 1, 2, 3, 4 or 5 positions corresponding to amino acids 270, 273, 284, 286 and/or 288 of the Tox-GT antigen sequence of SEQ ID NO: 18. In particular, 1, 2, 3, 4 or 5 amino acids at positions corresponding to amino acids 270, 273, 284, 286 and/or 288 of the ToxB-GT antigen sequence of SEQ ID NO:18 may be substituted, preferably by alanine residues. Where amino acids 270, 273, 284, 286 and/or 288 of SEQ ID NO: 18 are substituted, the substitutions are preferably D270A, R273A, Y284A, D286A and/or D288A, most preferably D270A, R273A, Y284A, D286A and D288A. These substitutions correspond to substitutions D270A, R273A, Y284A, D286A and D288A of SEQ ID NO: 2. The amino acid sequence of a detoxified ToxB-GT antigen having alanine substitutions at these positions is provided in SEQ ID NO: 60.

Where the ToxB-GT comprises two amino acid substitutions, the substitutions are preferably not at amino acid positions 102 and 278, or amino acid positions 102 and 288, of the ToxB-GT antigen sequence of SEQ ID NO:18. The detoxified ToxB-GT antigen included in the compositions of the invention may thus be a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 60 ; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 60, or of a polypeptide having 50% or more identity to SEQ ID NO: 60, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 540, or more). Amino acid fragments of detoxified ToxB-GT may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, or up to 540, consecutive amino acid residues of SEQ ID NO: 60. Preferred fragments comprise an epitope of SEQ ID NO: 60. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 60 while retaining at least one epitope of SEQ ID NO: 60.

The abbreviation "ToxB-ED" refers to the enzymatic domain of TcdB. The ToxB-ED domain (SEQ ID NO: 17, encoded by the nucleic acid sequence of SEQ ID NO: 46) is a fragment of TcdB that corresponds to amino acids 1-767 of SEQ ID NO: 2. The ToxB-ED domain of TcdB thus comprises the ToxB-GT domain. The ToxB-GT antigen included in the composition of the invention may thus be a ToxB-ED antigen.

The ToxB-ED antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 17; and/or (b) that is a fragment of at least "n" consecutive amino acids of

SEQ ID NO: 17, or of a polypeptide having 50% or more identity to SEQ ID NO:17, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 650, 700, 750, or more). Preferred fragments comprise an epitope of SEQ ID NO: 17. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 17 while retaining at least one epitope of SEQ ID NO:17.

Amino acid fragments of ToxB-ED may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, up to 550, up to 600, up to 650, up to 700, or up to 750 consecutive amino acid residues of SEQ ID NO: 17.

The ToxB-ED antigen included in the compositions of the invention may be detoxified. Detoxification may be achieved by mutating the amino acid sequence or the encoding nucleic acid sequence of the wild-type ToxB-ED antigen using any appropriate method known in the art *e.g.* site-directed mutagenesis. Preferably, the ToxB-ED antigen comprises one or more amino acid substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, or more mutations), relative to the wild-type ToxB-ED antigen sequence of SEQ ID NO:17. For example, the ToxB-ED antigen comprises one or more amino acid substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more mutations), *e.g.* at amino acid positions 17, 102, 139, 269, 270, 273, 284, 286, 288, 384, 449, 444, 445, 448, 449, 450, 451, 452, 455, 461, 463, 472, 515, 518, and/or 520, relative to the wild-type ToxB-ED antigen sequence of SEQ ID NO:17. For example, the ToxB-ED antigen may comprise substitutions at 1, 2, 3, 4 or 5 positions corresponding to amino acids 270, 273, 284, 286 and/or 288 of the ToxB-ED antigen sequence of SEQ ID NO:17. In particular, 1, 2, 3, 4 or 5 amino acids at positions corresponding to amino acids 270, 273, 284, 286 and/or 288 of the ToxB-ED antigen sequence of SEQ ID NO:17 may be substituted, preferably by alanine residues. The ToxB-ED antigen may also comprise substitutions at 1, 2, or 3 positions corresponding to amino acids 587, 653, and/or 698 of the ToxB-ED antigen sequence of SEQ ID NO:17. In particular, 1, 2, or 3 amino acids at positions corresponding to amino acids 587, 653, and/or 698 of the ToxB-ED antigen sequence of SEQ ID NO:17 may be substituted, preferably by alanine or asparagine residues. Where amino acids 587, 653, and/or 698 of SEQ ID NO: 17 are substituted, the substitutions are preferably D587N, H653A, and/or C698A, most preferably D587N, H653A, and C698A. These substitutions correspond to substitutions D587N, H653A, and C698A of SEQ ID NO: 2. The amino acid sequences of a detoxified ToxB-ED antigen having substitutions at positions 270, 273, 284, 286, 288, 587, 657 and 698 (relative to SEQ ID NO: 2) is provided in SEQ ID NO: 58.

Where the ToxB-ED comprises two amino acid substitutions, the substitutions are preferably not at amino acid positions 102 and 278, or amino acid positions 102 and 288, of the ToxB-ED antigen sequence of SEQ ID NO:17.

The detoxified ToxB-ED antigen included in the compositions of the invention may thus be a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 58 ; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO 58: or of a polypeptide having 50% or more identified to SEQ ID NO: 58, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 650, 700, 750, or more). Amino acid fragments of detoxified ToxB-ED may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, up to 550, up to 600, up to 650, up to 700, or up to 750 consecutive amino acid residues of SEQ ID NO: 58.

Preferred fragments comprise an epitope of SEQ ID NO: 58. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 58 while retaining at least one epitope of SEQ ID NO: 58.

ToxB-GT antigens and ToxB-ED antigens included in the compositions of the invention may also include the ToxB-CP and or ToxB-T domains defined below which are present in the full-length TcdB antigen. ToxB-GT antigens and ToxB-ED antigens may, for example, comprise *n* amino acids from the N-terminal region of the ToxB-T domain described below, wherein *n* = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 ,19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1025, 1050, 1051, 1052, 1053, 1054, 1055, 1056, 1057, 1058, 1059, 1060, 1061, 1062, 1063, 1064, or 1065.

ToxB-GT and ToxB-ED antigens included in the compositions of the invention preferably do not comprise the binding domain of TcdB. In particular, the ToxB-GT and ToxB-ED preferably do not comprise the ToxB-B domain described in more detail below or fragments of this domain, *e.g.* the ToxB-B2 and/or ToxB-B7 domains described in more detail below.

ToxA-GT antigens

The full-length TcdA antigen (also referred to herein as ToxA and Toxin A) comprises the amino acid sequence of SEQ ID NO: 1 (encoded by the nucleic acid sequence of SEQ ID NO: 30). Detoxified TcdA antigen is referred to herein as Toxoid A.

The abbreviation "ToxA-GT" refers to the glucosyl transferase domain of TcdA, which is located within the N-terminal region of the enzymatic domain (ED). The ToxA-GT domain (SEQ ID NO: 4,

encoded by the nucleic acid sequence of SEQ ID NO: 33) is a fragment of TcdA that corresponds to amino acids 1-541 of SEQ ID NO: 1.

The ToxA-GT antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 4; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 4, or of a polypeptide having 50% or more identity to SEQ ID NO:4, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 540, or more). Preferred fragments comprise an epitope of SEQ ID NO: 4. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 4 while retaining at least one epitope of SEQ ID NO:4.

Amino acid fragments of ToxA-GT may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, or up to 540, consecutive amino acid residues of SEQ ID NO: 4.

The ToxA-GT antigen included in the compositions of the invention may be detoxified. Detoxification may be achieved by mutating the amino acid sequence or the encoding nucleic acid sequence of the wild-type ToxA-GT antigen using any appropriate method known in the art *e.g.* site-directed mutagenesis. Preferably, the ToxA-GT antigen comprises one or more amino acid substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, or more mutations), relative to the wild-type ToxA-GT antigen sequence of SEQ ID NO:4. For example, the ToxA-GT antigen may comprise substitutions at 1, 2 or 3 positions corresponding to amino acids 283, 285 and 287 of the ToxA-GT antigen sequence of SEQ ID NO:4. In particular, 1, 2, or 3 amino acids at positions corresponding to amino acids 283, 285 and 287 of the ToxA-GT antigen sequence of SEQ ID NO:4 may be substituted, preferably by alanine residues (*i.e.* Y283A, D285A, D287A). These mutations correspond to positions 283, 285 and 287 of SEQ ID NO: 1. The amino acid sequence of a detoxified ToxA-GT antigen having alanine substitutions at these positions is provided in SEQ ID NO: 56.

Where the ToxA-GT antigen comprises one amino acid substitution, the substitution is preferably not at amino acid position 278 of the ToxA-GT antigen sequence of SEQ ID NO: 4. Where the ToxA-GT antigen comprises two amino acid substitutions, the substitutions are preferably not at amino acid positions 101 and 278, of the ToxA-GT antigen sequence of SEQ ID NO:4. Where the ToxA-GT antigen comprises three amino acid substitutions, the substitutions are preferably not at amino acid positions 101, 278 and 519, or amino acid positions 101, 287 and 519, of the ToxA-GT antigen sequence of SEQ ID NO:4.

The detoxified ToxA-GT antigen included in the compositions of the invention may thus be a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 56; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 56, or of a polypeptide having 50% or more identity to SEQ ID NO: 56, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 540, or more). Preferred fragments comprise an epitope of SEQ ID NO: 56. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 56 while retaining at least one epitope of SEQ ID NO: 56. Amino acid fragments of detoxified ToxB-GT may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, or up to 540, consecutive amino acid residues of SEQ ID NO: 56.

The abbreviation "ToxA-ED" refers to the enzymatic domain of TcdA. The ToxA-ED domain (SEQ ID NO: 3, encoded by the nucleic acid sequence of SEQ ID NO: 32) is a fragment of TcdA that corresponds to amino acids 1-769 of SEQ ID NO: 1. The ToxA-ED domain of TcdA thus comprises the ToxA-GT domain. The ToxA-GT antigen included in the composition of the invention may thus be a ToxA-ED antigen.

The ToxA-ED antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 3; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 3, or of a polypeptide having 50% or more identity to SEQ ID NO:3, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, or more). Preferred fragments comprise an epitope of SEQ ID NO: 3. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 3 while retaining at least one epitope of SEQ ID NO:3.

Amino acid fragments of ToxA-ED may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, up to 550, up to 600, up to 650, up to, 700, or up to 750, consecutive amino acid residues of SEQ ID NO: 3.

The ToxA-ED antigen included in the compositions of the invention may be detoxified. Detoxification may be achieved by mutating the amino acid sequence or the encoding nucleic acid sequence of the wild-type ToxA-ED antigen using any appropriate method known in the art *e.g.* site-directed mutagenesis. Preferably, the ToxA-ED antigen comprises one or more amino acid

substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,15, 16, 17, 18, 19, 20, 25, 30, or more mutations), relative to the wild-type ToxA-ED antigen sequence of SEQ ID NO:3. For example, the ToxA-ED antigen may comprise substitutions at 1, 2 or 3 positions corresponding to amino acids 283, 285 and 287 of the ToxA-ED antigen sequence of SEQ ID NO: 3. In particular, 1, 2, or 3 amino acids at positions corresponding to amino acids 283, 285 and 287 of the ToxA-ED antigen sequence of SEQ ID NO: 3 may be substituted, preferably by alanine residues. The amino acid sequence of a detoxified ToxA-ED antigen having alanine substitutions at these positions is provided in SEQ ID NO: 54.

The ToxA-ED antigen may also comprise substitutions at 1, 2, or 3 positions corresponding to amino acids 589, 655, and/or 700 of the ToxA-ED antigen sequence of SEQ ID NO:3. In particular, 1, 2, or 3 amino acids at positions corresponding to amino acids 589, 655, and/or 700 of the ToxA-ED antigen sequence of SEQ ID NO:3 may be substituted, preferably by alanine or asparagine residues. Where amino acids 589, 655 and/or 700 are substituted, the substitutions are preferably D589N, H655A and/or C700A, most preferably D589N, H655A and C700A.

Where the ToxA-ED antigen comprises one amino acid substitution, the substitution is preferably not at amino acid position 278 of the ToxA-ED antigen sequence of SEQ ID NO: 3. Where the ToxA-ED antigen comprises two amino acid substitutions, the substitutions are preferably not at amino acid positions 101 and 278, of the ToxA-ED antigen sequence of SEQ ID NO:3. Where the ToxA-ED antigen comprises three amino acid substitutions, the substitutions are preferably not at amino acid positions 101, 278 and 519, or amino acid positions 101, 287 and 519, of the ToxA-ED antigen sequence of SEQ ID NO:3.

The detoxified ToxA-ED antigen included in the compositions of the invention may thus be a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 54 ; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 54 or of a polypeptide having 50% or more identified to SEQ ID NO: 54, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, or more). Preferred fragments comprise an epitope of SEQ ID NO: 54. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 54 while retaining at least one epitope of SEQ ID NO: 54. Amino acid fragments of detoxified ToxA-ED may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, up to 550, up to 600, up to 650, up to, 700, or up to 750, consecutive amino acid residues of SEQ ID NO: 54.

ToxA-GT antigens and ToxA-ED antigens included in the compositions of the invention may also include the ToxA-CP and or ToxA-T domains defined below which are present in the full-length TcdA antigen. ToxA-GT antigens and ToxA-ED antigens may, for example, comprise *n* amino acids from the N-terminal region of the ToxA-T domain described below, wherein *n* = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 ,19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1025, 1050, 1051, 1052, 1053, 1054, 1055, 1056, 1057, 1058, 1059, 1060, 1061, 1062, 1063, 1064, or 1065.

ToxA-GT and ToxA-ED antigens included in the compositions of the invention preferably do not comprise the binding domain of TcdA. In particular, the ToxA-GT and ToxA-ED preferably do not comprise the ToxA-B domain described in more detail below or fragments of this domain, e.g. the ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6 domains described in more detail below.

TcdA antigens

Compositions of the invention may comprise a TcdA antigen. The TcdA antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 1; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 1 or of a polypeptide having 50% or more identified to SEQ ID NO:1, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, 2500, or more). Amino acid fragments of TcdA may comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, up to 550, up to 600, up to 650, up to, 700, up to 750, up to 1000, up to 1250, up to 1500, up to 1750, up to 2000, up to 2250, or up to 2500,consecutive amino acid residues of SEQ ID NO: 1. Preferred fragments of TcdA comprise an epitope of SEQ ID NO: 1. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 1 while retaining at least one epitope of SEQ ID NO: 1. Other fragments of TcdA omit one or more protein domains. Protein domains that may be omitted can include functional protein domains, such as the "B", "T", "GT" ,"CP", "ToxA-ED", "ToxA-GT", "ToxA-CP", "ToxA-T", "ToxA-T4", "ToxA-PTA2", "ToxA-P5-7", "ToxA-P5-6", "ToxA-P9-10", "ToxA-B2", "ToxA-B3", "ToxA-B5", and "ToxA-B6" domains discussed herein.

Fragments of the TcdA antigen that may be included in the compositions of the invention are preferably selected from the group consisting of: "ToxA-ED", "ToxA-GT", "ToxA-CP", "ToxA-T", "ToxA-T4", "ToxA-PTA2", "ToxA-P5-7", "ToxA-P5-6", "ToxA-P9-10", "ToxA-B2", "ToxA-B3", "ToxA-B5" and "ToxA-B6". This set of fragments is referred to herein as the "TcdA antigen group".

Thus, compositions of the invention may comprise one or more (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15) TcdA antigens selected from the group consisting of: (1) a ToxA-ED antigen, (2) a ToxA-GT antigen, (3) a ToxA-CP antigen, (4) a ToxA-T antigen, (5) a ToxA-T4 antigen, (6) a ToxA-B antigen, (7) a ToxA-PTA2 antigen, (8) a ToxA-P5-7 antigen, (9) a ToxA-P5-6 antigen, (10) a ToxA-P9-10 antigen, (11) a ToxA-B2 antigen, (12) a ToxA-B3 antigen, (13) a ToxA-B5 antigen, (14) a ToxA-B6 antigen, and (15) a full-length TcdA antigen. Wherein compositions of the invention comprise one TcdA fragment, the one TcdA fragment is preferably not a ToxA-CP antigen alone.

The *(1) ToxA-GT antigen, (2) ToxA-ED antigen,* and *(15) full-length TcdA antigen* are defined above. The remaining antigens are defined in more detail below.

(3) ToxA-CP antigen

The ToxA-CP domain (SEQ ID NO: 5, encoded by the nucleic acid sequence of SEQ ID NO: 34) corresponds to amino acids 542-769 of SEQ ID NO: 1. The abbreviation "ToxA-CP" refers to the cysteine protease domain of TcdA, which is located within the C-terminal region of the enzymatic domain.

The ToxA-CP antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 5; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 5, or of a polypeptide having 50% or more identity to SEQ ID NO:5, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 225or more). Preferred fragments comprise an epitope of SEQ ID NO: 5. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 5 while retaining at least one epitope of SEQ ID NO:5. Amino acid fragments of ToxA-CP may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, or up to 225 , consecutive amino acid residues of SEQ ID NO: 5.

The ToxA-CP antigen included in the compositions of the invention may be detoxified. Detoxification may be achieved by mutating the amino acid sequence or the encoding nucleic acid sequence of the wild-type ToxA-CP antigen using any appropriate method known in the art *e.g.* site-directed mutagenesis. Preferably, the ToxA-CP antigen comprises one or more amino acid substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,15, 16, 17, 18, 19, 20, 25, 30, or more mutations), relative to the wild-type ToxA-CP antigen sequence of SEQ ID NO:5. For example, the ToxA-CP antigen may comprise substitutions at 1, 2 or 3 positions corresponding to amino acids 48, 114 and 159 of the ToxA-CP antigen sequence of SEQ ID NO:5. In particular, 1, 2, or 3 amino acids at positions corresponding to amino acids 48, 114 and 159 of the ToxA-CP antigen sequence of SEQ

ID NO:5 may be substituted, preferably by alanine or asparagine residues. Where amino acids 48, 114 and/or 159 of SEQ ID NO: 5 are substituted, the substitutions are preferably D48N, H114A and/or A159A, most preferably D48N, H114A and A159A. These substitutions correspond to substitutions D589N, H655A and C700A of SEQ ID NO: 1. The amino acid sequence of a detoxified ToxA-CP antigen having alanine or asparagine substitutions at these positions is provided in SEQ ID NO: 62.

Amino acid fragments of detoxified ToxA-CP may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, or up to 225, consecutive amino acid residues of SEQ ID NO: 62.

Where compositions of the invention contain only one TcdA antigen, the one TcdA antigen is preferably not ToxA-CP alone. Where compositions of the invention comprise a ToxA-CP antigen, the antigen may be a ToxA-ED antigen.

(4) ToxA-T antigen

The ToxA-T domain (SEQ ID NO: 6, encoded by the nucleic acid sequence of SEQ ID NO: 35) corresponds to amino acids 770-1808 of SEQ ID NO: 1. The abbreviation "ToxA-T" refers to the translocation domain of TcdA.

The ToxA-T antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 6; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 6, or of a polypeptide having 50% or more identity to SEQ ID NO:6, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500, 550, 600, 700, 800, 900, 1000, or more). Preferred fragments comprise an epitope of SEQ ID NO: 6. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 6 while retaining at least one epitope of SEQ ID NO:6. Amino acid fragments of ToxA-T may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, up to 500, up to 550, up to 600, up to 700, up to 800, up to 900, or up to 1000, consecutive amino acid residues of SEQ ID NO: 6.

(5) ToxA-T4

The ToxA-T4 domain (SEQ ID NO: 7, encoded by the nucleic acid sequence of SEQ ID NO: 36) corresponds to amino acids 1510-1775 of SEQ ID NO: 1. The abbreviation "ToxA-T4" refers to a region within TcdA. The ToxA-T4 region was found to be insoluble.

The ToxA-T4 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%,

80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 7; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 7, or of a polypeptide having 50% or more identity to SEQ ID NO:7, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, or more). Preferred fragments comprise an epitope of SEQ ID NO: 7. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 7 while retaining at least one epitope of SEQ ID NO:7. Amino acid fragments of ToxA-T4 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, or up to 260, consecutive amino acid residues of SEQ ID NO: 7.

(6) ToxA-B antigen

The ToxA-B domain (SEQ ID NO: 8, encoded by the nucleic acid sequence of SEQ ID NO: 37) corresponds to amino acids 1809-2710 of SEQ ID NO: 1. The abbreviation "ToxA-B" refers to a fragment of the binding domain of TcdA. The binding domain of TcdA (denoted "B" in Figure 1) is responsible for toxin binding to the surface of epithelial cells. The inventors have found that fragments of the binding domain are effective in combination with GT antigens at eliciting an immune response. Compositions of the invention thus employ fragments of the binding domain (*e.g.* ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6).

The ToxA-B antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 8; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 8, or of a polypeptide having 50% or more identity to SEQ ID NO:8, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 700, 800, 900, or more). Preferred fragments comprise an epitope of SEQ ID NO: 8. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 8 while retaining at least one epitope of SEQ ID NO:8. Amino acid fragments of ToxA-B may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, up to 500, up to 550, up to 600, up to 700, up to 800, or up to 900, consecutive amino acid residues of SEQ ID NO: 8.

(7) ToxA-PTA2

The ToxA-PTA2 domain (SEQ ID NO: 9, encoded by the nucleic acid sequence of SEQ ID NO: 38) corresponds to amino acids 1995-2198 of SEQ ID NO: 1. The abbreviation "ToxA-PTA2" refers to a

region within the binding domain of TcdA and was found to be insoluble. As described in WO98/59053, the ToxA-PTA2 fragment comprises 8 tandem repeat sequences from within the C-terminal repeat region of Toxin A.

The ToxA-PTA2 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 9; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 9, or of a polypeptide having 50% or more identity to SEQ ID NO:9, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, or more). Preferred fragments comprise an epitope of SEQ ID NO: 9. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 9 while retaining at least one epitope of SEQ ID NO:9. Amino acid fragments of ToxA-PTA2 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, or up to 200, consecutive amino acid residues of SEQ ID NO: 9.

(8) ToxA-P5-7 antigen

The ToxA-P5-7 antigen (SEQ ID NO: 10, encoded by the nucleic acid sequence of SEQ ID NO: 39) corresponds to amino acids 2249-2706 of SEQ ID NO: 1. The abbreviation "ToxA-P5-7" refers to a region within the binding domain of TcdA. As described in WO98/59053, the ToxA-P5-7 fragment comprises 20 tandem repeat sequences from within the C-terminal repeat region of Toxin A.

The ToxA-P5-7 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 10; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 10, or of a polypeptide having 50% or more identity to SEQ ID NO:10, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 450, or more). Preferred fragments comprise an epitope of SEQ ID NO: 10. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 10 while retaining at least one epitope of SEQ ID NO:10. Amino acid fragments of ToxA-P5-7 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, or up to 450, consecutive amino acid residues of SEQ ID NO: 10.

(9) ToxA-P5-6 antigen

The ToxA-P5-6 domain (also referred to as "P5-6") (SEQ ID NO: 11, encoded by the nucleic acid sequence of SEQ ID NO: 40) corresponds to amino acids 2387-2706 of SEQ ID NO: 1. The abbreviation "ToxA-P5-6" refers to a region within the binding domain of TcdA. As described in WO98/59053, the ToxA-P5-6 fragment comprises 14 tandem repeat sequences from within the C-terminal repeat region of Toxin A.

The ToxA-P5-6 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 11; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 11, or of a polypeptide having 50% or more identity to SEQ ID NO:11, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, or more). Preferred fragments comprise an epitope of SEQ ID NO: 11. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 11 while retaining at least one epitope of SEQ ID NO:11. Amino acid fragments of ToxA-P5-6 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, or up to 300, consecutive amino acid residues of SEQ ID NO: 11.

The ToxA-p5-6 antigen may comprise a mutation in at least one amino acid (for example 1, 2, 3, 5, 6, 7, 8, 9, 10 or more) relative to SEQ ID NO:11. A mutation preferably involves a single amino acid and is preferably a point mutation. The mutations may each independently be a deletion, an insertion or a substitution,. For example, a mutated ToxA-p5-6 antigen may include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) single amino acid deletions relative to the ToxA-p5-6 sequence SEQ ID NO: 11. By way of further example, a mutated ToxA-p5-6 antigen may include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) insertions (*e.g.* each of 1, 2, 3, 4 or 5 amino acids) relative to the ToxA-p5-6 sequence SEQ ID NO: 11. Deletions, substitutions or insertions may be at the N-terminus and/or C-terminus, or may be between the two termini. Thus a truncation is an example of a deletion. Truncations may involve deletion of up to 40 (or more) amino acids at the N-terminus and/or C-terminus. Particular insertions include the addition of two amino acids at the C-terminal, for example Leucine (L) and Glutamic acid (E) as shown in SEQ ID NO: 84.

Preferred mutations are amino acid substitutions. Amino acid substitutions may be from one amino acid to any one of the other nineteen naturally occurring amino acids. A conservative substitution is commonly defined as a substitution introducing an amino acid having sufficiently similar chemical properties, e.g. having a related side chain (*e.g.* a basic, positively charged amino acid should be replaced by another basic, positively charged amino acid), in order to preserve the structure and the biological function of the molecule. Genetically-encoded amino acids may be divided into five

families: (1) acidic *i.e.* aspartate, glutamate; (2) basic *i.e.* lysine, arginine, histidine; (3) non-polar *i.e.* alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; (4) charged i.e.aspartic acid, glutamic acid, arginine, lysine, histidine and (5) uncharged polar *i.e.* glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In general, substitution of single amino acids within these families does not have a major effect on the biological activity. Particularly, substitutions may be made at positions 41 and/or 42 of the ToxA-p5-6 antigen numbered according to SEQ ID 11. Particularly the histidine (H) at position 41 may be substituted with aspartic acid (D) as shown in SEQ ID NO: 101 (a substitution named H41D).Particularly asparagine (N) at position 42 may be substituted by alanine (A) as shown in SEQ ID NO: 102 (a substitution named N42A). Yet more particularly, the ToxA-P5-6 antigen may comprise both of these two mutations H41D and N42A as exemplified in SEQ ID NO: 103.

The ToxA-p5-6 antigen may be part of a hybrid polypeptide of formula: A-Bp5-6-C wherein:

A is an optional N-terminal additional amino acid sequence. The additional amino acid sequence may be either derived from vector sequences, from MCS or the sequences could be from extraneous polypeptides that aid in hyper expression of proteins. The additional amino acids could be used for affinity purification or for antibody detection. The additional amino acid sequence may be any known in the art such as GST tag, His tag, T7 tag Trx tag, MBP tag, His-GM tag etc. Particularly, the additional amino acid sequence comprises the sequence MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSSRITR (SEQ ID NO: 104)

B is ToxA-p5-6 antigen having an amino acid sequence selected from the group consisting of SEQ ID NO 11, SEQ ID NO 84, SEQ ID NO 101, SEQ ID NO 102 and SEQ ID NO: 103.

C is an optional C-terminal amino sequence having the following sequence TESTCRXQA (SEQ ID NO: 105) wherein X is one of the twenty naturally occurring amino acids.

Examples of hybrid polypeptides comprising ToxA-p5-6 antigen are shown in SEQ ID NOs: 106, 107, 108, 109, 110, and 111. Seq ID NO: 111 is encoded by the nucleic acid sequence of SEQ ID NO: 112. Preferred ToxA-p5-6 antigens for use in combinations of the invention include SEQ ID NO:11 and SEQ ID NO: 111.

(10) ToxA-P9-10 antigen

The ToxA-P9-10 domain (SEQ ID NO: 12, encoded by the nucleic acid sequence of SEQ ID NO: 41) corresponds to amino acids 1843-2706 of SEQ ID NO: 1. The abbreviation "ToxA-P9-10" refers to a region within the binding domain of TcdA. As described in WO98/59053, the ToxA-P9-10 fragment comprises all 36 tandem repeat sequences from within the C-terminal repeat region of Toxin A.

The ToxA-P9-10 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 12; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 12, or of a polypeptide having 50% or more identity to SEQ ID NO:12, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 700, 800, 850, or more). Preferred fragments comprise an epitope of SEQ ID NO: 12. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 12 while retaining at least one epitope of SEQ ID NO:12. Amino acid fragments of ToxA-P9-10 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, up to 500, up to 550, up to 600, up to 700, up to 800, or up to 850, consecutive amino acid residues of SEQ ID NO: 12.

(11) ToxA-B2 antigen

The ToxA-B2 domain (SEQ ID NO: 13, encoded by the nucleic acid sequence of SEQ ID NO: 42) corresponds to amino acids 2303-2706 of SEQ ID NO: 1. The abbreviation "ToxA-B2" refers to a region within the binding domain of TcdA. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E). ToxA-B2 was designed to include 6 of the 13 putative structural units forming the binding domain (see Figure 2).

The ToxA-B2 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 13; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 13, or of a polypeptide having 50% or more identity to SEQ ID NO:13, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400or more). Preferred fragments comprise an epitope of SEQ ID NO: 13. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 13 while retaining at least one epitope of SEQ ID NO:13. Amino acid fragments of ToxA-B2 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, consecutive amino acid residues of SEQ ID NO: 13.

(12) ToxA-B3 antigen

The ToxA-B3 domain (SEQ ID NO: 14, encoded by the nucleic acid sequence of SEQ ID NO: 43) corresponds to amino acids 1839-2710 of SEQ ID NO: 1. The abbreviation "ToxA-B3" refers to a

region within the binding domain of TcdA. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E). ToxA-B3 was designed to include 12 of the 13 putative structural units forming the binding domain (see Figure 3).

The ToxA-B3 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 14; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 14, or of a polypeptide having 50% or more identity to SEQ ID NO:14, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 700, 800, 850, or more). Preferred fragments comprise an epitope of SEQ ID NO: 14. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 14 while retaining at least one epitope of SEQ ID NO:14. Amino acid fragments of ToxA-B3 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, up to 500, up to 550, up to 600, up to 700, up to 800, or up to 850, consecutive amino acid residues of SEQ ID NO: 14.

(13) ToxA-B5 antigen

The ToxA-B5 domain (SEQ ID NO: 15, encoded by the nucleic acid sequence of SEQ ID NO: 44) corresponds to amino acids 1964-2706 of SEQ ID NO: 1. The abbreviation "ToxA-B5" refers to a region within the binding domain of TcdA. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E). ToxA-B5 was designed to include 10.5 of the 13 putative structural units forming the binding domain (see Figure 4).

The ToxA-B5 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 15; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 15, or of a polypeptide having 50% or more identity to SEQ ID NO:15, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 700, 740 or more). Preferred fragments comprise an epitope of SEQ ID NO: 15. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 15 while retaining at least one epitope of SEQ ID NO:15. Amino acid fragments of ToxA-B5 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to

175, up to 200, up to 250, up to 300, up to 400, up to 500, up to 550, up to 600, up to 700, or up to 740, consecutive amino acid residues of SEQ ID NO: 15.

(14) ToxA-B6 antigen

The ToxA-B6 domain (SEQ ID NO: 16, encoded by the nucleic acid sequence of SEQ ID NO: 45) corresponds to amino acids 1890-2706 of SEQ ID NO: 1. The abbreviation "ToxA-B6" refers to a region within the binding domain of TcdA. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E). ToxA-B6 was designed to include 11.5 of the 13 putative structural units forming the binding domain (see Figure 5).

The ToxA-B6 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 16; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 16, or of a polypeptide having 50% or more identity to SEQ ID NO:16, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 700, 800 or more). Preferred fragments comprise an epitope of SEQ ID NO: 16. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 16 while retaining at least one epitope of SEQ ID NO:16. Amino acid fragments of ToxA-B6 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, up to 500, up to 550, up to 600, up to 700, up to 800, or up to 850, consecutive amino acid residues of SEQ ID NO: 16.

The TcdB antigens

Compositions of the invention may comprise a TcdB antigen. The TcdB antigen included in the polypeptides of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 2; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 2 or of a polypeptide having 50% or more identified to SEQ ID NO:2, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, 2400, or more).). Amino acid fragments of TcdB may comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 350, up to 400, up to 450, up to 500, up to 550, up to 600, up to 650, up to, 700, up to 750, up to 1000, up to 1250, up to 1500, up to 1750, up to 2000, up to 2250, or up to 2400,consecutive amino acid residues of SEQ ID NO: 2. Preferred fragments of TcdB comprise an epitope of SEQ ID NO: 2. Other preferred fragments lack one or

more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 2 while retaining at least one epitope of SEQ ID NO: 2. Other fragments of TcdB omit one or more protein domains. Protein domains that may be omitted can include functional protein domains, such as the "B", "T", "GT", "CP", ""ToxB-ED", "ToxB-GT", "ToxB-CP", "ToxB-T", "ToxB-B", "ToxB-B2" and "ToxB-B7" domains discussed herein.

The TcdB fragments that may be included in the composition of the invention are preferably selected from the group consisting of: "ToxB-ED", "ToxB-GT", "ToxB-CP", "ToxB-T", "ToxB-B", "ToxB-B2", and ToxB-B7. This set of antigens is referred to herein as the "TcdB antigen group".

Thus, compositions of the invention may comprise one or more (*i.e.* 1, 2, 3, 4, 5, 6, 7, or all 8) TcdB antigens selected from the group consisting of: (1) a ToxB-ED antigen, (2) a ToxB-GT antigen, (3) a ToxB-CP antigen, (4) a ToxB-T antigen, (5) a ToxB-B antigen, (6) a ToxB-B2 antigen, (7) a ToxA-B7 antigen and (8) a full-length TcdB antigen. Wherein compositions of the invention comprise only one TcdB fragment, the one TcdB fragment is preferably not ToxB-CP alone.

The *(1) ToxB-GT antigen, (2) ToxB-ED antigen* and *(8) full-length TcdB antigen* are defined above. The remaining antigens are defined in more detail below.

(3) ToxB-CP antigen

The ToxB-CP domain (SEQ ID NO: 19, encoded by the nucleic acid sequence of SEQ ID NO: 48) corresponds to amino acids 544-767 of SEQ ID NO: 2. The abbreviation "ToxB-CP" refers to the cysteine protease domain of TcdB, which is located within the C-terminal region of the enzymatic domain.

The ToxB-CP antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 19; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 19, or of a polypeptide having 50% or more identity to SEQ ID NO:19, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 230, or more). Preferred fragments comprise an epitope of SEQ ID NO: 19. Amino acid fragments of ToxB-CP may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, or up to 230, consecutive amino acid residues of SEQ ID NO: 19. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 19 while retaining at least one epitope of SEQ ID NO:19.

The ToxB-CP antigen included in the compositions of the invention may be detoxified. Detoxification may be achieved by mutating the amino acid sequence or the encoding nucleic acid sequence of the wild-type ToxB-CP antigen using any appropriate method known in the art *e.g.* site-directed mutagenesis. Preferably, the ToxB-CP antigen comprises one or more amino acid substitutions (*i.e.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,15, 16, 17, 18, 19, 20, 25, 30, or more mutations), relative to the wild-type ToxB-CP antigen sequence of SEQ ID NO: 19. For example, the ToxB-CP antigen may comprise substitutions at 1, 2 or 3 positions corresponding to amino acids 44, 110 and 155 of the ToxB-CP antigen sequence of SEQ ID NO:19. In particular, 1, 2, or 3 amino acids at positions corresponding to amino acids 44, 110 and 155 of the ToxB-CP antigen sequence of SEQ ID NO:19 may be substituted, preferably by alanine or asparagine residues. Where amino acids 44, 110, and/or 155 of SEQ ID NO: 19 are substituted, the substitutions are preferably D44N, H110A, and/or C155A, most preferably D44N, H110A, and/or C155A. The amino acid sequence of a detoxified ToxB-CP antigen having alanine or asparagine substitutions at these positions is provided in SEQ ID NO: 64. These substitutions correspond to substitutions D587N, H653A, and C698A of SEQ ID NO: 2. Amino acid fragments of detoxified ToxB-CP may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, or up to 225, consecutive amino acid residues of SEQ ID NO: 64.

Where compositions of the invention contain only one TcdB antigen, the one TcdB antigen is preferably not ToxB-CP alone. Where compositions of the invention comprise a ToxB-CP antigen, the antigen may be a ToxB-ED antigen.

(4) ToxB-T antigen

The ToxB-T domain (SEQ ID NO: 20, encoded by the nucleic acid sequence of SEQ ID NO: 49) corresponds to amino acids 768-1833 of SEQ ID NO: 2. The abbreviation "ToxB-T" refers to the translocation domain of TcdB.

The ToxB-T antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 20; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 20, or of a polypeptide having 50% or more identity to SEQ ID NO:20, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, 550, 600, 700, 800, 900, 1000, 1050, or more). Preferred fragments comprise an epitope of SEQ ID NO: 20. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 20 while retaining at least one epitope of SEQ ID NO:20. Amino acid fragments of ToxB-T may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to

150, up to 175, up to 200, up to 250, up to 300, up to 400, up to 500, up to 550, up to 600, up to 700, up to 800, 900, 1000, or up to 1050, consecutive amino acid residues of SEQ ID NO: 20.

(5) ToxB-B antigen

The ToxB-B domain (SEQ ID NO: 21, encoded by the nucleic acid sequence of SEQ ID NO: 50) corresponds to amino acids 1853-2366 of SEQ ID NO: 2. The abbreviation "ToxB-B" refers to a fragment of the binding domain of TcdB. The inventors have found that fragments of the binding domain are effective in combination with GT antigens at eliciting an immune response. Compositions of the invention thus employ fragments of the binding domain (*e.g.* ToxB-B, ToxB-B2 antigen, and/ or ToxA-B7).

The ToxB-B antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 21; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 21, or of a polypeptide having 50% or more identity to SEQ ID NO:21, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 250, 300, 400, 500, or more). Preferred fragments comprise an epitope of SEQ ID NO: 21. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 21 while retaining at least one epitope of SEQ ID NO:21. Amino acid fragments of ToxB-B may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, up to 300, up to 400, up to 450, or up to 500 consecutive amino acid residues of SEQ ID NO: 21.

(6) ToxB-B2 antigen

The ToxB-B2 domain (SEQ ID NO: 22, encoded by the nucleic acid sequence of SEQ ID NO: 51) corresponds to amino acids 2157-2366 of SEQ ID NO: 2. The abbreviation "ToxA-B2" refers to the C-terminal region of the binding domain of TcdB. The three-dimensional structure of the TcdB binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E). ToxB-B2 was designed to include 4 of the 9 putative structural units forming the binding domain (see Figure 6).

The ToxB-B2 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 22; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 22, or of a polypeptide having 50% or more identity to SEQ ID NO:22, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 175, 200, or more). Preferred fragments comprise an epitope of SEQ ID NO: 22. Other preferred fragments lack

one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 22 while retaining at least one epitope of SEQ ID NO:22.

Amino acid fragments of ToxB-B2 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, or up to 200 consecutive amino acid residues of SEQ ID NO: 22.

(7) ToxB-B7 antigen

The ToxB-B7 domain (SEQ ID NO: 23, encoded by the nucleic acid sequence of SEQ ID NO: 52) corresponds to amino acids 2056-2366 of SEQ ID NO: 2.

The ToxB-B7 antigen included in the compositions of the invention is a polypeptide that comprises or consists of an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.8%, 99.9%, or more) to SEQ ID NO: 23; and/or (b) that is a fragment of at least "n" consecutive amino acids of SEQ ID NO: 23, or of a polypeptide having 50% or more identity to SEQ ID NO:23, wherein "n" is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300or more). Preferred fragments comprise an epitope of SEQ ID NO: 23. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 23 while retaining at least one epitope of SEQ ID NO:23.

Amino acid fragments of ToxB-B7 may thus comprise an amino acid sequence of *e.g.* up to 30, up to 40, up to 50, up to 60, up to 70, up to 80, up to 90, up to 100, up to 125, up to 150, up to 175, up to 200, up to 250, or up to 300 consecutive amino acid residues of SEQ ID NO: 23.

Antigen combinations

Compositions of the invention may comprise a ToxB-GT antigen and one or more TcdA antigens (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 TcdA antigens selected from (1) a ToxA-ED antigen, (2) a ToxA-GT antigen, (3) a ToxA-CP antigen, (4) a ToxA-T antigen, (5) a ToxA-T4 antigen, (6) a ToxA-B antigen, (7) a ToxA-PTA2 antigen, (8) a ToxA-P5-7 antigen, (9) a ToxA-P5-6 antigen, (10) a ToxA-P9-10 antigen, (11) a ToxA-B2 antigen, (12) a ToxA-B3 antigen, (13) a ToxA-B5 antigen, (14) a ToxA-B6 antigen, and (15) a full-length TcdA antigen, as described above). Such compositions may further comprise one or more additional TcdB antigens (e.g. 1, 2, 3, 4, 5, 6, 7, or 8 TcdB antigens selected from the group consisting of: (1) a ToxB-ED antigen, (2) a ToxB-GT antigen, (3) a ToxB-CP antigen, (4) a ToxB-T antigen, (5) a ToxB-B antigen, (6) a ToxB-B2 antigen, (7) a ToxA-B7 antigen and (8) a full-length TcdB antigen, as TcdB described above).

Alternatively, compositions of the invention may comprise a ToxA-GT antigen and one or more TcdB antigens (e.g. 1, 2, 3, 4, 5, 6, 7, or 8 TcdB antigens selected from the group consisting of: (1) a

ToxB-ED antigen, (2) a ToxB-GT antigen, (3) a ToxB-CP antigen, (4) a ToxB-T antigen, (5) a ToxB-B antigen, (6) a ToxB-B2 antigen, (7) a ToxA-B7 antigen and (8) a full-length TcdB antigen, as TcdB described above). Such compositions may further comprise one or more additional TcdA antigens (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 TcdA antigens selected from (1) a ToxA-ED antigen, (2) a ToxA-GT antigen, (3) a ToxA-CP antigen, (4) a ToxA-T antigen, (5) a ToxA-T4 antigen, (6) a ToxA-B antigen, (7) a ToxA-PTA2 antigen, (8) a ToxA-P5-7 antigen, (9) a ToxA-P5-6 antigen, (10) a ToxA-P9-10 antigen, (11) a ToxA-B2 antigen, (12) a ToxA-B3 antigen, (13) a ToxA-B5 antigen, (14) a ToxA-B6 antigen, and (15) a full-length TcdA antigen, as described above).

Specific examples of combinations of antigens that may be included in the compositions of the invention are set out below.

In some embodiments, the immunogenic composition comprises a combination of i) one ToxB-GT antigen and one TcdA antigen or ii) one ToxA-GT antigen and one TcdB antigen.

In some embodiments, the composition comprises ToxA-GT and one antigen from the TcdB antigen group *e.g.* ToxA-GT + ToxB-ED, ToxA-GT + ToxB-GT, ToxA-GT + ToxB-CP, ToxA-GT + ToxB-T, ToxA-GT + ToxB-B, ToxA-GT + ToxB-B2, ToxA-ED + ToxB-B7, ToxA-ED + ToxB-ED, ToxA-ED + ToxB-GT, ToxA-ED + ToxB-CP, ToxA-ED + ToxB-T, ToxA-ED + ToxB-B, ToxA-ED + ToxB-B2, and ToxA-ED + ToxB-B7.

In some embodiments, the composition comprises ToxB-GT and one antigen from the TcdA antigen group *e.g.* ToxB-GT + ToxA-ED, ToxB-GT + ToxA-GT, ToxB-GT + ToxA-CP, ToxB-GT + ToxA-T, ToxB-GT + ToxA-T4, ToxB-GT + ToxA-PTA2, ToxB-GT + ToxA-P5-7, ToxB-GT + ToxA-P5-6, ToxB-GT + ToxA-P9-10, ToxB-GT + ToxA-B2, ToxB-GT + ToxA-B3, ToxB-GT + ToxA-B5, ToxB-GT + ToxA-B6, ToxB-ED + ToxA-ED, ToxB-ED + ToxA-GT, ToxB-ED + ToxA-CP, ToxB-ED + ToxA-T, ToxB-ED + ToxA-T4, ToxB-ED + ToxA-PTA2, ToxB-ED + ToxA-P5-7, ToxB-ED + ToxA-P5-6, ToxB-ED + ToxA-P9-10, ToxB-ED + ToxA-B2, ToxB-ED + ToxA-B3, ToxB-ED + ToxA-B5, and ToxB-ED + ToxA-B6. Preferably, the composition comprises (a) ToxB-GT + ToxA-P5-6, (b) ToxB-GT + ToxA-B2, (c) ToxB-GT + ToxB-B + ToxA-B2, or (d) ToxB-GT + ToxB-B + ToxA-P5-6.

In another embodiment, the immunogenic composition comprises 3 antigens. Such an immunogenic composition may comprise a combination of i) one ToxB-GT antigen and two TcdA antigens; ii) one ToxA-GT antigen and two TcdB antigens; or iii) one ToxB-GT antigen, one ToxA-GT antigen and one further TcdA or TcdB antigen, *e*.g. ToxB-GT + ToxA-B2 + ToxB-B, ToxB-GT + ToxB-B+ToxA-P5-6.

The immunogenic composition may comprise four antigens. For example, the composition may comprise a ToxB-GT antigen, a ToxA-GT antigen and two additional antigens from the TcdA and/or TcdB antigen groups, *e.g.* ToxB-GT + ToxB-B + ToxA-GT + ToxA-B2.

It has been found that combinations comprising the ToxA-GT and/or ToxB-GT antigens are surprisingly effective when combined with fragments derived form the binding domains of both TcdA and TcdB. In particular, the composition may therefore comprise a combination of i) a ToxA-GT antigen or a ToxB-GT antigen and (ii) at least one TcdA antigen selected from ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6; and at least one TcdB antigen selected from ToxB-B, ToxB-B2 antigen, and/ or ToxA-B7. The composition may also comprise a combination of i) a ToxA-GT antigen and a ToxB-GT antigen and (ii) at least one TcdA antigen selected from ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6; and at least one TcdB antigen selected from ToxB-B, ToxB-B2 antigen, and/ or ToxA-B7.

The composition may further comprise *e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more additional fragments. Such further fragments are preferably selected from TcdA antigen group and/or from the TcdB antigen group.

Hybrid polypeptides

The antigens in the composition may be present as individual separate polypeptides and/or "hybrid" polypeptides. In some embodiments, none of the antigens are in the form of hybrid polypeptides. In some embodiments, none of the antigens are in the form of hybrid polypeptides. Hybrid polypeptides (also referred to herein as chimeras, or chimeric proteins) are described in more detail below.

The antigens may be present in the compositions of the invention as individual separate polypeptides (*i.e.* mixed together). As an alternative, compositions of the invention comprise a "hybrid" polypeptide, where at least two (*e.g.* 2, 3, 4, 5, or more) antigens are expressed as a single polypeptide chain. Compositions of the invention may also comprise at least one individual separate polypeptide antigens and at least one hybrid polypeptide. Hybrid polypeptides offer two main advantages: first, a polypeptide that may be unstable or poorly expressed on its own can be assisted by adding a suitable hybrid partner that overcomes the problem; second, commercial manufacture is simplified as only one expression and purification need be employed in order to produce two polypeptides which are both antigenically useful.

Hybrid polypeptides may comprise a ToxB-GT antigen and one or more TcdA antigens. The hybrid polypeptide thus comprises two or more antigens that are not the same. Thus, the hybrid polypeptide may comprise amino acid sequences from *i.e.* 2, 3, 4, 5, 6, 7, 8, 9, 10 or more different antigens, and may comprise multiple copies of each antigen *i.e.* 2, 3, 4, 5, 6, 7, 8, 9, 10, or more copies.

Hybrid polypeptides may comprise a ToxA-GT antigen and one or more TcdB antigens. The hybrid polypeptide thus comprises two or more antigens that are not the same. Thus, the hybrid polypeptide may comprise amino acid sequences from *i.e.* 2, 3, 4, 5, 6, 7, 8, 9, 10 or more different antigens, and may comprise multiple copies of each type of fragment *i.e.* 2, 3, 4, 5, 6, 7, 8, 9, 10, or more copies.

The TcdA antigens are preferably selected from the TcdA antigen group, *e.g.* the hybrid polypeptide may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 of the antigens in the TcdA antigen group. The TcdB antigens are preferably selected from the TcdB antigen group, *e.g.* the hybrid polypeptide may comprise 1, 2, 3, 4, 5, 6, 7 or 8 of the antigens in the TcdB antigen group.

Different hybrid polypeptides may be mixed together in a single formulation. Hybrids may be combined with non-hybrid antigens. Within such combinations, a TcdA/TcdB antigen may be present in more than one hybrid polypeptide and/or as a non-hybrid polypeptide. Preferably, a TcdA/TcdB antigen is present either as a hybrid or as a non-hybrid, but not as both.

The hybrid polypeptides can also be combined with conjugates or non- *C. difficile* antigens.

Hybrid polypeptides can be represented by the formula NH_2-A-{-X-L-}$_n$-B-COOH, wherein: X is an amino acid sequence of a toxin fragment, preferably a toxoid fragment, as described above; L is an optional linker amino acid sequence; A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; *n* is an integer of 2 or more (*e.g.* 2, 3, 4, 5, 6, *etc.*). Usually *n* is 2 or 3.

If a -X- moiety has a leader polypeptide sequence in its wild-type form, this may be included or omitted in the hybrid protein. In some embodiments, the leader polypeptides will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein *i.e.* the leader polypeptide of X_1 will be retained, but the leader polypeptides of X_2 … X_n will be omitted. This is equivalent to deleting all leader polypeptides and using the leader polypeptide of X_1 as moiety -A-.

For each *n* instances of {-X-L-}, linker amino acid sequence -L- may be present or absent. For instance, when *n*=2 the hybrid may be NH_2-X_1-L_1-X_2-L_2-COOH, NH_2-X_1-X_2-COOH, NH_2-X_1-L_1-X_2-COOH, NH_2-X_1-X_2-L_2-COOH, *etc.* Linker amino acid sequence(s) -L- will typically be short (*e.g.* 20 or fewer amino acids *i.e.* 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short polypeptide sequences which facilitate cloning, poly-glycine linkers (*i.e.* comprising Gly_n where *n* = 2, 3, 4, 5, 6, 7, 8, 9, 10 or more), and histidine tags (*i.e.* His_n where *n* = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG (SEQ ID NO:25) or GSGSGGGG (SEQ ID NO:26), with the Gly-Ser dipeptide being formed from a *Bam*HI restriction site, thus aiding cloning and manipulation, and the $(Gly)_4$ tetrapeptide being a typical poly-glycine linker. Other suitable linkers, particularly for use as the final L_n are a Leu-Glu dipeptide or SEQ ID NO: 27.

-A- is an optional N-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short polypeptide sequences which facilitate cloning or purification (*e.g.* histidine tags *i.e.* His_n where *n* = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X_1 lacks its own N-terminus

methionine, -A- is preferably an oligopeptide (*e.g.* with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides a N-terminus methionine *e.g.* Met-Ala-Ser, or a single Met residue.

-B- is an optional C-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include sequences to direct protein trafficking, short polypeptide sequences which facilitate cloning or purification (*e.g.* comprising histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art.

For example, the invention provides a hybrid polypeptide ("B4 hybrid") consisting of ToxB-GT (SEQ ID NO: 18) fused to ToxA-P5-6 (SEQ ID NO: 11) via a peptide linker (SEQ ID NO: 25). A schematic representation of the B4 hybrid is provided in Figure 7 (SEQ ID NO: 24, encoded by the nucleic acid sequence of SEQ ID NO: 53).

The hybrid polypeptides of the invention are typically not holotoxins, *i.e.* they do not comprise all of the functional domains (GT, CP, T and B) present in a native toxin or holotoxin. For example, where a hybrid polypeptide comprising a ToxB-GT antigen also comprises a binding domain fragment of TcdB (*e.g.* ToxB-B, ToxB-B2 and/or ToxB-B7), the hybrid does not comprise the CP and T domains of Tcd B in the order in which they are found in a native toxin B. Similarly, where a hybrid polypeptide comprising a ToxA-GT antigen also comprises a binding domain fragment of TcdA (*e.g.* ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6), the hybrid does not comprise the CP and T domains of TcdA in the order in which they are found in a native toxin A.

In some embodiments, the functional domains in a hybrid polypeptide are in a different order from N-terminus to C-terminus to the order of the functional domains found in the native toxin *e.g.* the T domain may be N-terminal of the GT domain.

Similarly, the TcdA and TcdB fragments may be in any order. For example, where a hybrid polypeptide comprises two TcdA antigens and one TcdB antigen, they may be in the order A-A-B, A-B-A, B-A-A from N-terminus to C-terminus, or where a hybrid polypeptide comprises two TcdB antigens and one TcdA antigen, they may be in the order B-B-A, B-A-B, A-B-B from N-terminus to C-terminus. In general, TcdA and TcdB antigens may alternate *e.g.* A-B-A or B-A-B.

In particular, the hybrid polypeptide preferably does not comprise the ToxB-ED and ToxB-T domains of TcdB fused to the ToxA-B domain of TcdA, wherein the B-domain of TcdA is fused to the C-terminus of the T-domain of TcdB, either directly or via a linker (*e.g.* a modified full length TcdB, wherein the B-domain of TcdB is substituted for the B-domain of TcdA). The hybrid polypeptide preferably does not comprise the GT domain of TcdB fused to the CP, T and B domains (in N-C direction) of TcdA, wherein the GT-domain of TcdB is fused to the C-terminus of the CP-

domain of TcdA, either directly or via a linker (*e.g.* a modified full length TcdA, wherein the GT-domain of TcdA is substituted for the GT-domain of TcdB). The hybrid polypeptide preferably does not comprise the B-domain of TcdA fused to the GT, CP and T domains (in N-C direction) of TcdB, wherein the B-domain of TcdA is fused to the C-terminus of the GT-domain of TcdB, either directly or via a linker.

Preparing compositions of the invention

The invention also provides a process for preparing a composition of the invention comprising a step of mixing antigens of any of the combinations of antigens as defined above. For example, the invention provides a process comprising a step of mixing (i) a ToxA-GT antigen and (ii) one or more (*i.e.* 1, 2, 3, or 4) TcdB antigens, and optionally (iii) one or more (*i.e.* 1, 2, 3, or 4) further TcdA antigens. For example, the process may comprise a step of mixing a ToxA-GT antigen and one or more antigens selected from the TcdB antigen group and optionally one or more antigens selected from the TcdA antigen group.

The invention also provides a process comprising a step of mixing (i) a ToxB-GT antigen and (ii) one or more (*i.e.* 1, 2, 3, or 4) TcdA antigens, and optionally (iii) one or more (*i.e.* 1, 2, 3, or 4) TcdB antigens. For example, the process may comprise a step of mixing a polypeptide comprising a ToxB-GT antigen and one or more antigens selected from the TcdA antigen group and optionally one or more antigens selected from the TcdB antigen group.

A process according to the invention for preparing a mixture of TcdA and TcdB antigens may comprise a further step of formulating the mixture of a combination of TcdA and TcdB antigens of the invention as a medicament, *e.g.* as a vaccine. Such processes may further comprise a step of packaging the formulation for storage or distribution as a medicament, *e.g.* as a vaccine.

Polypeptides used with the invention

Polypeptides used with the invention can take various forms (*e.g.* native, fusions, glycosylated, non-glycosylated, lipidated, non-lipidated, phosphorylated, non-phosphorylated, myristoylated, non-myristoylated, monomeric, multimeric, particulate, denatured, *etc.*).

Polypeptides used with the invention can be prepared by various means (*e.g.* recombinant expression, purification from cell culture, chemical synthesis, *etc.*). Recombinantly-expressed proteins are preferred, particularly for hybrid polypeptides.

Polypeptides used with the invention are preferably provided in purified or substantially purified form *i.e.* substantially free from other polypeptides (*e.g.* free from naturally-occurring polypeptides), particularly from other *C. difficile* or host cell polypeptides, and are generally at least about 50% pure (by weight), and usually at least about 90% pure *i.e.* less than about 50%, and more preferably less than about 10% (*e.g.* 5%) of a composition is made up of other expressed polypeptides. Thus the antigens in the compositions are separated from the whole organism with which the molecule is expressed.

Polypeptides used with the invention are preferably *C. difficile* polypeptides.

Polypeptides used with the invention are preferably isolated or purified.

The term "polypeptide" refers to amino acid polymers of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labelling component. Also included are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art. Polypeptides can occur as single chains or associated chains.

The invention provides polypeptides comprising a sequence -P-Q- or -Q-P-, wherein: -P- is an amino acid sequence as defined above and -Q- is not a sequence as defined above *i.e.* the invention provides fusion proteins. Where the N-terminus codon of -P- is not ATG, but this codon is not present at the N-terminus of a polypeptide, it will be translated as the standard amino acid for that codon rather than as a Met. Where this codon is at the N-terminus of a polypeptide, however, it will be translated as Met. Examples of -Q- moieties include, but are not limited to, histidine tags (*i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more), maltose-binding protein, or glutathione-S-transferase (GST).

The invention also provides a process for producing a polypeptide of the invention, comprising the step of culturing a host cell transformed with nucleic acid of the invention under conditions which induce polypeptide expression.

Although expression of the polypeptides of the invention may take place in a *C. difficile*, the invention will usually use a heterologous host for expression (recombinant expression). The heterologous host may be prokaryotic (*e.g.* a bacterium) or eukaryotic. It may be *E.coli*, but other suitable hosts include *Brevibacillus chosinensis, Bacillus subtilis, Vibrio cholerae, Salmonella typhi, Salmonella typhimurium, Neisseria lactamica, Neisseria cinerea, Mycobacteria* (*e.g. M.tuberculosis*), yeasts, *etc.* Compared to the wild-type *C. difficile* genes encoding polypeptides of the invention, it is helpful to change codons to optimise expression efficiency in such hosts without affecting the encoded amino acids.

The invention provides a process for producing a polypeptide of the invention, comprising the step of synthesising at least part of the polypeptide by chemical means.

Nucleic acids

The invention also provides compositions comprising nucleic acids (*e.g.* combinations of nucleic acids, vectors, or vector combinations) encoding the combinations of polypeptides or hybrid polypeptides of the invention described above.

Nucleotide sequences encoding combinations of antigens of the invention are known or may be designed according to the genetic code. Thus, in the context of the present invention, such a nucleotide sequence may encode one or more of the polypeptide sequences disclosed herein, or may encode an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more, *e.g.* 90% identity or more, or 95% identity or more, or 99% identity or more, to any of said polypeptides; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of any of said polypeptides: 1, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more; *e.g.* 20 or more; or *e.g.* 50 or more; or *e.g.* 80 or more).

The native nucleotide sequences of the nucleic acids encoding all of the TcdA and TcdB antigens described above are given in the sequence listing and summarised in the sequence listing table. The nucleotide sequences encoding some of these antigens has been optimised using a codon optimisation process and optimised nucleotide sequences are also provided in some cases. Examples of codon optimised sequences include nucleic acid sequences comprising the nucleotide sequences of SEQ ID NOs: 55, 57, 59, 61, 63 and 65-69). The invention includes compositions comprising nucleic acids identified in the sequence listing table encoding the combinations of antigens described above.The invention also provides nucleic acid which can hybridize to these nucleic acids. Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction of widely known and published in the art (*e.g.* page 7.52 of [42]). Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C, 55°C and 68°C; buffer concentrations of 10 x SSC, 6 x SSC, 1 x SSC, 0.1 x SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 x SSC, 1 x SSC, 0.1 x SSC, or de-ionized water. Hybridization techniques and their optimization are well known in the art [43, 44, 42, 45, *etc.*].

A nucleic acid may hybridize to a target under low stringency conditions; in other embodiments it hybridizes under intermediate stringency conditions; in preferred embodiments, it hybridizes under high stringency conditions. An exemplary set of low stringency hybridization conditions is 50°C and 10 x SSC. An exemplary set of intermediate stringency hybridization conditions is 55°C and 1 x SSC. An exemplary set of high stringency hybridization conditions is 68°C and 0.1 x SSC.

The invention includes nucleic acid comprising sequences complementary to these sequences (*e.g.* for antisense or probing, or for use as primers).

Nucleic acid according to the invention can take various forms (*e.g.* single-stranded, double-stranded, vectors, primers, probes, labelled *etc.*). Nucleic acids of the invention may be circular or branched, but will generally be linear. Unless otherwise specified or required, any embodiment of the invention that utilizes a nucleic acid may utilize both the double-stranded form and each of two complementary

single-stranded forms which make up the double-stranded form. Primers and probes are generally single-stranded, as are antisense nucleic acids.

Nucleic acids encoding antigens described herein are preferably provided in purified or substantially purified form *i.e.* substantially free from other nucleic acids (*e.g.* free from naturally-occurring nucleic acids), particularly from other *C. difficile* or host cell nucleic acids, generally being at least about 50% pure (by weight), and usually at least about 90% pure. Nucleic acids of the invention are preferably *C. difficile* nucleic acids.

Nucleic acids encoding antigens described herein may be prepared in many ways *e.g.* by chemical synthesis (*e.g.* phosphoramidite synthesis of DNA) in whole or in part, by digesting longer nucleic acids using nucleases (*e.g.* restriction enzymes), by joining shorter nucleic acids or nucleotides (*e.g.* using ligases or polymerases), from genomic or cDNA libraries, *etc.*

Nucleic acids may be attached to a solid support (*e.g.* a bead, plate, filter, film, slide, microarray support, resin, *etc.*). Nucleic acids may be labelled *e.g.* with a radioactive or fluorescent label, or a biotin label. This is particularly useful where the nucleic acid is to be used in detection techniques *e.g.* where the nucleic acid is a primer or as a probe.

The term "nucleic acid" includes in general means a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and/or their analogs. It includes DNA, RNA, DNA/RNA hybrids. It also includes DNA or RNA analogs, such as those containing modified backbones (*e.g. polypeptide* nucleic acids (PNAs) or phosphorothioates) or modified bases. Thus the invention includes mRNA, tRNA, rRNA, ribozymes, DNA, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors, probes, primers, *etc.* Where nucleic acid of the invention takes the form of RNA, it may or may not have a 5' cap.

Nucleic acids encoding antigens described herein may be part of a vector *i.e.* part of a nucleic acid construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors" which are designed for isolation, propagation and replication of inserted nucleotides, "expression vectors" which are designed for expression of a nucleotide sequence in a host cell, "viral vectors" which is designed to result in the production of a recombinant virus or virus-like particle, or "shuttle vectors", which comprise the attributes of more than one type of vector. Preferred vectors are plasmids. A "host cell" includes an individual cell or cell culture which can be or has been a recipient of exogenous nucleic acid. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. Host cells include cells transfected or infected *in vivo* or *in vitro* with nucleic acid of the invention.

The term "complement" or "complementary" when used in relation to nucleic acids refers to Watson-Crick base pairing. Thus the complement of C is G, the complement of G is C, the complement of A

is T (or U), and the complement of T (or U) is A. It is also possible to use bases such as I (the purine inosine) *e.g.* to complement pyrimidines (C or T).

Nucleic acids encoding antigens described herein can be used, for example: to produce polypeptides; as hybridization probes for the detection of nucleic acid in biological samples; to generate additional copies of the nucleic acids; to generate ribozymes or antisense oligonucleotides; as single-stranded DNA primers or probes; or as triple-strand forming oligonucleotides.

The invention provides a process for producing nucleic acid encoding antigens described herein, wherein the nucleic acid is synthesised in part or in whole using chemical means.

The invention provides vectors comprising nucleotide sequences encoding antigens described herein (*e.g.* cloning or expression vectors) and host cells transformed with such vectors.

For certain embodiments of the invention, nucleic acids are preferably at least 7 nucleotides in length (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300 nucleotides or longer).

For certain embodiments of the invention, nucleic acids are preferably at most 500 nucleotides in length (*e.g.* 450, 400, 350, 300, 250, 200, 150, 140, 130, 120, 110, 100, 90, 80, 75, 70, 65, 60, 55, 50, 45, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15 nucleotides or shorter).

Strains and variants

Antigens are defined above by reference to *C. difficile* ToxA and ToxB from *C. difficile* strain 630. The basic reference sequence for ToxA and ToxB can easily be found in public gene databases. For instance, GenBank accession number AM180355 is the complete *C. difficile* genome sequence, and the individual ToxA and ToxB sequences are given as "locus_tag" entries in the genome sequence's "features" section. Functional annotations are also given in the databases.

Immunogenic compositions of the invention are useful for immunisation against CDAD caused by multiple different strains of *C. difficile*. The invention is not limited to compositions comprising fragments only from the 630 strain. Sequences of several strains of *C. difficile* are available, including those of *C.* difficile strains R20291(SM), *C. difficile* strain 196, *C. difficile* strain BI1, *C. difficile* strain BI/NAP1/027 (ribotype 027), *C. difficile* strain M120 and *C. difficile* strain M68, strain 855, strain QCD-63q42, strain ATCC43255. Standard search and alignment techniques can be used to identify in any further genome sequences the homolog of any particular toxin sequence from the *C. difficile* strain 630. For example in strain ATCC43255, strain CIP107932, strain QCD-23m63, strain QCD-32g58, strain QCD-37x79, strain QCD-63q42, strain QCD-66c26, strain QCD-76w55, strain QCD-97b34, strain CD196, strain CDBI1, strain CDCF5, strain CDSM, strain CDM68, strain CDM120 or strain R20291. Moreover, the available sequences from the *C. difficile* strain 630 can be

used to design primers for amplification of homologous sequences from other strains. Thus the invention is not limited to polypeptides from this strain, but rather encompasses such variants and homologs from other strains of *C. difficile*, as well as non-natural variants. In general, suitable variants of a particular SEQ ID NO include its allelic variants, its polymorphic forms, its homologs, its orthologs, its paralogs, its mutants, *etc.*

Thus, for instance, polypeptides used with the invention may, compared to the strain 630 reference sequence, include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) amino acid substitutions, such as conservative substitutions (*i.e.* substitutions of one amino acid with another which has a related side chain). Genetically-encoded amino acids are generally divided into four families: (1) acidic *i.e.* aspartate, glutamate; (2) basic *i.e.* lysine, arginine, histidine; (3) non-polar *i.e.* alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar *i.e.* glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In general, substitution of single amino acids within these families does not have a major effect on the biological activity. The polypeptides may also include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) single amino acid deletions relative to the strain 630 sequences. The polypeptides may also include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) insertions (*e.g.* each of 1, 2, 3, 4 or 5 amino acids) relative to the TcdA and/or TcdB sequences.

Similarly, a polypeptide used with the invention may comprise an amino acid sequence that:

(a) is identical (*i.e.* 100% identical) to a sequence disclosed in the sequence listing;

(b) shares sequence identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) with a sequence disclosed in the sequence listing;

(c) has 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 (or more) single amino acid alterations (deletions, insertions, substitutions), which may be at separate locations or may be contiguous, as compared to the sequences of (a) or (b); and

(d) when aligned with a particular sequence from the sequence listing using a pairwise alignment algorithm, each moving window of x amino acids from N-terminus to C-terminus (such that for an alignment that extends to p amino acids, where $p>x$, there are $p-x+1$ such windows) has at least $x \cdot y$ identical aligned amino acids, where: x is selected from 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200; y is selected from 0.50, 0.60, 0.70, 0.75, 0.80, 0.85, 0.90, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99; and if $x \cdot y$ is not an integer then it is rounded up to the nearest integer. The preferred pairwise alignment algorithm is the Needleman-Wunsch global alignment algorithm [46], using default parameters (*e.g.* with Gap opening penalty = 10.0, and with Gap extension penalty = 0.5, using the EBLOSUM62 scoring matrix). This algorithm is conveniently implemented in the *needle* tool in the EMBOSS package [47].

In general, when a polypeptide of the invention comprises a sequence that is not identical to a complete *C difficile* sequence from the sequence listing (*e.g.* when it comprises a sequence listing

with <100% sequence identity thereto, or when it comprises a fragment thereof) it is preferred in each individual instance that the polypeptide can elicit an antibody that recognises its respective toxin (either TcdA or TcdB), preferably the complete *C. difficile* sequence provided in the sequence listing.

Where hybrid polypeptides are used, the individual antigens within the hybrid (*i.e.* individual -X- moieties) may be from one or more strains. Where n=2, for instance, X_2 may be from the same strain as X_1 or from a different strain. Where n=3, the strains might be (i) $X_1=X_2=X_3$ (ii) $X_1=X_2\neq X_3$ (iii) $X_1\neq X_2=X_3$ (iv) $X_1\neq X_2\neq X_3$ or (v) $X_1=X_3\neq X_2$, *etc.*

Within group (c), deletions or substitutions may be at the N-terminus and/or C-terminus, or may be between the two termini. Thus a truncation is an example of a deletion. Truncations may involve deletion of up to 40 (or more) amino acids at the N-terminus and/or C-terminus.

Immunogenic compositions and medicaments

The term "immunogenic" in the context of an antigen described herein is used to mean that the antigen is capable of eliciting an immune response, such as a cell-mediated and/or an antibody response, against the wild-type *C. difficile* protein from which it is derived, for example, when used to immunise a subject (preferably a mammal, more preferably a human or a mouse).

An immunogenic composition of the invention comprises an antigen according to the invention. Immunogenic compositions of the invention may be useful as vaccines. Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat infection), but will typically be prophylactic. The term "protected against infection" means that the immune system of a subject has been primed (*e.g.* by vaccination) to to trigger an immune response and repel the infection. A vaccinated subject may thus get infected, but is better able to repel the infection than a control subject.

Compositions may thus be pharmaceutically acceptable. They will usually include components in addition to the antigens *e.g.* they typically include one or more pharmaceutical carrier(s) and/or excipient(s). A thorough discussion of such components is available in [48].

Compositions will generally be administered to a mammal in aqueous form. Prior to administration, however, the composition may have been in a non-aqueous form. For instance, although some vaccines are manufactured in aqueous form, then filled and distributed and administered also in aqueous form, other vaccines are lyophilised during manufacture and are reconstituted into an aqueous form at the time of use. Thus a composition of the invention may be dried, such as a lyophilised formulation.

The composition may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccine should be substantially free from (*i.e.* less than 5μg/ml) mercurial material

e.g. thiomersal-free. Vaccines containing no mercury are more preferred. Preservative-free vaccines are particularly preferred.

To improve thermal stability, a composition may include a temperature protective agent. Further details of such agents are provided below.

To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml *e.g.* about 10±2mg/ml NaCl. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride, *etc.*

Compositions will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg.

Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer (particularly with an aluminium hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the 5-20mM range.

The pH of a composition will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0 *e.g.* 6.5 and 7.5, or between 7.0 and 7.8.

The composition is preferably sterile. The composition is preferably non-pyrogenic *e.g.* containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The composition is preferably gluten free.

The composition may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a 'multidose' kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose compositions, the compositions may be contained in a container having an aseptic adaptor for removal of material.

Human vaccines are typically administered in a dosage volume of about 0.5ml, although a half dose (*i.e.* about 0.25ml) may be administered to children.

Immunogenic compositions of the invention may also comprise one or more immunoregulatory agents. Preferably, one or more of the immunoregulatory agents include one or more adjuvants. The adjuvants may include a TH1 adjuvant and/or a TH2 adjuvant, further discussed below.

Adjuvants which may be used in compositions of the invention include, but are not limited to:

- mineral salts, such as aluminium salts and calcium salts, including hydroxides (*e.g.* oxyhydroxides), phosphates (*e.g.* hydroxyphosphates, orthophosphates) and sulphates, *etc.* [*e.g.* see chapters 8 & 9 of ref. 49];

- oil-in-water emulsions, such as squalene-water emulsions, including MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer) [Chapter 10 of ref.49, see also ref. 50-53, chapter 10 of ref. 54 and chapter 12 of ref. 55], complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA);
- saponin formulations [chapter 22 of ref. 49], such as QS21 [56] and ISCOMs [chapter 23 of ref. 49];
- virosomes and virus-like particles (VLPs) [57-63];
- bacterial or microbial derivatives, such as non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), Lipid A derivatives [64, 65], immunostimulatory oligonucleotides [66-71], such as IC-31™ [72] (deoxynucleotide comprising 26-mer sequence 5'-$(IC)_{13}$-3' (SEQ ID NO:28) and polycationic polymer polypeptide comprising 11-mer amino acid sequence KLKLLLLLKLK (SEQ ID NO:29)) and ADP-ribosylating toxins and detoxified derivatives thereof [73 - 82];
- human immunomodulators, including cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 [83 , 84], interferons (*e.g.* interferon-γ), macrophage colony stimulating factor, and tumor necrosis factor;
- bioadhesives and mucoadhesives, such as chitosan and derivatives thereof, esterified hyaluronic acid microspheres [85] or mucoadhesives, such as cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides and carboxymethylcellulos [86];
- microparticles (*i.e.* a particle of ~100nm to ~150μm in diameter, more preferably ~200nm to ~30μm in diameter, and most preferably ~500nm to ~10μm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, *etc.*);
- liposomes [Chapters 13 & 14 of [49, 87-89];
- polyoxyethylene ethers and polyoxyethylene esters [90];
- PCPP formulations [91 and 92];
- muramyl polypeptides, including N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-l-alanyl-d-isoglutamine (nor-MDP), and N-acetylmuramyl-l-alanyl-d-isoglutaminyl-l-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE); and
- imidazoquinolone compounds, including Imiquamod and its homologues (*e.g.* "Resiquimod 3M") [93 and 94].

Immunogenic compositions and vaccines of the invention may also comprise combinations of aspects of one or more of the adjuvants identified above. For example, the following adjuvant

compositions may be used in the invention: (1) a saponin and an oil-in-water emulsion [95]; (2) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) [96]; (3) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) + a cholesterol; (4) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) [97]; (5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [98]; (6) SAF, containing 10% squalane, 0.4% Tween 80™, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion. (7) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); and (8) one or more mineral salts (such as an aluminum salt) + a non-toxic derivative of LPS (such as 3dMPL).

Other substances that act as immunostimulating agents are disclosed in chapter 7 of [49].

The use of an aluminium hydroxide and/or aluminium phosphate adjuvant is particularly preferred, and antigens are generally adsorbed to these salts. Calcium phosphate is another preferred adjuvant. Other preferred adjuvant combinations include combinations of Th1 and Th2 adjuvants such as CpG & alum or resiquimod & alum. A combination of aluminium phosphate and 3dMPL may be used (this has been reported as effective in pneumococcal immunisation [99]).

The compositions of the invention may elicit both a cell mediated immune response as well as a humoral immune response. This immune response will preferably induce long lasting (*e.g.* neutralising) antibodies and a cell mediated immunity that can quickly respond upon exposure to *C. difficile*.

Two types of T cells, CD4 and CD8 cells, are generally thought necessary to initiate and/or enhance cell mediated immunity and humoral immunity. CD8 T cells can express a CD8 co-receptor and are commonly referred to as Cytotoxic T lymphocytes (CTLs). CD8 T cells are able to recognized or interact with antigens displayed on MHC Class I molecules.

CD4 T cells can express a CD4 co-receptor and are commonly referred to as T helper cells. CD4 T cells are able to recognize antigenic polypeptides bound to MHC class II molecules. Upon interaction with a MHC class II molecule, the CD4 cells can secrete factors such as cytokines. These secreted cytokines can activate B cells, cytotoxic T cells, macrophages, and other cells that participate in an immune response. Helper T cells or CD4+ cells can be further divided into two functionally distinct subsets: TH1 phenotype and TH2 phenotypes which differ in their cytokine and effector function.

Activated TH1 cells enhance cellular immunity (including an increase in antigen-specific CTL production) and are therefore of particular value in responding to intracellular infections. Activated TH1 cells may secrete one or more of IL-2, IFN-γ, and TNF-β. A TH1 immune response may result in local inflammatory reactions by activating macrophages, NK (natural killer) cells, and CD8

cytotoxic T cells (CTLs). A TH1 immune response may also act to expand the immune response by stimulating growth of B and T cells with IL-12. TH1 stimulated B cells may secrete IgG2a.

Activated TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. Activated TH2 cells may secrete one or more of IL-4, IL-5, IL-6, and IL-10. A TH2 immune response may result in the production of IgG1, IgE, IgA and memory B cells for future protection.

An enhanced immune response may include one or more of an enhanced TH1 immune response and a TH2 immune response.

A TH1 immune response may include one or more of an increase in CTLs, an increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFN-γ, and TNF-β), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. Preferably, the enhanced TH1 immune response will include an increase in IgG2a production.

A TH1 immune response may be elicited using a TH1 adjuvant. A TH1 adjuvant will generally elicit increased levels of IgG2a production relative to immunization of the antigen without adjuvant. TH1 adjuvants suitable for use in the invention may include for example saponin formulations, virosomes and virus like particles, non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), immunostimulatory oligonucleotides. Immunostimulatory oligonucleotides, such as oligonucleotides containing a CpG motif, are preferred TH1 adjuvants for use in the invention.

A TH2 immune response may include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10), or an increase in the production of IgG1, IgE, IgA and memory B cells. Preferably, the enhanced TH2 immune response will include an increase in IgG1 production.

A TH2 immune response may be elicited using a TH2 adjuvant. A TH2 adjuvant will generally elicit increased levels of IgG1 production relative to immunization of the antigen without adjuvant. TH2 adjuvants suitable for use in the invention include, for example, mineral containing compositions, oil-emulsions, and ADP-ribosylating toxins and detoxified derivatives thereof. Mineral containing compositions, such as aluminium salts are preferred TH2 adjuvants for use in the invention.

Preferably, the invention includes a composition comprising a combination of a TH1 adjuvant and a TH2 adjuvant. Preferably, such a composition elicits an enhanced TH1 and an enhanced TH2 response, *i.e.*, an increase in the production of both IgG1 and IgG2a production relative to immunization without an adjuvant. Still more preferably, the composition comprising a combination of a TH1 and a TH2 adjuvant elicits an increased TH1 and/or an increased TH2 immune response relative to immunization with a single adjuvant (*i.e.*, relative to immunization with a TH1 adjuvant alone or immunization with a TH2 adjuvant alone).

The immune response may be one or both of a TH1 immune response and a TH2 response. Preferably, immune response provides for one or both of an enhanced TH1 response and an enhanced TH2 response.

The enhanced immune response may be one or both of a systemic and a mucosal immune response. Preferably, the immune response provides for one or both of an enhanced systemic and an enhanced mucosal immune response. Preferably the mucosal immune response is a TH2 immune response. Preferably, the mucosal immune response includes an increase in the production of IgA.

The compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared (*e.g.* a lyophilised composition or a spray-freeze dried composition). The composition may be prepared for topical administration *e.g.* as an ointment, cream or powder. The composition may be prepared for oral administration *e.g.* as a tablet or capsule, as a spray, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration *e.g.* as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration *e.g.* as drops. The composition may be in kit form, designed such that a combined composition is reconstituted just prior to administration to a patient. Such kits may comprise one or more antigens in liquid form and one or more lyophilised antigens.

Where a composition is to be prepared extemporaneously prior to use (*e.g.* where a component is presented in lyophilised form) and is presented as a kit, the kit may comprise two vials, or it may comprise one ready-filled syringe and one vial, with the contents of the syringe being used to reactivate the contents of the vial prior to injection.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of antigen(s), as well as any other components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Where more than one antigen is included in a composition then two antigens may be present at the same dose as each other or at different doses.

As mentioned above, a composition may include a temperature protective agent, and this component may be particularly useful in adjuvanted compositions (particularly those containing a mineral adjuvant, such as an aluminium salt). As described in reference 100, a liquid temperature protective agent may be added to an aqueous vaccine composition to lower its freezing point *e.g.* to reduce the

freezing point to below 0°C. Thus the composition can be stored below 0°C, but above its freezing point, to inhibit thermal breakdown. The temperature protective agent also permits freezing of the composition while protecting mineral salt adjuvants against agglomeration or sedimentation after freezing and thawing, and may also protect the composition at elevated temperatures *e.g.* above 40°C. A starting aqueous vaccine and the liquid temperature protective agent may be mixed such that the liquid temperature protective agent forms from 1-80% by volume of the final mixture. Suitable temperature protective agents should be safe for human administration, readily miscible/soluble in water, and should not damage other components (*e.g.* antigen and adjuvant) in the composition. Examples include glycerin, propylene glycol, and/or polyethylene glycol (PEG). Suitable PEGs may have an average molecular weight ranging from 200-20,000 Da. In a preferred embodiment, the polyethylene glycol can have an average molecular weight of about 300 Da ('PEG-300').

Compositions of the invention may be formed by mixing (i) an aqueous composition comprising two or more (*e.g.* 2, 3, or 4) antigen(s) of the antigen combinations of the invention with (ii) a temperature protective agent. The mixture may then be stored *e.g.* below 0°C, from 0-20°C, from 20-35°C, from 35-55°C, or higher. It may be stored in liquid or frozen form. The mixture may be lyophilised. The composition may alternatively be formed by mixing (i) a dried composition comprising two or more (*e.g.* 2, 3, or 4) antigen(s) of the antigen combinations of the invention, with (ii) a liquid composition comprising the temperature protective agent. Thus component (ii) can be used to reconstitute component (i).

Methods of treatment, and administration of the vaccine

The invention also provides a method for raising an immune response in a mammal comprising the step of administering an effective amount of a composition of the invention.

The invention also provides an immunogenic composition comprising a combination of *Clostridium difficile* antigens, said combination comprising a) a ToxB-GT antigen and a TcdA antigen (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more polypeptide fragments of TcdA); and/or b) ToxA-GT antigen and a TcdB antigen (*e.g.* 1, 2, 3, 4, 5, 6, 7, or more polypeptide fragments of TcdB) for use as a medicament *e.g.* for use in raising an immune response in a mammal. Particular immunogenic compositions comprise a combination of *Clostridium difficile* antigens, said combination comprising (i) ToxB-GT antigen and ToxA-P5-6 antigen or (ii) ToxB-GT antigen and ToxA-B2 antigen for use as a medicament *e.g.* for use in raising an immune response in a mammal.

The invention also provides an immunogenic composition comprising a combination of *Clostridium difficile* antigens, said combination comprising a) a ToxB-GT antigen and a TcdA antigen (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more polypeptide fragments of TcdA); and/or b) a ToxA-GT antigen and a TcdB antigen (*e.g.* 1, 2, 3, 4, 5, 6, 7, or more polypeptide fragments of TcdB) in the manufacture of a medicament for raising an immune response in a mammal. Particular immunogenic compositions comprise a combination of *Clostridium difficile* antigens, said combination comprising

(i) ToxB-GT antigen and ToxA-P5-6 antigen or (ii) ToxB-GT antigen and ToxA-B2 antigen in the manufacture of a medicament for raising an immune response in a mammal.

The immune response is preferably protective and preferably involves antibodies and/or cell-mediated immunity. The method may raise a booster response.

By raising an immune response in the mammal by these uses and methods, the mammal can be protected against *C. difficile* infection. More particularly, the mammal may be protected against CDAD, including one or more of diarrhoea, antibiotic associated diarrhoea (AAD), abdominal pain, fever, leukocytosis, pseudomembranous colitis or toxic megacolon. Compositions of the invention are effective against *C. difficile* of various different serotypes. Compositions of the invention may be useful in protecting against CDAD resulting from *C. difficile* strains 630, B1, B1/NAP1/027 (ribotype 027), R20291(SM), 196, BI1, M120 M68, 855, QCD-63q42, ATCC43255, CIP107932, QCD-23m63, QCD-32g58, QCD-37x79, QCD-63q42, QCD-66c26, QCD-76w55, QCD-97b34, CD196, CDBI1, CDCF5, CDSM, CDM68, CDM120 or R20291 *etc.*

The invention also provides a kit comprising a first component and a second component wherein neither the first component nor the second component is a composition of the invention as described above, but wherein the first component and the second component can be combined to provide a composition of the invention as described above. The kit may further include a third component comprising one or more of the following: instructions, syringe or other delivery device, adjuvant, or pharmaceutically acceptable formulating solution.

The invention also provides a delivery device pre-filled with an immunogenic composition of the invention.

The mammal is preferably a human, a large veterinary mammal (*e.g.* horses, cattle, deer, goats, pigs) and/or a domestic pet (*e.g.* dogs, cats, gerbils, hamsters, guinea pigs, chinchillas). Most preferably, the mammal is preferably a human. Immunogenic compositions according to the invention may be used to treat both children and adults. Thus a human patient may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred patients for receiving the vaccines are the elderly (*e.g.* $\geq$50 years old, $\geq$60 years old, and preferably $\geq$65 years), the young (*e.g.* $\leq$5 years old), hospitalised patients, healthcare workers, armed service and military personnel, pregnant women, the chronically ill, or immunodeficient patients. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population.

One way of checking efficacy of therapeutic treatment involves monitoring *C. difficile* infection after administration of the compositions of the invention. One way of checking efficacy of prophylactic treatment involves monitoring immune responses, systemically (such as monitoring the level of IgG1 and IgG2a production) and/or mucosally (such as monitoring the level of IgA production), against the antigens in the compositions of the invention after administration of the composition. Typically, antigen-specific serum antibody responses are determined post-immunisation but pre-challenge

whereas antigen-specific mucosal antibody responses are determined post-immunisation and post-challenge.

Another way of assessing the immunogenicity of the compositions of the present invention is to express the proteins recombinantly for screening patient sera or mucosal secretions by immunoblot and/or microarrays. A positive reaction between the protein and the patient sample indicates that the patient has mounted an immune response to the protein in question. This method may also be used to identify immunodominant antigens and/or epitopes within antigens.

The efficacy of vaccine compositions can also be determined *in vivo* by challenging animal models of *C. difficile* infection, *e.g.*, hamsters, guinea pigs or mice, with the vaccine compositions. One such model is described herein.

Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or mucosally, such as by rectal, oral (*e.g.* tablet, spray), vaginal, topical, transdermal or transcutaneous, intranasal, ocular, aural, pulmonary or other mucosal administration.

The invention may be used to elicit systemic and/or mucosal immunity, preferably to elicit an enhanced systemic and/or mucosal immunity.

Preferably the enhanced systemic and/or mucosal immunity is reflected in an enhanced TH1 and/or TH2 immune response. Preferably, the enhanced immune response includes an increase in the production of IgG1 and/or IgG2a and/or IgA.

Dosage can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes *e.g.* a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, *etc.* Multiple doses will typically be administered at least 1 week apart (*e.g.* about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, *etc.*).

Vaccines produced by the invention may be administered to patients at substantially the same time as (*e.g.* during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines *e.g.* at substantially the same time as a pneumonia vaccine, measles vaccine, a mumps vaccine, a rubella vaccine, a MMR vaccine, a varicella vaccine, a MMRV vaccine, a diphtheria vaccine, a tetanus vaccine, a pertussis vaccine, a DTP vaccine, a conjugated *H.influenzae* type b vaccine, an inactivated poliovirus vaccine, a hepatitis B virus vaccine, a meningococcal conjugate vaccine (such as a tetravalent A-C-W135-Y vaccine), a respiratory syncytial virus vaccine, *etc.*

Mucosal immunisation

The invention provides an immunogenic composition comprising (i) a polypeptide comprising ToxB-GT and one or more polypeptide fragments of TcdA (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more polypeptide fragments of TcdA) and/or a polypeptide comprising ToxA-GT and one or more polypeptide fragments of TcdB (*e.g.* 1, 2, 3, 4, 5, 6, 7, or more polypeptide fragments of TcdB), and (ii) a bacterial ADP-ribosylating toxins and or detoxified derivative thereof. The invention also provides a method for raising an immune response in a mammal comprising the step of administering an effective amount of such an immunogenic composition to the mammal.

Further antigenic components of compositions of the invention

The invention also provides compositions further comprising at least one further *C. difficile* antigen. Further *C. difficile* antigens include, for example, saccharide antigens. Saccharide antigens may be conjugated to peptides of the invention using standard conjugation techniques known in the art. A preferred saccharide antigen for use in compositions of the invention is the cell wall polysaccharide II (referred to herein as "PS-II"), thought to be a conserved surface antigen in *C. difficile*. The structure of the PS-II repeating unit is described in [101]:

[→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Glc*p*-(1→4)-[β-D-Glc*p*-(1→3]-β-D-Gal*p*NAc-(1→3)-α-D-Man*p*-(1→P]

For example, a polypeptide described above (such as ToxB-GT) may be chemically conjugated to *e.g.* PS-II.

The invention also provides compositions further comprising at least one antigen that is not a *C. difficile* antigen.

In particular, the invention also provides a composition comprising a polypeptide or the invention and one or more of the following further antigens:

- a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y (preferably all four).
- a saccharide or polypeptide antigen from *Streptococcus pneumoniae* [*e.g.* 102, 103, 104].
- an antigen from hepatitis A virus, such as inactivated virus [*e.g.* 105, 106].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [*e.g.* 106, 107].
- a diphtheria antigen, such as a diphtheria toxoid [*e.g.* chapter 3 of ref. 108] or the CRM_{197} mutant [*e.g.* 109].
- a tetanus antigen, such as a tetanus toxoid [*e.g.* chapter 4 of ref. 108].
- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [*e.g.* refs. 110 & 111].
- a saccharide antigen from *Haemophilus influenzae* B [*e.g.* 112].

- polio antigen(s) [*e.g.* 113, 114] such as IPV.
- measles, mumps and/or rubella antigens [*e.g.* chapters 9, 10 & 11 of ref. 108].
- influenza antigen(s) [*e.g.* chapter 19 of ref. 108], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [*e.g.* 115].
- an protein antigen from *Streptococcus agalactiae* (group B streptococcus) [*e.g.* 116, 117].
- a saccharide antigen from *Streptococcus agalactiae* (group B streptococcus).
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [*e.g.* 117, 118, 119].
- an antigen from *Staphylococcus aureus* [*e.g.* 120].

The composition may comprise one or more of these further antigens.

Toxic protein antigens may be detoxified where necessary (*e.g.* detoxification of pertussis toxin by chemical and/or genetic means [111]).

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens. DTP combinations are thus preferred.

Saccharide antigens are preferably in the form of conjugates. Carrier proteins for the conjugates include diphtheria toxin, tetanus toxin, the *N.meningitidis* outer membrane protein [121], synthetic polypeptides [122,123], heat shock proteins [124,125], pertussis proteins [126,127], protein D from *H.influenzae* [128], cytokines [129], lymphokines [129], streptococcal proteins, hormones [129], growth factors [129], toxin A or B from *C.difficile* [130], iron-uptake proteins [131], *etc.* A preferred carrier protein is the CRM197 mutant of diphtheria toxin [132].

Antigens in the composition will typically be present at a concentration of at least 1μg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

As an alternative to using proteins antigens in the immunogenic compositions of the invention, nucleic acid (preferably DNA *e.g.* in the form of a plasmid) encoding the antigen may be used.

Antigens are preferably adsorbed to an aluminium salt.

Antibodies

Antibodies against *C. difficile* TcdA and TcdB can be used for passive immunisation [*e.g.* 133, 134, 135, 136, 137, 138 and 139]. Thus the invention provides combinations of antibodies corresponding to, and specific to, the antigen combinations of the invention as disclosed herein. Preferably, the composition comprises an antibody that is specific to a ToxB-GT antigen, and/or an epitope thereof and an antibody that is specific to a TcdA antigen, and/or an epitope thereof; and/or an antibody that is specific to a ToxA-GT antigen, and/or an epitope thereof and an antibody that is specific to a TcdB

antigen, and/or an epitope thereof. Combinations of antibodies according to the invention are provided for simultaneous, separate or sequential administration. The invention also provides and immunogenic and pharmaceutical compositions comprising such antibodies. Herein, in the context of the invention, the term "antibody" or "antibodies" comprises the combinations of antibodies of the invention. The invention also provides compositions comprising combinations of antibodies of the invention.

The invention also provides the use of antibodies of the invention in medicine and in therapy, *.e.g.* for passive immunisation against CDAD. The invention also provides a method for treating a mammal comprising the step of administering an effective amount such a composition. As described above for immunogenic compositions, these methods and uses allow a mammal to be protected against CDAD. In particular, antibodies of the invention may be used in methods of treating or preventing infections by *C. difficile*, comprising the step of administering to the mammal an effective amount of a combination of antibodies as described herein, or a composition comprising such a combination. In these methods, the at least two (*e.g.* 2, 3, or 4) antibodies of the invention may be administered simultaneously, separately or sequentially.

The term "antibody" includes intact immunoglobulin molecules, as well as fragments thereof which are capable of binding an antigen. These include hybrid (chimeric) antibody molecules [140, 141]; F(ab′)2 and F(ab) fragments and Fv molecules; non-covalent heterodimers [142, 143]; single-chain Fv molecules (sFv) [144]; dimeric and trimeric antibody fragment constructs; minibodies [145, 146]; humanized antibody molecules [147-149]; and any functional fragments obtained from such molecules, as well as antibodies obtained through non-conventional processes such as phage display. Preferably, the antibodies are monoclonal antibodies. Methods of obtaining monoclonal antibodies are well known in the art. Humanised or fully-human antibodies are preferred. Antibodies and antibody combinations of the invention may be purified or isolated.

The invention also provides a process for preparing a mixture of a combination of antibodies of the invention, said process comprising a step of mixing antibodies of any of the combinations of antibodies as defined above. For example, the invention provides a process comprising a step of mixing at least two (*i.e.* 2, 3, or 4) antibodies selected from: (a) an antibody which recognises a ToxA-GT antigen, and/or an epitope thereof, and an antibody which a TcdB antigen and/or an epitope thereof. For example, the process may comprise a step of mixing an antibody which recognises a ToxA-GT antigen, and/or an epitope thereof, and an antibody which recognises a TcdB antigen and/or an epitope thereof. The invention also provides a process comprising a step of mixing at least two (*i.e.* 2, 3, or 4) antibodies selected from: (a) an antibody which recognises a ToxB-GT antigen, and/or an epitope thereof, and an antibody which recognises a TcdA antigen, and/or an epitope thereof. For example, the process may comprise a step of mixing an antibody which recognises a ToxB-GT antigen, and/or an epitope thereof, and an antibody which recognises a TcdA antigen and/or an epitope thereof. A process according to the invention for preparing a mixture of antibodies may comprise a further step of formulating the mixture as a medicament. Such processes

may further comprise a step of packaging the formulation for storage or distribution as a medicament.

General

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, [150-157, *etc]*.

Where the invention concerns an "epitope", this epitope may be a B-cell epitope and/or a T-cell epitope. Such epitopes can be identified empirically (*e.g.* using PEPSCAN [158,159] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [160], matrix-based approaches [161], MAPITOPE [162], TEPITOPE [163,164], neural networks [165], OptiMer & EpiMer [166, 167], ADEPT [168], Tsites [169], hydrophilicity [170], antigenic index [171] or the methods disclosed in [172-176, *etc.*]. Epitopes are the parts of an antigen that are recognised by and bind to the antigen binding sites of antibodies or T-cell receptors, and they may also be referred to as "antigenic determinants".

The terms "antigen" and "amino acid sequence", as they are used in this document, should be taken to include reference to each of the above sequences, as well as to their fragments, homologues, derivatives and variants. The term "toxin" refers to a poisonous substance, especially a protein, that is produced by living cells or organisms and is capable of causing disease when introduced into the tissues of a subject and is often capable of inducing production of neutralizing antibodies or antitoxins in a subject.

The term "toxoid" refers to a toxin or fragment thereof which has undergone "detoxification" or "toxoiding" (e.g. by recombinant means, by chemical modification etc.) but has maintained its ability to combine with, or induce production of anti-toxin antibodies e.g. when administered to a subject.

The term "neutralising titer" refers to a composition comprising "neutralising peptides" or "neutralising antibodies" that inhibit or neutralise the biological effect of an infectious body (*e.g.* a toxin).

Where an antigen "domain" is omitted, this may involve omission of a signal polypeptide, of a cytoplasmic domain, of a transmembrane domain, of an extracellular domain, *etc.*

The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.
The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do

not materially alter the basic and novel characteristics of the claimed composition, method or structure.

The term "about" in relation to a numerical value *x* means, for example, $x \pm 10\%$.

References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. 177. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 178.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Schematic representation of the recombinant toxin fragments used in this study. All polypeptides were expressed in *Escherichia coli*, except ToxA_GT, which was expressed in *Brevibacillus choshinensis*. ED = enzymatic domain; GT = glucosyl-transferase domain; CP = cysteine protease domain; T = translocation domain; B = binding domain. All domains are soluble, with the exception of the T4 and PTA2 domains of TcdA, which are insoluble.

Figure 2. ToxA-B2 was designed to include 6 of the 13 putative structural units forming the binding domain. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E).

Figure 3. ToxA-B3 was designed to include 12 of the 13 putative structural units forming the binding domain. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E).

Figure 4. ToxA-B5 was designed to include 10.5 of the 13 putative structural units forming the binding domain. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E).

Figure 5. ToxA-B6 was designed to include 11.5 of the 13 putative structural units forming the binding domain. The three-dimensional structure of the TcdA binding domain was predicted by computer modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E).

Figure 6. ToxB-B2 was designed to include 4 of the 9 putative structural units forming the binding domain The three-dimensional structure of the TcdB binding domain was predicted by computer

modelling using the crystal structure of the C-terminal fragment as template (see reference 41, PDB code 2F6E).

Figure 7. Schematic representation of the B4 hybrid. ToxB_GT (SEQ ID NO: 18) is fused to ToxA-P5-6 (SEQ ID NO: 11) via a linker peptide (SEQ ID NO: 25).

Figure 8. Flow chart summarizing the experimental strategy used for the identification of candidate fragments.

Figure 9. Geometric mean titres (GMTs) of antibodies directed against sub-domains of TcdA (A) and TcdB (B), as determined by ELISA.

Figure 10. Example of an *in vitro* neutralization experiment showing the TcdA/B-induced cell rounding and the neutralization by serum against ToxA_B2+ToxB-GT.

Figure 11. Schematic representation of the toxin domain fragments used in hamster studies. ED = enzymatic domain; GT = glucosyl transferase domain; CP = cysteine protease domain; T = translocation domain; B = binding domain.

Figure 12. Toxoid A + Toxoid B. Average bacterial shedding in faeces. Challenged with B1.

Figure 13. Toxoid A + Toxoid B – Post- infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)).

Figure 14. ToxB_B + P5_6 - post- infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)).

Figure 15. Graphical representation of the data provided in Figure 29.

Figure 16. Bacterial shedding of *C. Difficile* spores in 100mg faeces from hamsters immunised with P5_6 + ToxB_B or controls. Challenged with B1 strain.

Figure 17. P5_6 + ToxB_B or controls. Challenged with B1 strain. Post- infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)).

Figure 18. Immunisation with ToxB_GT + P5+6. Colonisation in faeces of vaccinated animals per (per hamster (upper panel) and average (lower panel)). Challenge with 630 strain.

Figure 19. ToxB_GT + P5_6 Terminal colonisation results. Challenge with 630 strain.

Figure 20. ToxB_GT + P5_6. Colonisation in faeces of vaccinated animals over time (days). Challenge with B1 strain.

Figure 21. ToxB_GT + P5_6. Infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)). Challenge with B1 strain.

Figure 22. ToxA-P5-6 + ToxB_GT (reduced dose). Colonisation in faeces of vaccinated animals over time (days). Challenge with B1 strain.

Figure 23. ToxA-P5-6 + ToxB_GT (reduced dose). Infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)). Challenge with B1 strain.

Figure 24. Immunisation with ToxB_GT(PSII) + P5_6. Colonisation in faeces of vaccinated animals over time (days). Challenge with 630 strain. Some animals are missing specific time points, *e.g.* where the animal failed to produce faeces on the day of collection (especially after periods of diarrhoea) or had diarrhoea on a specific time point.

Figure 25. Average number of *C. difficile* being shed in faeces from surviving vaccinated animals (ToxB_GT(PSII) + P5_6 (H1-H6), or ToxB_GT + P5_6 (H7-8)).

Figure 26 (a and b). ToxB_GT(PSII) + P5_6 results – infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)).

Figure 27. ToxB_GT + ToxA_B2. Colonisation in faeces of vaccinated animals over time (days). Challenge with B1 strain.

Figure 28 (a and b). ToxB_GT + ToxA_B2. Infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)). Challenge with B1 strain.

Figure 29. ToxB_GT + ToxB_B + P5_6. Average colonisation in faeces of vaccinated animals.

Figure 30. ToxB_GT + ToxB_B + P5_6. Challenge with B1. Post- infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)).

Figure 31. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 – average bacterial shedding of *C. Difficile* spores in 100mg faeces. Challenge with B1.

Figure 32. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 – colonisation at culling. Challenge with B1.

Figure 33. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 (lower dose) - average bacterial shedding of *C. Difficile* spores in 100mg faeces. Challenge with B1.

Figure 34. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 (lower dose) - infection analysis of *C. difficile* recovered in faecal material - localisation of bacteria (x = axis is location: "Col" = colon; "Cae" = caecum; "LA" = lumen associated; "TA" = tissue associated. Y= axis is number of *C. difficile* (CFU/ml)) Challenge with B1.

Figure 35. Microflora changes after clindamycin treatment.

Figure 36. Modification to microbiota in vaccinated animals.

Figure 37. SDS-PAGE gel of recombinant purified fragments.

Figure 38. Anti-ToxA (A) and ToxB (B) IgG titers (UE/mL), adjuvanted with Alum or MF59. IgG response after mice immunised with recombinant ToxA (A) and ToxB fragments (B). Anti toxin A and toxin B IgG titers were measured by ELISA in sera from mice immunized i.p. with each fragment with Al(OH)3 (left colmn in pair) or MF59 (right column in pair) adjuvant. Results are shown as geometric mean ± SD on at least three experiments.

Figure 39: IgG antibodies against toxin A (A) and toxin B (B) in ceacum samples from hamsters vaccinated with ToxA-P5-6 + ToxB-GT. Dot blots were carried out on filtered caecum samples taken from vaccinated animals in the acute phase of infection (48 hours post-challenge) (hamsters 1-2) and at experimental endpoint (14 days post-challenge) (hamsters 3-8). Control animals were treated with adjuvant only and infected in the same experimental conditions (hamsters 9-10).

Figure 40: Toxin A and B levels in hamsters vaccinated with ToxA-P5-6 + ToxB-GT combination. Values are the fold dilution required for cell rounding. Filtered caecum samples were taken in vaccinated animals in the acute phase of infection (48 hours post-challenge) and at experimental endpoint (14 days post-challenge). Control animals were treated with adjuvant only and infected in the same experimental conditions.

MODES FOR CARRYING OUT THE INVENTION

The inventors identified recombinant fragments of TcdA and TcdB which may be used as immunogens for use in a vaccine to prevent CDAD.

Fragments

A schematic representation of the experimental approach is provided in Figure 8. The inventors designed a panel of toxin-based fragments of TcdA and TcdB (11 fragments of TcdA and 6 fragments of TcdB). The toxin-based fragments used in this study are described in Figure 1. These fragments were chosen to cover as far as possible the whole lengths of TcdA and TcdB, and the

boundaries of these fragments were determined on the basis of their crystal structures. In the case of the cell binding domains of ToxA and ToxB (see Figure 1 for summary), computer models were used. By using recombinant techniques, it is possible to use an expression system *e.g. E. coli, Brevibacillus choshinensis, etc.,* to easily generate polypeptide fragments and which are more stable and resistant to degradation than inactivated toxoids, as well also avoid many of the safety concerns regarding the use of inactivated toxoids. Fragments used in the examples comprising ToxA-GT or ToxB-GT were detoxified. A Coomassie-stained SDS-PAGE gel of peptides used for immunization is shown in Figure 37.

Cloning, expression and purification of recombinant toxin fragments

Sequences were cloned into the pet15b+ vector (Nterm-HIS tag) using the Polymerase Incomplete Primer Extension (PIPE) method. Normal PCR generates mixtures of incomplete extension products. Using simple primer design, short overlapping sequences were introduced at the ends of these incomplete extension mixtures which allow complementary strands to anneal and produce hybrid vector/insert combinations. All hybrids were transformed directly into *E.coli* HK100 recipient cells. Single ampicillin resistant colonies were selected and checked for the presence of recombinant plasmid by colony PCR. Competent *E.coli* BL21(DE3) cells were transformed with the plasmids purified from positive clones (ToxA_GT, was expressed in *Brevibacillus choshinensis*). The ToxA-p5-6 antigen was expressed as a hybrid polypeptide comprising the N-terminal amino acid sequence of SEQ ID NO 104 and the C-terminal amino acid sequence of SEQ ID NO 105. The amino acid sequence of this hybrid polypeptide is shown in SEQ ID NO 111. SEQ ID NO: 111 is encoded by the nucleic acid sequence of SEQ ID NO: 112.

The PIPE method was employed to generate ToxA-GT (Y283A, D285A, D287A), TcdA-CP (D589A, H655A and C700A, numbered relative to SEQ ID NO: 1), ToxB-GT (D270A, R273A, Y284A, D286A and D288A) and ToxB-CP (D587A, H653A and C698A) mutants with abrogated enzymatic activity.

Protein expression was induced by addition of 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) to the culture during exponential growth phase, followed by incubation for 4 hours at 25°C. Cell extracts were loaded onto SDS- PAGE gels to check for protein expression (data not shown).

For ToxA_GT, the catalytic domain (residues 1-541) of the WT *C. difficile* Toxin A was cloned into pNI-His vector (Takara Bio). Site-directed mutagenesis was performed to obtain the (Y283A, D285A, D287A) ToxA-GT mutant. The plasmid was electroporated into the *B. choshinensis* HPD31-SP3 strain (Takara Bio). *B. choshinensis* expression cells were grown in TMNm at 25◦C, 1600 rpm for 48 hours. The protein was purified by IMAC chromatography and than buffer-exchanged into PBS using PD-10 desalting column (GE). Protein quantification was performed by BCA assay.

For some experiments, PSII was conjugated to two different carrier proteins (*C.difficile* recombinant proteins: ToxA_B2 and ToxB_GT) after chemical modification of the mannose sugar of the

repeating unit at the reducing end. First step was the mannose sugar reduction with 50 mM $NaBH_4$ (Sigma) in 10 mM NaPi buffer pH 9.0 at room temperature for 2 hours; the reduced PSII was purified by Sephadex G25 chromatography (G&E Healthcare) in water and then oxidized with 15 equivalent of $NaIO_4$ (Sigma) in 10 mM NaPi buffer pH 7.2 at room temperature for 2 hours at the dark. The oxidized PSII was then purified by Sephadex G25 chromatography (G&E Healthcare) in water. The oxidized PSII (10 mg/ml) was then conjugated to carrier proteins using a stoichiometry of 4:1 (weight PSII per weight Protein) in 200 mM NaPi/1M NaCl buffer pH 8.0, and in presence of $NaBH_3CN$ (2:1, weight PSII per weight $NaBH_3CN$). The mixture was incubated for 48-72 hours at 37°C, mixing very gently with a magnetic stirrer. Conjugates were purified from excess of unconjugated PSII using size exclusion Superdex 75 chromatography (G&E Healthcare) in 10 mM NaPi/10 mM NaCl buffer pH7.2. Conjugates were characterized by SDS-PAGE using 7% Tris-Acetate gels (NuPAGE, from Invitrogen) in NuPAGE Tris-Acetate SDS running buffer (20x, Invitrogen). Protein concentration was determined by MicroBCA protein assay kit (Thermo Scientific). Total saccharide concentration was determined by HPAEC-PAD analysis. Unconjugated saccharide was separated by SPE C4 hydrophobic interaction column (0.5 mL resin, Bioselect, Grace Vydac) and subsequently estimated by HPAEC-PAD analysis.

Purification and inactivation of toxoid A and toxoid B

C. difficile strain VPI 10463 spore stocks were inoculated on BHIS plates (brain heart infusion supplemented with yeast extract [5 mg/ml] and L-cysteine [0.1%]) and incubated at 36°C for 2 days. Colonies were added from prepared plate to the Tryptone-Yeast Extract- Mannitol (TYM) media and incubated for 16 hours at 35 C° in anaerobic chamber. 200 µl of 90% glycerol was added together with 800 µl of the *C. difficile* culture (1 OD at 590 nm) to the 1-ml cryogenic vial. The vial was immediately placed in a −80°C freezer for storage. 100 µl of glycerol stock was added to 10 ml TYM media and incubated for 16 Hours at 35 °C in anaerobic chamber. Each 1 liter of Tryptone-Yeast Extract (TY) media was inoculated with seed culture (1/100 dilution).Culture was incubated at 35 °C for 5 days in anaerobic chamber. Samples were then centrifuged at 3000g for 15 minutes at 4°C, and filtered through a 0.22µm pore size filter. The supernatant was then concentrated by tangential flow filtration.

Fraction I: AS50

80.03g of ammonium sulphate were added to 6 x concentrated culture supernatant from strain VPI 10463 in Tryptone Yeast extract medium (265 ml) over the course of 2 h at 0°C (50 % saturation). Stirring was continued for 3 h at 0 °C, then the precipitate was sedimented by centrifugation at 10000 rpm, for 30 minutes at 4°C. The pellet was resuspended in buffer A and dialyzed at 4°C against two changes of buffer (A: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl; B: 50 mM Tris-HCl, pH7.5, 1 M NaCl) (2 x 1 l) yielding a final volume of 27 ml (Fraction I) conc. 6.118 mg/ml, mini BCA.

Fraction II: HiTrap Q HP, pH 7.5

ToxA and ToxB were separated by chromatography on 2 x 5 ml HiTrap Q HP columns, connected in series. A linear gradient from 0-100 % B was applied with 30 CV, 2 ml/min. ToxA elutes around 20 % B, ToxB around 50 % B (data not shown). 20 µl of fractions I, 4, 14-44 were analyzed on 7 % PAA gels in Tris-Acetate buffer (data not shown).

Fraction IIIb: HiTrap Q HP, pH 5.0

ToxB (Fraction IIb) was further purified by chromatography on HiTrap Q HP at pH 5.0. Buffer included C: 20 mM Piperazine-HCl, pH 5.0, 50 mM NaCl; D, 20 mM Piperazine-HCl, pH 5.0, 1 M NaCl. A segmented gradient was applied, 30-60 % D, 15 CV. ToxB elutes at 40% D (data not shown). 10 µl of each fraction was analyzed on a 7 % PAA gel in Tris-Acetate buffer (data not shown).

Fraction IIIa: HiTrap Q HP, pH 7.5

B was applied with 30 CV, 2 ml/min to a segmented gradient from 2-20 %. ToxA elutes around 15 % B. 20 µl of each fraction was analyzed on a 7 % PAA gel in Tris-Acetate buffer (data not shown). Pool was dialyzed against 2 l 50 mM Tris-HCl, 50 mM NaCl, 4°C over night. Final volume : 52 ml (Fraction IIIa).

A final quality control was performed.

For Western blots, preparations were loaded on a 7 % Tris Acetate SDS-PAA gel, and transferred to nitrocellulose using the I-Blot machine (12 min transfer). Membranes were washed 3 x in TBST, and blocked over night with 1 % BSA (Promega) in TBST. Primary antibodies were added at 1 : 5000 for 1 h in TBST. Membranes were washed 3 times for 5 min in TBST. Secondary antibody (Promega anti-rabbit AP conjugate) was added at 1 :8000 in TBST for 45 min. The blot was washed three times in TBST and two times for 5 min in MilliQ water. Blots were developed for 20 sec in stabilized AP substrate (Promega) (data not shown).

ToxA and Tox B preparations were found to be completely free of cross contamination.

For permanent storage, dialysis was performed. Dialysis buffer comprised 50 mM Tris-HCl, 500 mM NaCl and 10% Glycerol. Samples were dialyzed against 2 changes of 500 ml buffer. Samples were quantified after dialysis (data not shown).

Detoxification of toxoids

Preparations were dialyzed against PBS. Tris could react with formaldehyde. Starting with 1.5 mg of each protein (ToxA: 1.5 mg corresponded. to 9.375 ml (9.7 ml) and ToxB: 1.5 mg corresponded to 4.411ml (4.5 ml)). Samples were dialyzed against 1 l each of PBS, 40h, 4 °C. Tox A was dialyzed for 4 h against 20% PEG 20.000 in PBS. Volume was reduced to 3.7 ml

Formylation of ToxA and ToxB

MW of ToxA and B: is approximately 300 kDa. Preparations included 0.25 mg/ml of ToxA and o.35 mg/ml of ToxB. Lysin stock comprised 1 M lysine·HCl in PBS. Summaries are included in Table 1 (a and b) below:

	ToxA	*Final Conc.*
Protein	3500 ul	0.58. µM
Lysin (1 M)	279.6	56 mM
Formaldehyde (36.5%)	8.2	10 mM
PBS	1212.2	
Total	5000	

Table 1(a). Summary of Toxoid A formulation

	ToxB	*Final Conc.*
Protein	4000 ul	0.93 µM
Lysin (1 M)	50	10 mM
Formaldehyde (0.4%)	150	3.9 mM
PBS	800	
Total	5000	

Table 1(b). Summary of Toxoid B formulation

After 120 h at 37 °C on a rotary shaker 1 ml each was withdrawn and dialyzed against 2 x 500 ml PBS for 2 x 24 h. Samples were confirmed as being activated using a cell-based toxicity assay (data not shown).

Immunisation of mice

Fragments were then used to immunize mice, to determine whether the fragments are immunogenic.

For each antigen, two groups of 8 female CD1 mice were used. Each group was immunised with 10 ugrs of antigen, formulated in Alum adjuvant (group 1) or Freund's adjuvant. Immunisations were performed intraperitoneally at days 0, 21, and 35. Final bleeding and culling was performed at day 49. Total antibody response of mice immunised with toxin fragments was then determined by ELISA. Microtiter plates were coated with TcdA and TcdB and incubated with antibodies against fragments, followed by alkaline phosphatase-conjugated secondary antibodies. After addition of the substrate, (p-nitrophenyl phosphate or pNPP), plates were analyzed by a plate reader at a dual wavelength of 405/620-650 nm. Antibody titres were quantified via interpolation against a reference standard curve.

ELISA studies showing total antibody responses of mice immunized with toxin fragments are shown in Figure 9. Interestingly, the ToxB-GT fragment was as immunogenic as the full length TcdB-ED domain. With the exception of ToxA-CP, all toxin fragments are immunogenic. The IgG responses (adjuvanted with Alum or MF59) are shown in Figure 38.

In vitro cell rounding neutralization assay

The *in vitro* neutralization assay is based on evidence that *C. difficile* toxins destabilize the actin cytoskeleton causing a cytopathic effect with a typical cell rounding. Anti-toxin antibodies can neutralize the cytotoxicity, thus preventing the cell rounding. Immune sera were therefore used to evaluate the ability of the fragments to neutralize *in vitro* the toxic effects of TcdA and TcdB.

Human fibroblasts (IMR-90) were grown to 80-90% confluence. Each cell line has a different sensitivity to toxins, and so the minimal doses of TcdA and TcdB required to cause 100% cell rounding in 24 hours (CTU_{100}) were determined. $CTU_{100}$was established as 20 ng/mL for TcdA and 10 pg/mL for TcdB. Two-fold dilutions of sera from 1:8 to 1:32,000 were pre-incubated with 1 CTU_{100} of each toxin for 90 min at 37°C. Mixtures of sera plus toxins were then added to the cells, followed by observation after 16-18 hours. The endpoint titers represent the reciprocal of the highest dilution able to inhibit cell rounding. Positive controls were sera α-toxoid A and B and negative controls were the pre-immune sera and the sera from mice treated with adjuvant alone.

Results

Neutralization titers are summarized in Tables 2 and 3, and the results of a typical neutralization experiment are shown in Figure 10. Soluble fragments of ToxA binding domain were found to induce strong neutralizing antibodies, irrespective of whether they were adjuvanted with MF59 or Alum. Insoluble fragments, ToxA-PTA2, ToxA-CP and ToxA-T4 did not induce neutralizing antibodies. ToxA-CP (which was not identified as being immunogenic) also did not induce neutralizing antibodies. Sera raised against ToxA did not cross-neutralise ToxB (Table 2).

	ToxA 20ng/ml		ToxB10pg/ml	
Antigen	Alum	MF59	Alum	MF59
p5_8	2000	2000	0/16	0/16
ToxA_B2	8000	8000	0	0
ToxA-B5	4000	4000	0	0
ToxA-B8	2000	2000	0	0
ToxA-B3	4000	2000	0	0
ToxA-PTA2	0		0	
ToxA-T4	0		0	
ToxA-GT	1000	256	0	0
p5_8 +ToxA-GT	4000	4000	0	0
ToxA-CP	0	0	0	0
ToxoidA	16000	16000	0	

Table 2. Neutralisation titers of sera raised against sub-domains of TcdA (differences in experimental repeats are denoted by "/").

	ToxA 20ng/ml		ToxB10pg/ml	
Antigen	Alum	MF59	Alum	MF59
ToxB-B	0	0	128/256	128/256
ToxB-B2	0	0	0	0
ToxB-B7	0	0	0	0
ToxB-ED	0	0	128*	128*
ToxB-GT	0	0	128*/256	256
ToxB-CP	0	0	0	0
ToxB-GT+ToxB-B	0	0	512	256
Toxoid B			2000	2000

Table 3. Neutralisation titers of sera raised against sub-domains of TcdB. * indicates 50% neutralization (differences in experimental repeats are denoted by "/").

ToxB-B, ToxB-ED and ToxB-GT induced a weak neutralizing antibody response, which were similar when adjuvanted with MF59 or Alum. By contrast, Tox-B2, ToxB-B7, ToxB-CP did not induce neutralizing antibodies. Sera raised against ToxB did not cross-neutralise ToxA (Table 3).

Thus, antibodies directed to TcdA are not able to cross-neutralize TcdB and vice versa.

The mouse immunization studies (above) and the results obtained from the *in vitro* cell rounding assay collectively suggest that the N-terminal region of the ED of TcdA and/or TcdB (*i.e.* the GT domain) is immunogenic and important for raising neutralizing antibodies against its respective toxin. Moreover, the neutralizing antibody response induced by the ToxB-GT fragment (and also the ToxB-ED fragment, comprising the ToxB-GT sequence) was the same, or better, than the neutralizing antibody response obtained using the majority of the binding domain of TcdB (*i.e.* the ToxB-B fragment).

The toxicity test was also performed to confirm whether the D270A, Y284A, D286A and D288A mutations in ToxB-GT led to a decrease in toxicity, as compared to the native, full length toxin B. A range of 10 concentrations ranging from 20ng/ml to 40 ugr/ml were tested using the assay protocol outlined above. Fibroblasts incubated with mutated ToxB-GT did not show any morphological alterations at the concentrations tested, while native full-length toxin B caused cell rounding at 10 pg/ml under the same experimental conditions (data not shown). Therefore, the D270A, Y284A, D286A and D288A mutations led to loss of toxicity under the experimental conditions tested.

Combinations of fragments

To determine whether it is possible to obtain sera capable of inducing concomitant neutralization of both toxin A and toxin B, the inventors then combined the most promising toxin fragments. Neutralization titres of the sera against the toxin combinations are summarized in Table 4.

	20 ToxAng/mL		10 ToxBpg/mL	
Antigen	Alum	MF59	Alum	MF59
p5_6 + ToxB-B	8000	4000	256	128
p5_6 + ToxB-B2	8000	4000	0	0
p5_6 + ToxB-GT	8000	4000	128	64/128
p5_6 + PTA2 + ToxB-GT+ToxB-B	8000	4000	1000	64
p5_6 + ToxB-GT+ToxB-B	2000	2000	256	128
ToxA_B2 + ToxB-B	4000	2000/4000	256	256
ToxA_B2 + ToxB-GT	8000	8000	256	256
ToxA_B2 + ToxB-B7	2000	2000	0	0
ToxA_B3 + ToxB-B	4000	4000	128	128
ToxA_B3 + ToxB-GT	4000	2000	128	128
ToxA_B3 + ToxB-B + ToxB-GT	4000	2000	256	256
ToxA_B3 + ToxB-B2	4000	2000	0	0
ToxA_B5 + ToxB-GT	4000	4000	512	32
ToxA_B6 + ToxB-B7	2000	1000	0	0
Chimera	**4000**	**4000**		
ToxoidA+ToxoidB	**16000**	**16000**	**2000**	**2000**

Table 4. Neutralization titres of sera raised against combinations of single sub-domains of TcdA and TcdB. (differences in experimental repeats are denoted by "/").

The inventors found that antibodies directed to several of the tested combinations are able to cross-neutralize TcdB and vice versa. By contrast, combinations of P5_6 + ToxB-B2, ToxA_B2 + ToxB-B7, ToxA_B3 + ToxB_B2 and ToxA_B6 + ToxB-B7 did not cross-neutralise. ToxA-P5_6+ToxB-B, and ToxA-B2+ToxB-GT emerged as most promising fragment combinations, further highlighting the ability of ToxB-GT to functionally substitute the ToxB-B region.

Interestingly, all combinations comprising ToxB-GT were able to induce neutralization titers against Toxin A and Toxin B, and combinations as well as equivalent combinations comprising the majority of the binding domain of TcdB (*i.e.* ToxB-B).

Chimeric proteins

The inventors designed chimeric proteins combining different TcdA and TcdB domains into a single polypeptide (summarised in Table 5). In "B1", ToxB-ED is N-terminal of ToxA-P5-6; in "B1 small", ToxB-CP is N-terminal of ToxA-P5-6; and in "B4", ToxB-GT is N-terminal of ToxA-P5-6. *In vitro* neutralization studies were performed using these chimeras under the same experimental conditions as for the fragments. Results are summarised in Table 5. Three chimeric proteins containing the p5_6 fragment fused to the enzymatic domains of TcdB induce sera able to neutralize TcdA but not TcdB.

Interestingly, the ToxA neutralizing activity induced by p5_6 is variable across the three p5_6 chimerae. This is likely due to changes in folding and/or immunogenicity. Similarly, the B4 chimera which contains fragments of binding domains of TcdA and TcdB induced antibodies with efficient neutralizing activity against TcdA but not against TcdB (Table 5).

	Tox A 20ng/ml		Tox B 10pg/ml	
Antigen	Alum	MF59	Alum	MF59
B1 (ToxB-ED/p5_6)	256*		0	
B1small (ToxB-CP/p5_6)	8000	8000	0	0
B4 (ToxB-GT/p5_6)	4000		0	
ToxoidA + ToxoidB	16000	16000	2000	2000

Table 5. Neutralization titres of sera raised against chimeric proteins.

These data suggest that compositions comprising a combination of a) a polypeptide comprising ToxB-GT and one or more polypeptide fragments of TcdA or b) a polypeptide comprising ToxA-GT and one or more polypeptide fragments of TcdB perform better than chimeric polypeptides comprising sequences from ToxA and ToxB.

Efficacy testing in hamsters

Hamster immunisation studies typically involved 10 Golden Syrian Hamsters. Within each group, 6 hamsters were immunized via intra peritoneal (i.p.) route with four doses of antigen (50 ugr of each antigen formulated in MF59 adjuvant) at days 1, 14, 28 and 36. Two untreated animals and two vaccinated with MF59 adjuvant alone were always included as negative controls. On day 60, all hamsters were treated with clindamycin (30mg/kg hamster body weight) to remove intestinal commensal flora. After 12 hours, animals were challenged by oral gavage of approximately 250 *C. difficile* spores. Body temperature and presence of clinical symptoms such as diarrhoea were monitored for 14 days after the challenge. Body temperature drop to 35^0C was taken as the humane endpoint of the experiment, at which point, the animal was culled. Body temperature was measured telemetrically using a chip inserted into the body cavity of the animals 3 weeks prior to infection. Table 6 shows the time after infection with the B1 or 630 strain before the body temperature drops by 2°C (5 hamsters were assessed per *C. difficile* strain).

Hamster	B1 infected	630 infected
1	31h	46h
2	33h 30 min	49h
3	33h 45 min	48h 45 min
4	32h 30min	46h 30 min
5	32h	45h 50 min

Table 6. Time from infection with the B1 strain or 630 strain to 2^oC loss of hamster body temp.

Post-infection analyses involved confirmation that the hamsters had been infected specifically with the infection strain (using multiple locus variable tandem repeat analysis (MVLA), based on banding patterns from 7 repeat regions), as well as bacterial counts in fecies and gut.

Infection was found not to interfere with anti-toxin immune response, since a sample serum from vaccinated animals with ToxA-B2 + ToxA-GT + ToxB-B3 + ToxB-GT mutants, collected before the challenge, showed comparable neutralization titers to those measured after challenge (data not shown).

Terminal colonization analyses were also performed on vaccinated and control hamsters. Control animals were culled at day 2 (post-challenge) when endpoint of body temperature of 35°C was reached. Vaccinated animals were culled at day 15 (post challenge, at the end of experiment). Guts were removed and bacterial counts on recovered bacteria determined. To enumerate the total bacterial load (spores and vegetative cells), each section was opened longitudinally, and the contents were removed by gentle washing in two changes of 10 ml PBS. Tissues were homogenized in 5 ml of PBS for 1 min using a Stomacher, and viable counts were determined for the homogenates. Serial 10-fold dilutions were plated on CCFA blood agar plates containing 20 g/ml amphotericin B to suppress yeast growth. To estimate the numbers of spores present in the samples, the samples were heated for 10 min at 56°C, and the numbers of spores present were determined by the viable count method as described above. Organisms not intimately associated with the mucosa are described herein as "lumen associated" (LA). Organisms more intimately associated (*i.e.* not removed by simple washing) are described herein as "tissue associated" (TA).

Assessments of toxin content in the gut were also performed. Gut washes were filtered through a 0.22 μm filter to remove bacterial cells. Filtered washes were then placed on confluent Vero cells at 10-fold decreasing concentrations (5-fold for the colon) for 24 hours. After incubation, cells were washed, fixed, and then coloured with Giemsa stain. If toxin was present then cell rounding caused detachment and the absence of colour. Toxin-content data represents the dilutions at which the cells remained attached (stained).

A number of toxin domain fragments were then tested in a hamster model. Details of the tested fragments are provided in Figure 11. Details of antigen combinations and an overview of the results obtained are provided in Table 7. Results from each trial are described in more detail below.

Antigen	**Challenge Strain**	**Protection**
ToxA-P5/6	B1	0 out of 6
ToxB_B	B1	0 out of 6
ToxB_B + ToxA-P5/6	630	6 out of 6
ToxB_B + Toxoid A	5 animals with B1	3 out of 3
	5 animals with 630	3 out of 3

Chimera B4	630	3 out of 5
ToxB-GT + ToxA-P5-6	630	6 out of 6
ToxB-B + ToxA-P5-6	B1	5 out of 6
ToxB-B + ToxA-P5-6 + ToxB-GT	B1	5 out of 6
Toxoid A + Toxoid B	B1	5 out of 6
ToxB-GT-PSII + ToxA-P5-6	630	5 out of 6 and 2 out of 2
ToxA-GT + ToxB-GT + ToxB-B + ToxA-B2	B1	5 out of 6
ToxB-GT + ToxA-P5-6	B1	6 out of 6
ToxB-GT + ToxA-B2	B1	6 out of 6
ToxB-GT + ToxA-B2 (20 ugrs)	B1	3 out of 8
ToxB-GT + ToxA-P5-6 (20 ugrs)	B1	6 out of 6 and 8 out of 8
ToxA-GT + ToxB-GT + ToxB-B + ToxA-B2 (20 ugrs)	B1	6 out of 7

Table 7. Summary of hamster vaccination experiments. [1] = technical issues led to increased volume and a higher measured number of spores.

Two different *C. difficile* strains were used in these studies, namely the 630 strain (genome sequence is publicly available at NCBI), and the B1 strain. The 630 strain is known to cause prolonged infection with less severe pathology and reduced amounts of toxin *in vivo*. The B1 strain is known to cause a severe pathology in hamsters (G. Douce, personal communication), with acute infection and high level of damage.

Toxoid A + Toxoid B

A combination of full length inactivated Toxoid A and Toxoid B was used as a positive control. This combination may be considered to represent a "gold standard" against which the combinations of the invention may be compared (see references 179 and 180). Toxoids were produced using fermentation, then purified and finally inactivated.

6 animals received 5µg of each toxoid (adjuvanted with MF59). 2 received adjuvant only and two were untreated. Amount to be administered was chosen on based on the literature. The main problem with using inactivated toxoids (*e.g.* using formaldehyde) is that the inactivation could be incomplete, thus posing a potential health risk when applied to a subject,

Animals were challenged with the B1 strain. All control animals died, and one vaccinated animal (H1) died shortly after the last control animal, showing body temperature profiles similar to controls (data not shown). All other vaccinated hamsters survived until the end of the experiment (Table 8).

		Time at cull	Temp at cull
H1	Vaccine	45h 50min	34.17°C
H2	Vaccine	14 days	
H3	Vaccine	14 days	
H4	Vaccine	14 days	
H5	Vaccine	14 days	
H6	Vaccine	14 days	
H7	Vaccine	29hr 10min	31.98°C
H8	Vaccine	31hr 1min	32.58°C
H9	Vaccine	30hr 36min	34.25°C
H10	Vaccine	28hr 37min	31.3°C

Table 8. Results for full length inactivated Toxoid A + Toxoid B. Challenge with B1.

Hamsters H2, H3 and H4 showed a short episode of diarrhoea during recovery, and H5 exhibited a longer period of diarrhoea and lethargy. H5 was administered rehydration therapy (sub-cutaneous administration of saline) which led to another episode of diarrhoea, followed by recovery. Therefore, immunisation with full length toxoids was found to protect 83% of hamsters against the B1 strain.

An analysis of bacterial shedding revealed that CFU are shed from vaccinated animals for several days (5, 9 and 11) after challenge, even when symptoms (diarrhoea) disappeared. H5 was dehydrated and so it was difficult to detect any faecal pellets at day 5, so, shedding was analysed only after 9 and 11 days. H4 had no detectable *C. difficile* in faeces after 11 days (Figure 12), although this animal was still colonised in the gut at the end of the experiment. An analysis of colonisation at culling is provided in Figure 13.

Assessment of toxin B content in the gut revealed that there is less toxin B present in the gut of surviving vaccinated hamsters after 15 days, compared to controls, which died after 2 days (Table 9) This result was also confirmed in the colon (Table 10). H1 is the vaccinated hamster which died during the acute phase of infection and has a high level of toxin B present, which is equivalent to the level of toxin B present in the control animals, which died.

Hamster	Vaccinated	Final dilution lysing cells
H1	Toxoid A + Toxoid B	10^8
H2	Toxoid A + Toxoid B	10^1
H3	Toxoid A + Toxoid B	10^1
H4	Toxoid A + Toxoid B	0
H5	Toxoid A + Toxoid B	10^1
H6	Toxoid A + Toxoid B	10^1
H7	Adjuvant only	10^5
H8	Adjuvant only	10^7
H9	None	10^4
H10	None	10^6

Table 9. Toxoid A + Toxoid B – toxin content in the caecal gut. Challenge with B1. Data are represented as dilutions at which cells remain attached.

Hamster	Vaccinated	Final dilution lysing cells
H1	Toxoid A + Toxoid B	1:390625
H2	Toxoid A + Toxoid B	0
H3	Toxoid A + Toxoid B	0
H4	Toxoid A + Toxoid B	0
H5	Toxoid A + Toxoid B	1:5
H6	Toxoid A + Toxoid B	0
H7	Adjuvant only	1:125
H8	Adjuvant only	1:3125
H9	None	1:15625
H10	None	1:15625

Table 10. Toxoid A + Toxoid B – toxin content in the colon. Challenge with B1. Data are represented as dilutions at which cells remain attached.

Overall, vaccination with 5µg of full length toxoid A and full length Toxoid B resulted in protection of 5 out of 6 animals against severe disease. However, vaccination did not protect against diarrhoea which, in the case of H5, lasted for a relatively long time. At the end of the experiment, lower amounts of spores were detectable in vaccinated animals, and three animals also showed lower levels of colonisation. Also, very low amounts of toxin B were detected in vaccinated animals at the end of the experiment, even though they were still colonised. This could be explained by, for example, toxin binding by antibodies and/or a decrease in bacterial toxin expression.

Individual fragments of ToxA-P5_6 or ToxB_B

Vaccination trials using recombinant fragments were first performed using single fragments of P5_6 or ToxB_B corresponding to portions of the cell binding domain of TcdA and TcdB respectively (50

ugr of antigen adjuvanted with MF59). In both cases, no protection was observed against challenge with approximately 100 spores of B1 strain (data not shown). Sample bleeds were taken from all animals at the experiment endpoint. All animals immunised with ToxA-P5_6 have high antibody titers to the p5-6 protein and Toxin A, as determined by ELISA (data not shown), but these antibodies were not protective against infection. Toxin A neutralising capacity was not assessed. All animals immunised with ToxB_B have high antibody titers to the ToxB_B protein, as determined by ELISA (data not shown). There was insufficient purified toxin B to test for reactogenicity of these sera against whole Toxin B. These antibodies were not protective against infection, and toxin B neutralising capacity was not assessed. Thus, individual antigens do not appear protective, despite the presence of antibodies.

Mixture of fragments of P5_6 and ToxB_B

Hamsters were then immunized with a mixture of 50 µg of P5_6 and 50 µg of ToxB_B (50 ugr of each antigen adjuvanted with MF59), followed by challenge with strain 630 (results shown in Table 11).

Hamster	Immunogen	Time to endpoint	Time to endpoint
1	ToxB_B + P5_6	Survived 9 days	
2	ToxB_B + P5_6	Survived 9 days	
3	ToxB_B + P5_6	Survived 9 days	
4	ToxB_B + P5_6	Survived 9 days*	
5	ToxB_B + P5_6	Survived 9 days*	
6	ToxB_B + P5_6	Survived 9 days	
7	MF59 alone		34h 36 min
8	MF59 alone		32h 36 min
9	None		65h 44 min
10	None		33h 36 min
	Mean		***41h 40 min***

Table11. Immunisation of hamsters with P5_6 plus ToxB_B. Challenge strain 630. * = hamsters showing intermittent diarrhoea with recovery. Challenge strain was 630.

Vaccinated animals were fully protected from death, but survivors showed mild diarrhoea during recovery. Post infection analyses are shown in Table 12 and Figure 14. Table 12 shows that the amount of *C. difficile* in faecal material from vaccinated mice remains high for up to a week, indicating that the anti-toxin response does not impact on colonisation.

Animal	Treatment	*C. difficile* recovered per 100mg of faecal material

		Day 2 post infection	Day 7 post infection	Day 14 post infection
1	Vaccinated	$6.9x10^5$	$1.8x10^5$	*ND
2	Vaccinated	$4.2x10^5$	$3x10^4$	ND
3	Vaccinated	$1.3x10^6$	$1.8x10^6$	ND
4	Vaccinated	$4.9x10^5$	$3.7x10^5$	ND
5	Vaccinated	$1.8x10^5$	$4.4x10^5$	857
6	Vaccinated	$1.6x10^5$	$7x10^4$	40

Table 12. ToxB_B + P5_6 - post- infection analysis of *C. difficile* recovered in faecal material. * = ND = bacteria were not detectable.

However, bacterial counts decrease over time, and can be entirely cleared within 14 days after infection. Figure 14 shows localisation of bacteria, and reveals that, at the experiment endpoint, controls have higher levels of lumen- and tissue-associated bacteria than vaccinated hamsters. Bacteria recovered post-infection were confirmed to be *C. difficile* strain 630 by MVLA (data not shown).

The combination of P5_6 and ToxB-B thus provides strong protection against *C. difficile* challenge. Interestingly, the anti-toxin response does not appear to impact significantly on colonisation, because all animals remain heavily colonised at *e.g.* 6 days post challenge. These data also suggest that a combination of (at least fragments of) ToxA and ToxB is necessary for protection.

P5_6 and ToxB_B

In view of the successful immunisation against strain 630, the inventors tested whether immunisation with P5_6 + ToxB_B protected against the more toxigenic B1 strain. 6 animals were immunised with 50 ugrs of each antigen (adjuvanted with MF59), followed by challenge with 10^3 spores of the B1 strain. As controls, 2 animals received adjuvant alone (H7-H8) and 2 animals received no vaccination (H9-H10). Following challenge, all control animals died. One vaccinated animal (H1) died shortly after the last control animal. All other vaccinated animals (H2 – H6) survived until the end of the experiment. (Table 13) This shows that 83% of the animals vaccinated with P5/6 + ToxB_B were protected against challenge with the B1 strain (also represented by Figure 15).

Animal	**Immunogen**	**Time at cull**	**Temp at cull**
H1	Vaccine	33hr 2min	34.16°C
H2	Vaccine	15 days	
H3	Vaccine	15 days	
H4	Vaccine	15 days	
H5	Vaccine	15 days	
H6	Vaccine	15 days	
H7	Adjuvant only	32hr 5min	34.87°C
H8	Adjuvant only	29hr 50min	34.54°C
H9	Control	30hr 36min	34.49°C

H10	Control	28hr 37min	33.64°C

Table 13. P5_6 + ToxB_B results. Challenge with B1 strain.

The number of colonies per 100mg faecal material was then determined (Figure 16). This revealed that the organisms are shed at high numbers for several days after challenge, even when symptoms (diarrhoea) have abated. Interestingly, the numbers shed actually increased for around 5 days post infection, before decreasing. At day 1 post-infection, only 1 out of the 6 vaccinated animals were shedding detectable *C. difficile* in their faeces. By day 3, all surviving vaccinated animals were shedding relatively high numbers of *C. difficile*. These animals shed high levels until day 11. On day 15, only 3 out of the 5 animals were shedding detectable *C. difficile* in their faeces (detection limit approx 200 spores).

An analysis of colonization at culling was also performed (Figure 17). Hamsters 4 and 5 showed heavy levels of contaminating flora on plated which obscured any low *C. difficile* spores present (these were also the 2 animals from which *C. difficile* could not be recovered from the faeces at day 11).

Assessment of toxin B content in the gut revealed that there is little or no toxin B present in the gut of surviving vaccinated hamsters after 15 days, compared to controls, which died after 2 days (Table 14). H1 is the vaccinated hamster which died during the acute phase of infection and has a high level of toxin present, which is equivalent to the level of toxin present in the control animals, which died. There is less toxin B present in the colon than the caecum in the animals which died during the acute phase of infection.

Hamster	Vaccinated	Final dilution lysing cells (caecum)	Final dilution lysing cells (colon)
H1	P5_6+ toxB_B	10^4	1:5
H2	P5_6+ toxB_B	10^3	1:5
H3	P5_6+ toxB_B	10^3	1:5
H4	P5_6+ toxB_B	0	1:5
H5	P5_6+ toxB_B	0	0
H6	P5_6+ toxB_B	0	0
H7	Adjuvant only	10^8	1:15625
H8	Adjuvant only	10^6	1:15625
H9	None	10^7	1:15625
H10	None	10^8	1:15625

Table 14. P5_6 + ToxB_B or controls. Challenged with B1 strain. Toxin content in the gut (caecum and colon). Data are represented as dilutions at which cells remain attached.

ToxB_B + P5_6 + PSII

Animals are immunized with a mixture of ToxB_B + P5_6 + PSII-CRM, in which the polysaccharide is conjugated to the CRM carrier protein. Protection studies are performed, along with an analysis of the faeces, and an assessment of toxin content in the gut.

Toxoid A + ToxB_B

The inventors then tested whether using fragments of the TcdA binding domain affected the protection afforded by using full length Toxoid A. Immunisation with a mixture of full length (inactivated) Toxoid A and ToxB_B (5 ugr of toxoid A and 50 ugr of ToxB_B adjuvanted with MF59) was found to protect against challenge with the 630 strain and also the B1 strain. Unvaccinated animals challenged with the 630 strain had strong diarrhoea and a temperature drop, at which point they were culled (Table 15). By contrast, immunised animals survived challenge with the 630 strain and only one of the vaccinated animals displayed only minor diarrhoea. Animals showed mild diarrhoea with recovery.

Hamster	Immunogen	End of the experiment	Time to endpoint
1	Toxoid A + Toxin B_B	Survived	
2	Toxoid A + Toxin B_B	Survived*	
3	Toxoid A + Toxin B_B	Survived	
4	MF59 alone		57h 52 min
5	No treatment		47h 4 min
	Mean		*52h 28 min*

Table 15. Toxoid A + Toxin B_B results. Challenge with 630. One of the animals got very limited diarrhoea (*). Unvaccinated animals had strong diarrhoea and temperature drop.

Unvaccinated animals challenged with the B1 strain also had strong diarrhoea and a temperature drop, at which point they were also culled (Table 16). Immunised animals survived the challenge, with two suffering mild diarrhoea during recovery. Therefore, immunisation with a mixture of Toxoid A and Toxoid B_B protected from death following challenge with the B1 strain, but did not protect against diarrhoea (Table 16).

Hamster	Immunogen	End of the experiment	Time to endpoint
1	Toxoid A + Toxin B_B	Survived*	
2	Toxoid A + Toxin B_B	Survived*	
3	Toxoid A + Toxin B_B	Survived	
4	MF59 alone		29h 47 min**
5	No treatment		32h 24 min**
	Mean		*31h 15 min*

Table 16. Toxoid A + Toxin B_B results. Challenge with B1. Mild recovery with diarrhoea (*); strong diarrhoea and temperature drop (**). Vaccinated animals are protected from death, but not temperature drop.

Further combinations

As discussed above, the inventors determined that fragments comprising the GT domain were immunogenic and capable of inducing neutralisation titers against their respective toxin. To test whether fragments comprising the GT domain are able to confer protection against CDAD, the

inventors tested a number of additional combinations using the 630 and B1challenge strains (summarised in Table 7).

ToxB_GT + P5_6 (630)

First, the inventors tested whether immunisation with a mixture of ToxB_GT and ToxA-P5_6 (adjuvanted with MF59) was found to protect against challenge with the 630 strain. ToxA-P5_6 in combination with the ToxB-B fragment was found to confer 100% protection against challenge with the 630 strain. In this experiment, none of the vaccinated animals showed diarrhoea when challenged with 630, and no clinical symptoms were observed (Table 17). Unvaccinated animals (hamster #9) challenged with the 630 strain had strong diarrhoea and a temperature drop, at which point they were culled.

Animal	Immunogen	Time to <35°C	Time at cull	Temp at cull
H1	ToxB_GT + P5_6		15 days	36.4°C
H2	ToxB_GT + P5_6		15 days	36.7°C
H3	ToxB_GT + P5_6		15 days	37.2°C
H4	ToxB_GT + P5_6		15 days	36.9°C
H5	ToxB_GT + P5_6		15 days	36.2°C
H6	ToxB_GT + P5_6		15 days	36.2°C
H7	Adjuvant only	35 hr 28min	41hr 34min	26.2°C
H8	Adjuvant only	37hr 37min	41hr 53min	25.9°C
H9	No treatment	38hr 15min	42hr	33.8°C

Table 17. ToxB_GT + P5+6 results. Challenge with 630. None of the vaccinated animals suffered from diarrhoea.

The number of colonies per 100mg faecal material was then determined (Figure 18), demonstrating that that the organisms are shed at high numbers for several days after challenge, even when symptoms (diarrhoea) have abated.

Assessment of terminal colonisation also revealed that all of the vaccinated animals showed a lower number of CFU and lower proportion of spores compared to controls. There were no detectable *C. difficile* spores in hamsters #4 and #5 (data not shown), and hamster #2 showed a ten-fold reduction in terminal colonisation compared to other vaccinated hamsters (Figure 19).

Assessment of toxin B content in the gut revealed that there is less toxin B present in the vaccinated hamsters after 15 days than in the controls, which died after 2 days (Table 18). This result was also confirmed in the colon (Table 19).

Hamster	Vaccinated	Final dilution lysing cells
H1	P5_6+ toxB_GT	10^2
H2	P5_6+ toxB_GT	10^1
H3	P5_6+ toxB_GT	10^1
H4	P5_6+ toxB_GT	10^1
H5	P5_6+ toxB_GT	0
H6	P5_6+ toxB_GT	10^1
H7	Adjuvant only	10^5
H8	Adjuvant only	10^4
H9	None	10^4

Table 18. ToxB_GT + P5_6 – toxin content in the caecal gut. Data are represented as dilutions at which cells remain attached. Challenge with 630 strain.

Hamster	Vaccinated	Final dilution lysing cells
H1	P5_6+ toxB_GT	1:5
H2	P5_6+ toxB_GT	0
H3	P5_6+ toxB_GT	0
H4	P5_6+ toxB_GT	0
H5	P5_6+ toxB_GT	0
H6	P5_6+ toxB_GT	0
H7	Adjuvant only	1:3125
H8	Adjuvant only	1:25
H9	None	1:625

Table 19. ToxB_GT + P5_6 – toxin content in the colon. Data are represented as dilutions at which cells remain attached. Challenge with 630 strain.

Therefore, immunisation with a mixture of ToxB_GT and ToxA-P5_6 provides strong protection against challenge with the 630 strain, which is as good as using the ToxB-B fragment in combination with ToxA-P5_6.

ToxB_GT + P5_6 (B1)

In view of the successful immunisation against strain 630, the inventors tested whether immunisation with P5_6 + ToxB_GT protected against the B1 strain. Animals (H1-H6) were immunized with a mixture of ToxB_GT + P5_6 (50 ugrs of each antigen, adjuvanted with MF59). The controls (adjuvant only) had strong diarrhoea and a temperature drop, at which point they were culled. All immunized animals survived against challenge with the B1 strain (6/6) (Table 20), exhibiting a single episode of diarrhoea. This is the first time that this has happened with any combination of recombinant antigens.

	Antigens	Time to	Time at cull	Temp at
H1	ToxB_GT + P5/6		14 days	
H2	ToxB_GT + P5/6		14 days	
H3	ToxB_GT + P5/6		14 days	
H4	ToxB_GT + P5/6		14 days	
H5	ToxB_GT + P5/6		14 days	
H6	ToxB_GT + P5/6		14 days	
H7	Adjuvant only	37h 21m	37h 36m	34.47 ºC
H8	Control	30h 1m	30h 56m	30.55 ºC

Table 20. ToxB_GT + P5_6 results. Challenge with B1 strain.

The number of colonies per 100mg faecal material was then determined (Figure 20), demonstrating that that the organisms are shed at high numbers for several days after challenge, even when symptoms (diarrhoea) have abated. All surviving animals shed high levels of *C. difficile* in their faeces. Only one animal (H4) had no detectable spores at day 11.

Hamster	Vaccinated	Final dilution lysing cells
H1	P5/6 + toxB_GT	10^4
H2	P5/6 + toxB_GT	10^3
H3	P5/6 + toxB_GT	10^3
H4	P5/6 + toxB_GT	0
H5	P5/6 + toxB_GT	10^2
H6	P5/6 + toxB_GT	10^2
H7	Adjuvant only	10^5
H8	Adjuvant only	10^6

Table 21. ToxB_GT + P5_6 – toxin content in the caecal gut. Data are represented as dilutions at which cells remain attached. Challenge with B1.

An analysis of colonization at culling was also performed (Figure 21). Results showed that all surviving hamsters, except H4, were colonized with *C. difficile* in the caecum and colon at the point of culling. All colonized surviving hamsters appear to have a higher vegetative cell: spore ratio than the animals which died in the acute stage of infection.

Hamster	Vaccinated	Final dilution lysing cells
H1	P5/6 + toxB_GT	1:25
H2	P5/6 + toxB_GT	1:5
H3	P5/6 + toxB_GT	0
H4	P5/6 + toxB_GT	0
H5	P5/6 + toxB_GT	1:5
H6	P5/6 + toxB_GT	0
H7	Adjuvant only	1:625
H8	Non-vaccinated control	1:625

Table 22. ToxB_GT + P5_6 – toxin content in the colon. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Assessment of toxin B content in the gut revealed that there is less toxin B present in the caecum of the vaccinated animals (H1-H6) than animals which died in the acute phase of infection (H7 and H8) (Table 21). H3 and H6 from the vaccinated group had higher levels of toxin B present than the other vaccinated animals. H4 had no toxin B present, which was expected because there were no detectable *C. difficile* in the gut at the point of culling. This result was also confirmed in the colon (Table 22). As seen in the gut washes from the caecum, there is little or no active toxin B present in the surviving hamsters after 14 days, as compared to the high levels in the control animals, which died. Also, there is apparently less toxin B in the colon than in the caecum, and the only animals showing a significant amount of toxin B in the colon were also the animals which died of acute disease. Again, this could be explained by, for example, toxin binding by antibodies and/or a decrease in bacterial toxin expression.

Overall, vaccination with ToxA-P5-6 + ToxB_GT provided 100% survival following challenge with the B1 strain. This combination did not protect the animals from diarrhoea following challenge with the B1 strain, although symptoms were relatively limited. By contrast, ToxA-P5_6 in combination with the ToxB-B fragment was found to confer only 83.3% protection against challenge with the B1 strain, and so using the ToxB-GT fragment in combination with a fragment of TcdA represents an improvement over using the ToxB-B fragment (see also Figure 40).

ToxB_GT + P5_6 (lower doses)

It was now tested whether a lower dose (20 ugrs per antigen) also conferred protection against the B1 strain. All vaccinated animals (H1-H8) survived challenge with the B1 strain, and the control animals (H7-H8) died (Table 23).

	Antigens	Time to <35°C	Time at cull	Temp at cull
H1	P5/6 + toxB_GT (20µg dose)		48h 28m	37.53 °C
H2	P5/6 + toxB_GT (20µg dose)		14 days	
H3	P5/6 + toxB_GT (20µg dose)		14 days	
H4	P5/6 + toxB_GT (20µg dose)		14 days	
H5	P5/6 + toxB_GT (20µg dose)		48h 44m	37.13 °C
H6	P5/6 + toxB_GT (20µg dose)		14 days	
H7	P5/6 + toxB_GT (20µg dose)		14 days	
H8	P5/6 + toxB_GT (20µg dose)		14 days	
H9	Control	29h 57m	30h 25m	34.15 °C
H10	Control	27h 46m	28h 15m	34.48 °C

Table 23. ToxA-P5-6 + ToxB_GT results (reduced dose). Challenge with B1 strain.

All vaccinated animals exhibited a single episode of diarrhoea, except H2 which showed no clinical symptoms. The non-vaccinated controls died shortly after the onset of diarrhoea and were culled when their body temperature dropped below 35°C. H1 and H5 were culled at 48 post-challenge, despite the fact that they had recovered from the diarrhoeal stage of infection (and based on experience would have survived). H1 and H5 were culled at this stage to provide some information

on the toxin B present and the damage to the gut, at this stage of infection. The number of colonies per 100mg faecal material was then determined (Figure 22), demonstrating that that the organisms are shed at high numbers for several days after challenge, even when symptoms (diarrhoea) have abated. The average shedding of C. difficile per vaccination group was calculated, and it appears that the shedding within these animals is reduced compared to any of the aforementioned experiments in which B1 was the challenge strain.

An analysis of colonization at culling was also performed (Figure 23). Results showed that all surviving hamsters were colonized with *C. difficile* in the caecum and colon at the point of culling. H7 and H8 had no detectable spores present in either the caecum or colon and had lower numbers of vegetative bacteria than the other vaccinated animals at cull, 14 days after challenge. H2, H3, H4 and H6 had a higher ratio of vegetative cells to spores at the time of cull. H6 was colonised to a higher extent than H2, H3 and H4. H1 and H5 were vaccinated animals which had recovered from the challenge and culled 48hr post challenge. H1 had a longer more severe episode of diarrhoea compared with H5 which only suffered a short, mild episode. Both the animals had recovered from diarrhoea and their tails were dry at the time of cull 48hr after challenge. H1 and H5 had a comparable numbers of vegetative bacteria and spores to the control animals which died in the acute phase of the infection.

Assessment of toxin B content in the gut revealed that there is little or no active toxin B in the caecum of vaccinated animals culled 14 days after challenge (Table 24).

Hamster	Vaccinated	Final dilution lysing cells
H1	P5/6 + toxB_GT	10^7
H2	P5/6 + toxB_GT	10^3
H3	P5/6 + toxB_GT	0
H4	P5/6 + toxB_GT	10^3
H5	P5/6 + toxB_GT	10^5
H6	P5/6 + toxB_GT	10^3
H7	P5/6 + toxB_GT	10^3
H8	P5/6 + toxB_GT	0
H9	Control	10^7
H10	Control	10^7

Table 24. ToxA-P5-6 + ToxB_GT (reduced dose) – toxin B content in the caecum. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Vaccinated animals which were killed 48h after challenge had high levels of toxin B present in the caecum and the amount was comparable to animals which died in the acute phase of the infection at roughly 29h after challenge. These observations were also confirmed in the colon (Table 25).

Hamster	Vaccinated	Final dilution lysing cells
H1	P5/6 + toxB_GT	1:3125
H2	P5/6 + toxB_GT	0
H3	P5/6 + toxB_GT	0
H4	P5/6 + toxB_GT	0
H5	P5/6 + toxB_GT	1:3125
H6	P5/6 + toxB_GT	1:5
H7	P5/6 + toxB_GT	0
H8	P5/6 + toxB_GT	0
H9	Non-vaccinated control	1:15625
H10	Non-vaccinated control	1:15625

Table 25. ToxA-P5-6 + ToxB_GT (reduced dose)- toxin B content in the colon. Data are represented as dilutions at which cells remain attached. Challenge with B1.

As seen in the gut washes from the caecum, there is little or no active toxin B present in the surviving hamsters after 14 days, as compared to the high levels in the control animals which died, or the two vaccinated animals culled at 48h after challenge. Also, there is less toxin B in the colon than in the caecum, and the only animals showing a significant amount of toxin B in the gut were the control animals, which died of acute disease. Those animals culled at 48h showed reduced levels whilst those animals culled at 14 days post challenge showed minimal or undetectable toxin B levels.

Levels of toxin A content in the gut were also assessed. Gut washes were filtered through a 0.22 μm filter to remove bacterial cells. Filtered washes were then placed on confluent HT29 cells at decreasing concentrations for 24 hours. After incubation, cells were washed, fixed, and then coloured with Giemsa stain. If toxin was present then cell rounding caused detachment and the absence of colour. Toxin-content data represents the dilutions at which the cells remained attached (stained). Assessment of toxin A content in the gut revealed that vaccinated animals culled 14 days after challenge had little or no toxin A present. Vaccinated animals H1 and H5 which were culled at 48 hr after challenge had a comparable amount of toxin A in the gut as the control animals (H9 and H10) which died in the acute phase of the infection (Table 26).

Hamster	Vaccinated	Final dilution lysing cells
H1	P5/6 + toxB_GT	10^4
H2	P5/6 + toxB_GT	10^3
H3	P5/6 + toxB_GT	0
H4	P5/6 + toxB_GT	10^3
H5	P5/6 + toxB_GT	10^3
H6	P5/6 + toxB_GT	10^1
H7	P5/6 + toxB_GT	10^1
H8	P5/6 + toxB_GT	0
H9	Non-vaccinated control	10^3
H10	Non-vaccinated control	10^3

Table 26. ToxA-P5-6 + ToxB_GT (reduced dose) – toxin A content in the caecum. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Overall, vaccination with p5/6 + toxB-GT at 20μg per antigen per dose protected the animals from death but did not prevent diarrhoea when challenged with *C.difficile* strain B1. All surviving animals were colonised throughout the experiment and shed *C. difficile* spores in their faeces. At the time of culling, all animals were still colonised with *C. difficile* with some only showing low levels of vegetative cells and others showing higher levels of spores and vegetative cells. Animals surviving to the end of the experiment, showed low levels of toxin (either A or B) in the gut lumen.

In contrast, the control animals succumbed to infection approximately 29h post infection. These animals showed high counts of both vegetative and spores in excised gut tissue and high level of toxin in filtered extracts. Interestingly, the 2 vaccinated animals that had recovered from the diarrhoeal phase of the disease, but were culled at 48h appeared to show counts and toxin levels that more closely mirrored that of the control animals that the vaccinated ones, with relatively high amounts of toxin present in the lumen. However, the fact that these animals were no longer displaying diarrhoea would suggest that antibodies produced and released from the circulation in response to damage protected the animals from the more fatal consequences of the disease.

Therefore, immunisation using a combination of ToxA-P5-6 + ToxB_GT provided 100% survival following challenge with the B1 strain, even when using a lower amount of antigen. Even when using 20 ugrs of each antigen, this combination out-performed ToxA-P5_6 in combination with the ToxB-B fragment, using 50ugrs of each antigen.

ToxA-P5-6 + ToxB-GT challenged with R20291 (SM)

In view of the high level of protection against the 630 and B1 strains by immunisation with a combination of ToxA-P5-6 + ToxB-GT, the inventors tested whether this combination is also protective against challenge with the R20291(SM) strain. Animals were therefore immunized with a mixture of ToxA-5-6 + ToxB-B, adjuvanted with MF59. Protection studies were performed, along with an analysis of the faeces, and an assessment of toxin content in the gut.

ToxB_GT-PSII + P5_6

The inventors then tested whether inclusion of PSII could induce an immune response able to reduce colonization. ToxB_GT was chemically conjugated to PSII, and this conjugate was able to induce PSII-specific antibodies (confirmed by ELISA, data not shown). Also, chemical conjugation to PSII was not found to impair neutralization activity.

The experiment consisted of three groups, which were challenged with strain 630: vaccination with ToxB_GT(PSII) + P5_6 (H1-H6), vaccination with ToxB_GT + P5_6 (H7-8) and treatment with adjuvant alone (H9-H10). Results are shown in Table 27. H1-H3 displayed no episodes of diarrhoea, but H4 had two episodes and was culled after the second episode. Of hamsters H1-H6, 5/6 survived. Of hamsters H7 and H8, 2/2 survived.

		Time to <35°C	Time at cull	Temp at cull
H1	P5/6 + toxB_GT-PSII		14 days	
H2	P5/6 + toxB_GT-PSII		14 days	
H3	P5/6 + toxB_GT-PSII		14 days	
H4	P5/6 + toxB_GT-PSII	89h 40m	89h 50m	34.55 °C
H5	P5/6 + toxB_GT-PSII		14 days	
H6	P5/6 + toxB_GT-PSII		14 days	
H7	P5/6 + toxB_GT		14 days	
H8	P5/6 + toxB_GT		14 days	
H9	Control	33h 37m	37h 48m	20.0°C
H10	Control	54h 43m	64h 0m	29.88°C

Table 27. ToxB_GT(PSII) + P5_6 results. Challenge strain 630.

The number of colonies per 100mg faecal material was then determined (Figure 24), demonstrating that that the organisms are shed at high numbers for several days after challenge, even when symptoms (diarrhoea) have abated. All surviving animals shed high levels of *C. difficile* in their faeces.

The average number of *C. difficile* being shed in faeces from surviving vaccinated animals (ToxB_GT(PSII) + P5_6 (H1-H6), or ToxB_GT + P5_6 (H7-8)) is shown in (Figure 25). This shows that there may be a slight advantage in including PS-II on colonization.

An analysis of colonization at culling was also performed (Figure 26(a and b)). Results showed low or no colonization of tissue-associated *C. difficile* in surviving (vaccinated) animals. Results obtained for H4 were comparable to negative controls.

Therefore, high colony counts were observed only in animals that did not survive.

Chimera B4

The inventors then tested the protectivity obtained using a hybrid protein comprising ToxB-GT + ToxA-P5-6 (the "B4" chimera). 6 animals (H1-6) were immunized with 50 ugr of the B4 chimera (adjuvanted with MF59). As controls, 2 animals received adjuvant alone (H7-H8) and 2 animals received no vaccination (H9-H10). Antigen was administered intraperitoneally. Animals were challenged with the 630 strain (H1 and H8 were culled prior to challenge). All control animals died, but 3/5 of the vaccinated animals survived until the end of the experiment (Table 28). Therefore, expressing ToxB-GT and ToxA-P5-6 as a chimera appears to reduce the effectiveness of this combination of antigens, compared to using a mixture of single antigens.

Hamster	**Immunogen**	**Time to endpoint**	**Time to**
1	**Culled prior to challenge***		
2	**B4 chimera**	**Survived until expt end**	
3	**B4 chimera**	**Survived**	
4	**B4 chimera**	**Survived**	

5	**B4 chimera**	**165h**	
6	**B4 chimera**	**155h**	
7	**MF59 alone**		**Approx 35h****
8	**Culled prior to challenge***		
9	**No treatment**		**62h 35 min**
10	**No treatment**		**37h 21 min**
	Mean		***44h 58min***

Table 28. Chimera B4 results. Challenge with 630 strain. Vaccinated animals were fully protected against death but not diarrhoea. * Animals were culled as a result of abscesses associated with chip insertion.

Assessment of colonisation of animals was determined by removal of faecal samples from cages at intervals after challenge. Faeces were weighed, re-suspended in sterile PBS and then plated on selective media. The number of colonies per 100mg faecal material was then determined (Table 29) demonstrating that that the organisms are shed at high numbers for several days after challenge (bacteria were not detectable in faeces of H6).

Animal	**Treatment**	***C. difficile* recovered per 100mg of faecal material**			
		Day 3 post infection	**Day 5 post infection**	**Day 11 post infection**	**Day 15 post infection**
2	**Vaccinated B4**	**$1.4x10^4$**	**$3.58x10^6$**	**$3.12x10^4$**	***ND**
3	**Vaccinated B4**	**83**	**$8.82x10^5$**	**$3.12x10^4$**	**ND**
4	**Vaccinated B4**	**122**	**$4.67x10^6$**	**$3.12x10^4$**	**ND**
5	**Vaccinated B4**	**333**	**No faeces**	**Dead**	
6	**Vaccinated B4**	**0**	**$2.76x10^6$**	**Dead**	

Table 29. Bacterial shedding of *C. Difficile* spores in 100mg faeces from hamsters immunised with Chimera B4 or controls. Challenged with 630 strain. *ND = Bacteria were not detectable.

ToxB_GT + ToxA_B2

The inventors then tested whether immunisation with ToxB_GT in combination with a different fragment of TcdA was capable of conferring the same high level of protection as for ToxB-GT + ToxA-P5/6. Animals were therefore immunised with a mixture of ToxB-GT + ToxA_B2, and challenged with the B1 strain. Animals (H1-H6) were immunized with a mixture of ToxB_GT + ToxA_B2 (adjuvanted with MF59). The controls (adjuvant only (H7 and H8) and no adjuvant (H9 and H10)) had strong diarrhoea and a temperature drop, at which point they were culled. All immunized animals survived against challenge with the B1 strain (6/6) (Table 30).

	Antigens	**Time to <35°C**	**Time at cull**	**Temp at cull**
H1	toxA_B2 + toxB_GT		14 days	
H2	toxA_B2 + toxB_GT		14 days	
H3	toxA_B2 + toxB_GT		14 days	

H4	toxA_B2 + toxB_GT		14 days	
H5	toxA_B2 + toxB_GT		14 days	
H6	toxA_B2 + toxB_GT		14 days	
H7	Adjuvant only	31h 17m	31h 40m	33.94ºC
H8	Adjuvant only	33h 8m	33h 20m	34.59 ºC
H9	Control	30h 2m	30h 45m	32.31 ºC
H10	Control	32h 7m	32h 35m	34.23 ºC

Table 30. ToxB_GT + ToxA_B2 results. Challenge with B1 strain.

All immunised animals exhibited a single episode of diarrhoea, apart from H3, which exhibited no diarrhoea (H1 exhibited the most severe diarrhoea of this batch and was monitored closely). All animals exhibiting clinical symptoms had shorter bouts of diarrhoea than observed in any of the aforementioned experiments, when challenged with the B1 strain.

The number of colonies per 100mg faecal material was then determined (Figure 27), demonstrating that that the organisms are shed at high numbers for several days after challenge, even when symptoms (diarrhoea) have abated. All surviving animals shed high levels of *C. difficile* in their faeces. By day 14, four immunised hamsters (H2, H3, H4, and H6) had no detectable *C. difficile* spores in their faeces.

An analysis of colonization at culling was also performed (Figure 28(a-b)). Results showed that all surviving hamsters were colonized with *C. difficile* in the caecum and colon at the point of culling, however the bacterial counts were very low and were approaching the lower end of the detection limit. H2 and H6 appeared to have no detectable bacteria associated with the tissue. All colonized surviving hamsters appear to have a higher vegetative cell: spore ratio than the animals which died in the acute stage of infection.

Assessment of toxin B content in the gut revealed that there is little (H5) or no active toxin B in the caecum of vaccinated animals (H5 had the highest number of bacteria in the gut at the point of culling of all vaccinated animal) (Table 31). This result was also confirmed in the colon (Table 32).

Hamster	**Vaccinated**	**Final dilution**
H1	toxA_B2 + toxB_GT	0
H2	toxA_B2 + toxB_GT	0
H3	toxA_B2 + toxB_GT	0
H4	toxA_B2 + toxB_GT	0
H5	toxA_B2 + toxB_GT	10^1
H6	toxA_B2 + toxB_GT	0
H7	Adjuvant only	10^4
H8	Adjuvant only	10^4
H9	Control	10^4
H10	Control	10^4

Table 31. B_GT + ToxA_B2 – toxin content in the caecal gut. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Hamster	Vaccinated	Final dilution lysing cells
H1	toxA_B2 + toxB_GT	0
H2	toxA_B2 + toxB_GT	1:5
H3	toxA_B2 + toxB_GT	0
H4	toxA_B2 + toxB_GT	0
H5	toxA_B2 + toxB_GT	1:5
H6	toxA_B2 + toxB_GT	0
H7	Adjuvant only	1:625
H8	Adjuvant only	1:25
H9	Non-vaccinated control	1:625
H10	Non-vaccinated control	1:25

Table 32. B_GT + ToxA_B2 - toxin content in the colon. Data are represented as dilutions at which cells remain attached. Challenge with B1.

As seen in the gut washes from the caecum, there is little or no active toxin B present in the surviving hamsters after 14 days, as compared to the high levels in the control animals, which died. Also, there is apparently less toxin B in the colon than in the caecum, and the only animals showing a significant amount of toxin B in the colon were also the animals which died of acute disease.

Overall, vaccination with ToxA-B2 + ToxB_GT provided 100% survival following challenge with the B1 strain, thereby out-performing the level of protection achieved using full length inactivated toxoids, and matching the high level of protection achieved using ToxB-GT + ToxA-P5-6. This combination did not protect the animals from diarrhoea following challenge with the B1 strain. Nevertheless, symptoms were relatively limited and were even less severe than with any of the aforementioned combinations tested, following challenge with the B1 strain. All surviving animals were colonised throughout the experiment, although 3/6 of the vaccinated animals showed no detectable *C. difficile* spores in the faeces at 14 days post infection (although detectable numbers of *C. difficile* could be cultured directly from the gut at that time). Also, vaccinated animals showed relatively low levels of toxin B in the gut at the end of the experiment. Again, this could be explained by, for example, toxin binding by antibodies and/or a decrease in bacterial toxin expression.

ToxA-B2 + ToxB_B + ToxB_GT

The inventors then tested whether vaccination with ToxB-GT + ToxB-B + ToxA-B2, has any effect on protection against CDAD.Animals were immunized with a mixture of ToxA-B2 + ToxB_B + ToxB-GT. Protection studies were performed, along with an analysis of the faeces, and an assessment of toxin content in the gut.

ToxB_B + ToxA-P5_6 + ToxB_GT

The inventors then tested whether vaccination with ToxB-GT + ToxA-P5-6 in combination with the ToxB-B fragment, has any further effect on protection against CDAD. Animals were therefore

immunized with a mixture of ToxB_B + P5_6 + ToxB_GT (adjuvanted with MF59). Unvaccinated controls had strong diarrhoea and a temperature drop, at which point they were culled. 5 of the 6 vaccinated hamsters (83%) survived challenge with the B1 strain (Table 33).

		Time to <35°C	Time at cull	Temp at cull
H1	Vaccine		15 days	
H2	Vaccine	50h 50m	50h 54 m	34.69°C
H3	Vaccine		15 days	
H4	Vaccine		15 days	
H5	Vaccine		15 days	
H6	Vaccine		15 days	
H7	Adjuvant only	28h 47m	28hr 47m	34.12°C
H8	Adjuvant only	28h 50m	28h 50m	33.46°C
H9	Control	30h 48m	30h 48m	34.9°C
H10	Control	29h 32m	29h 32m	32.2°C

Table 33. ToxB_GT + ToxB_B + P5_6 in MF59 adjuvant. Challenge with B1. 5 out of 6 vaccinated animals survived. H1 and H4 showed a single episode of diarrhoea lasting roughly one hour.

Hamsters H1 and H4 showed only a single episode of diarrhoea lasting roughly 20 hours, and hamsters H3, H5 and H6 had no episodes of diarrhea. An analysis of faeces (Figure 29) shows that *C. difficile* organisms are shed at very high numbers for several days following challenge, even when symptoms (diarrhea) have abated. An overview of terminal colonization is provided in Figure 30.

Assessment of toxin B content in the gut revealed that there is less toxin B present in 5 out of the 6 vaccinated hamsters after 15 days than in the controls, which died after 2 days (Table 34).

Hamster	Vaccinated	Final dilution lysing cells
H1	P5_6+ toxB_B + toxB-GT	10^1
H2	P5_6+ toxB_B + toxB-GT	10^6
H3	P5_6+ toxB_B + toxB-GT	0
H4	P5_6+ toxB_B + toxB-GT	0
H5	P5_6+ toxB_B + toxB-GT	0
H6	P5_6+ toxB_B + toxB-GT	10^1
H7	Adjuvant only	10^6
H8	Adjuvant only	10^5
H9	None	10^5
H10	None	10^5

Table 34. ToxB_B + P5_6 + ToxB_GT. Toxin content in the caecal gut. Data are represented as dilutions at which cells remain attached. Challenge with B1 strain.

This result was also confirmed in the colon (Table 35). H2 died at 50h 54 mins and had a similar amount of toxin B in the gut as the control animals, which died.

Hamster	Vaccinated	Final dilution lysing cells
H1	P5_6+ toxB_B + toxB-GT	0
H2	P5_6+ toxB_B + toxB-GT	1:25
H3	P5_6+ toxB_B + toxB-GT	0
H4	P5_6+ toxB_B + toxB-GT	0
H5	P5_6+ toxB_B + toxB-GT	0
H6	P5_6+ toxB_B + toxB-GT	0
H7	Adjuvant only	1:15625
H8	Adjuvant only	1:78125
H9	None	1:78125
H10	None	1:78125

Table 35. ToxB_B + P5_6 + ToxB_GT. Toxin content in the colon. Data are represented as dilutions at which cells remain attached. Challenge with B1 strain.

Therefore, immunisation with a mixture of ToxB_B + P5_6 + ToxB_GT provides strong protection against challenge with the 630 strain, but does not appear to confer any advantage over ToxB-GT + ToxA-P5-6.

ToxA_GT + ToxB_GT + ToxB_B + ToxA_B2

The inventors then tested the level of protectivity conferred by immunisation with a combination of antigens comprising ToxA-GT. Animals were therefore immunized with a mixture of ToxA-GT + ToxB-GT + ToxB-B + ToxA-B2. 6 animals received 50 µg of each antigen (adjuvanted with MF59), (H1-H6). 1 animal received adjuvant only (H7), 2 were untreated (H8-H9) and one was unchallenged (H10). The challenge strain used in this experiment was the B1 strain. All of the control animals died after challenge, exhibiting diarrhoea shortly before a drop in body temperature to the clinical endpoint. One vaccinated animal (H1) exhibited signs of sickness and dehydration, and died shortly after the last control animal, showing a similar body temperature profile to controls (data not shown). All other vaccinated animals survived until the end of the experiment (Table 36). H2-H6 showed a single short episode of diarrhoea during recovery. Therefore 83% of vaccinated subjects were protected from death when challenged with the B1 strain.

	Antigens	Time to <35°C	Time at cull	Temp at cull
H1	toxA_B2 + toxA_GT + toxB_B + toxB_GT	33h 59m	34h 50m	34.42 °C
H2	toxA_B2 + toxA_GT + toxB_B + toxB_GT		14 days	
H3	toxA_B2 + toxA_GT + toxB_B + toxB_GT		14 days	
H4	toxA_B2 + toxA_GT + toxB_B + toxB_GT		14 days	
H5	toxA_B2 + toxA_GT + toxB_B + toxB_GT		14 days	
H6	toxA_B2 + toxA_GT + toxB_B + toxB_GT		14 days	
H7	Adjuvant only	27h 30m	28h 3m	33.07 °C
H8	Control	26h 32m	26h 56m	34.08 °C
H9	Control		27h 20m	
H10	toxA_B2 + toxA_GT + toxB_B + toxB_GT	Not challenged – sera collected		

Table 36. ToxA_GT + ToxB_GT + ToxB_B + ToxA_B2 results. Challenge strain B1.

An analysis of bacterial shedding (Figure 31) revealed a decrease in *C. difficile* spores in faeces over time (decreasing considerably after day 7), and this trend is comparable to data obtained using full length toxoids. Data are unavailable for H4 on day 3, and H3 and H5 had no detectable spores on day 14. All surviving hamsters had a higher vegetative: spore ratio than the animals which died in the acute phase of infection. An analysis of colonization at culling is provided in Figure 32.

For hamsters that survived challenge with the B1 strain, toxin B content was analysed at day 14 (Table 37).

Hamster	Vaccinated	Final dilution
H1	toxA_B2 + toxA_GT + toxB_B + toxB_GT	10^4
H2	toxA_B2 + toxA_GT + toxB_B + toxB_GT	10^3
H3	toxA_B2 + toxA_GT + toxB_B + toxB_GT	0
H4	toxA_B2 + toxA_GT + toxB_B + toxB_GT	10^1
H5	toxA_B2 + toxA_GT + toxB_B + toxB_GT	0
H6	toxA_B2 + toxA_GT + toxB_B + toxB_GT	0
H7	Adjuvant only	10^4
H8	Non-vaccinated control	10^4
H9	Non-vaccinated control	10^4

Table 37. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 – toxin content in the caecal gut. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Vaccinated animals showed low toxin B levels in the gut despite being colonized by the bacteria (apart from H2, which had more detectable toxin B and which was more heavily colonized at the point of culling). The vaccinated hamster, H1, which died in the acute phase of infection had an equivalent amount of toxin B in the gut to the non-vaccinated and adjuvant-only controls, which dies 28h after infection. These observations were confirmed in the colon (Table 38). There is apparently less toxin B in the colon than in the caecum, and the only animals showing a significant amount of toxin B in the colon were also the animals which died of acute disease.

Hamster	Vaccinated	Final dilution lysing cells
H1	toxA_B2 + toxA_GT + toxB_B + toxB_GT	1:3125
H2	toxA_B2 + toxA_GT + toxB_B + toxB_GT	1:25
H3	toxA_B2 + toxA_GT + toxB_B + toxB_GT	0
H4	toxA_B2 + toxA_GT + toxB_B + toxB_GT	0
H5	toxA_B2 + toxA_GT + toxB_B + toxB_GT	0
H6	toxA_B2 + toxA_GT + toxB_B + toxB_GT	1:5
H7	Adjuvant only	1:625
H8	Non-vaccinated control	1:3125
H9	Non-vaccinated control	1:15625

Table 38. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 – toxin content in the colon. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Overall, vaccination with ToxA_GT + ToxB_GT + ToxB_B + ToxA_B2 protected 5 out of 6 animals from death when challenged with the B1 strain, but did not protect against diarrhoea. The level of protection achieved by including ToxA-GT in the combination was comparable to the level of protection achieved when immunising with full length inactivated toxoids. All surviving animals were colonised throughout the experiment and shed equivalent levels of *C. difficile* spores in the faeces. At the time of cull, all animals were still colonised with *C. difficile*. Surviving vaccinated animals showed a higher ratio of vegetative cells: spores in the gut, compared to controls. With one exception (H2), surviving animals showed low levels of toxin B activity in the guts. Again, this could be explained by, for example, toxin binding by antibodies and/or a decrease in bacterial toxin expression. Overall, this combination showed an efficacy comparable to that obtained using the gold standard immunisation with toxoids.

ToxA_B2 + ToxB_GT + ToxB_GT + ToxB_B + ToxA_GT lower doses

The inventors then tested whether using a lower antigen dose of ToxA_GT + ToxB_GT + ToxB_B + ToxA_B2 (20ugrs of each antigen) also conferred high level protection against the B1 strain.

All vaccinated animals (H1-H8) survived challenge with the B1 strain, and the control animals (H7-H8) were culled when their body temperature dropped below 35°C (Table 39). Note that one vaccinated animal (H2) was culled at 9 days after challenge due to loss of body condition, and not a drop in body temperature (the animal did not gain weight, was dehydrated, and normal gut function had not returned, as evidenced by the absence of formed faecal material).

	Antigens	Time to < 35°C	Time at cull	Temp at cull
H1	toxA_B2 + toxA_GT + toxB_GT + toxB_B (20ug)		14 days	
H2	toxA_B2 + toxA_GT + toxB_GT + toxB_B (20ug)		9 days	36.89 °C
H3	toxA_B2 + toxA_GT + toxB_GT + toxB_B (20ug)		14 days	
H4	toxA_B2 + toxA_GT + toxB_GT + toxB_B (20ug)		14 days	
H5	toxA_B2 + toxA_GT + toxB_GT + toxB_B (20ug)		14 days	
H6	toxA_B2 + toxA_GT + toxB_GT + toxB_B (20ug)		14 days	
H7	toxA_B2 + toxA_GT + toxB_GT + toxB_B (20ug)		14 days	
H8	Adjuvant only	28h 43m	28h 45m	34.85 °C
H9	Control	28h 37m	28h 26m	34.82 °C
H10	Control	26h 47m	26h 50m	34.82 °C

Table 39. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 (lower dose) results. Challenge with B1.

The number of colonies per 100mg faecal material was then determined (Figure 33), demonstrating that that the organisms are shed at high numbers for several days after challenge, even when symptoms (diarrhoea) have abated. The level of spores in faeces was found to drop considerably after day 8.

An analysis of colonization at culling was also performed (Figure 34). Results showed that all surviving hamsters were colonized with *C. difficile* in the gut at the point of culling. H2, which was culled at 9 days after challenge, had comparable amounts of vegetative cells and spores to control animals (H8, H9 and H10) which died in the acute phase of infection. H1, H3 and H4 had high levels of *C. difficile* but there were lower levels of spores present than in animals which died in the acute phase of infection. H5, H6 and H7 had lower numbers of *C. difficile* and lower levels of spores. H6 had no detectable spores associated with the tissue in the caecum or the colon.

Assessment of toxin B content in the caecum revealed that there is little or no active toxin in the caecum of vaccinated animals culled 14 days after challenge (Table 40). Interestingly, the control animal, H8, had little or no active toxin present, which is unexpected because this animal died during the acute phase of infection. H2, which was culled at 9 days after challenge, had a comparable amount of toxin present in the gut as the control animals (H9 and H10), which died in the acute phase of infection. H1 and H3 had active toxin present, whereas H4 and H6 had less toxin present. H5 and H7 had no active toxin present, which correlated with the low number of bacteria present.

Hamster	Antigen	Final dilution lysing cells
H1	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	10^4
H2	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	10^8
H3	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	10^4
H4	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	10^2
H5	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	0
H6	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	10^2
H7	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	0
H8	Control	10^1
H9	Control	10^8
H10	Control	10^8

Table 40. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 (lower dose). Toxin B content in the caecum. Data are represented as dilutions at which cells remain attached. Challenge with B1. Challenge with B1.

Similar toxin results were observed in the colon (Table 41), where H8 appeared to have no active toxin present in the gut. H9 and H10, which died during the acute phase of infection, had high levels of toxin present in the colon. H2, which was culled at 9 days after challenge, had active toxin present in the colon, whereas animals which were culled at 14 days after challenge had little or no active toxin present.

Hamster	Antigen	Final dilution lysing cells
H1	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	0
H2	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:125
H3	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:25
H4	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	0
H5	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	0
H6	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	0
H7	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	0
H8	Control	0
H9	Control	1:78125
H10	control	1:15625

Table 41. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 (lower dose). Toxin B content in the colon. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Levels of toxin A content in the caecum were also assessed, using the methodology outlined above. Assessment of toxin A content in the gut revealed that H9 and H10 had high levels of toxin A present. H8, which also died in the acute phase of infection, had little active toxin present, although this result is in agreement with the previous measurement of toxin B. H2, which was culled 9 days

after challenge, had a higher level of toxin A present in the caecum compared to animals which were culled 14 days after challenge, which had little or no active toxin present (Table 42).

Hamster	Antigen	Final dilution lysing cells
H1	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:25
H2	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:125
H3	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:25
H4	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:25
H5	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:5
H6	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:5
H7	toxA_B2+toxA_GT+toxB_GT+toxB_B (low dose)	1:5
H8	Control	1:5
H9	Control	1: 15625
H10	control	1:625

Table 42. ToxB_GT + ToxA_GT + ToxB_B + ToxA_B2 (lower dose). Toxin A content in the caecum. Data are represented as dilutions at which cells remain attached. Challenge with B1.

Overall, immunisation with a lower dose of ToxA-B2 + ToxA_GT + ToxB_GT + ToxB_B (20µg per antigen per dose) also protected the animals from death, but not diarrhoea. All animals which survived initial challenge recovered normal gut function except one (H2). Therefore, immunisation with a lower dose of ToxA-B2 + ToxA_GT + ToxB_GT + ToxB_B appears to provide a similar or better level of protection compared to the gold standard, using toxoids.

Neutralisation titres from vaccinated hamsters

Sera from vaccinated hamsters were analyzed by *in vitro* neutralization assay. Results are shown in Table 43.

Antigen	Challenge strain	Neutralisation titres against ToxA	Neutralisation titres against ToxB
ToxA-P5/6	B1	ND	ND
ToxB-B	B1	0	512
ToxA-P5-6 + ToxB-B	630	4000	512
ToxoidA + ToxB-B	B1 630	32000 32000	512 512
Chimera B4	630	2000	512
ToxA-P5-6 + ToxB-GT	630	8000	512
ToxA-P5-6 + ToxB-GT	B1	16000	256
ToxA-P5-6 + ToxB-GT (reduced antigen dose)	B1	8000	128
ToxA-B2 + ToxB-GT	B1	8000	256

ToxA-B2 + ToxB-GT (reduced antigen dose)	B1	16000 (8000)	128 (0)
ToxA-P5-6 + ToxB-B	B1	2000 32	256 / 512 32 16
ToxA-P5-6 + ToxB-B + ToxB-GT	B1	1000 64	2000 64
ToxoidA + Toxoid B	B1	32000 16000	512 512
ToxA-P5-6 + ToxB-GT-PSII ToxA-P5-6 + ToxB-GT	630	4000 256 2000	2000 512 2000
ToxA-B2+ ToxA-GT + ToxB-B + ToxB-GT	B1	4000 ND/512	512 ND 0
ToxA-B2+ ToxA-GT + ToxB-B + ToxB-GT (reduced antigen dose)	B1	8000 (512)	256 (0)

Table 43. *In vitro* neutralization titers from vaccinated hamsters. (differences in experimental repeats are denoted by "/"). Selected neutralisation titers shown for comparison. Reduced antigen dose is 20μg per antigen.

Animals immunised with a mixture of fragments comprising at least one fragment from ToxA and at least one fragment from ToxB, as well as full length Toxin A and Toxin B, generated neutralisation titers against both toxins. Of the tested combinations, only ToxA-B2 + ToxB-B + ToxB_GT did not generate neutralisation titers against both toxins, and was not found to be protective against the 630 strain. Also, animals immunised with single fragment generated neutralisation titres against only their respective toxin, and were not observed to be protective. These data suggest that protection against *C. difficile* requires the production of neutralisation titers against both toxins.

Analysis of microbiota

Vaccinated animals challenged with *C. difficile* which recover from a single episode of diarrhoea, continue to shed the organism in the faeces for at least 3 weeks. To analyse the impact of *C. difficile* infection on the microbiome, changes were monitored through 16S amplification of faecal material, pre- and post- infection.

First, the inventors assessed microflora changes after clindamycin treatment (Figure 35). An average of 3000 sequences returned from 454 sequencing per sample and phylum were assigned. In untreated normal hamsters, Bacteroidetes are the most abundant phyla (59%). Clindamycin treatment results in a dramatic contraction of Bacteroidetes, sequential expansion of Proteobacteria (84%) and loss of overall microbial diversity. Increased recovery of Fusobacteria was observed from day 2. A recovery

of diversity was observed by day 5, although by day 15 phylum microbial richness had still not completely returned.

The inventors then tested microflora changes in vaccinated animals. Vaccination protects hamsters from lethal challenge with toxinogenic *C. difficile* 630, despite bacterial growth and toxin production. As shown in Figure 36, surviving animals show microbiota changes that are consistent with those observed in clindamycin treated animals. At day 14 these phyla decreased but stayed higher than the other infection regimes. Microbial diversity declined to SDI 1.3 at day 4 but then increased similar to pre-clindamycin levels (SDI 1.7).

Overall, the inventors found that vaccination with toxin fragments that include the enzymatic domain of toxin B provide the highest level of protection against *C. difficile* infection. Administration of the broad-spectrum antibiotic clindamycin resulted in decreased microbial complexity. Whilst the microbiota diversity increased over time it never returned to pre-clindamycin levels. These data, together with clinical data, suggest that *C. difficile* toxin associated damage could enhance microbiota dysbiosis caused by antibiotics, and this may reveal why patients remain susceptible to relapse.

Investigaiton of toxin-specific IgG in the intestinal lumen

The presence of toxin-specific IgG in the intestinal lumen of animals vaccinated with ToxA-P5-6+ToxB-GT was investigated. Although response to ToxA was higher in the acute phase of infection, raising amounts of anti-ToxB antibodies were detectable at the endpoint (Figure 39).

To further evaluate the effects of vaccination with ToxA-P5-6+ToxB-GT, toxins levels produced *in vivo* were monitored and gut histology was performed.

High toxin levels were detected 48 hours post infection both in control and vaccinated hamsters (Figure 40), whilst severe gut inflammation accompanied by epithelial necrosis and polymorphonulcear (PMN) influx was only observed in control animals. Tissue from vaccinated animals showed less epithelial damage and limited PMN infiltrate. Hyperplasia associated with appearance of mucin-producing cells and crypt to tip length increase was observed, particularly in the lower colon.

Protected animals showed lower levels of toxin within the intestinal lumen 14 days after infection despite the presence of high numbers of C. diffcile colonies associated to the intestinal tissue. The gut epithelia appeared to revert to normality with absence of polymorph influx. Interestingly, whilst no alteration of caecum was evident, some hyperplasia persisted in the terminal colon of these animals.

Conclusion

The inventors have found that administration of combinations of *Clostridium difficile* antigens comprising ToxB-GT and TcdA fragments are able to provide high levels of protection against CDAD, comparable to, or better than using binding domain–based fragments for immunisation.

Hamster vaccination experiments led to the identification of combinations of fragments which were able to protect animals from the fatal outcome typically observed in absence of vaccination, even following challenge with the B1 strain.

Surprisingly, the inventors also found that immunisation with combinations of the invention as individual separate polypeptides (*i.e.* mixed together), confers much stronger protection against CDAD, than using hybrid polypeptides. This is exemplified by the "B4 chimera", which showed only a moderate level of protection against the milder 630 strain.

Combinations of the invention strongly reduced the clinical symptoms of CDAD, such as dehydration and diarrhoea. Moreover, the level of protection afforded by the combinations of the invention matched or surpassed the protection provided by using inactivated toxoids. By using recombinant polypeptides, the inventors were also able to overcome the plethora problems associated with vaccination using inactivated toxoids.

The inventors have thus provided multi-strain vaccine candidates against CDAD, which are safer and more easily produced than using inactivated toxoids, and which offer an alternative to binding domain-based immunisation against *C. difficile*.

Description of sequence	**SEQ ID NO:**
Peptides	
Full length TcdA	1
Full length TcdB	2
ToxA-ED	3
ToxA-GT	4
ToxA-CP	5
ToxA-T	6
ToxA-T4	7
ToxA-B	8
ToxA-PTA2	9
ToxA-P5-7	10
ToxA-P5-6	11
ToxA-P9-10	12
ToxA-B2	13
ToxA-B3	14
ToxA-B5	15
ToxA-B6	16
ToxB-ED	17

ToxB-GT	18
ToxB-CP	19
ToxB-T	20
ToxB-B	21
ToxB-B2	22
ToxB-B7	23
B4 hybrid	24
Linker	25
Linker	26
Linker	27
IC-31	28
Polycationic polymer	29
Nucleic acids	
Full length TcdA	30
Full length TcdB	31
ToxA-ED	32
ToxA-GT	33
ToxA-CP	34
ToxA-T	35
ToxA-T4	36
ToxA-B	37
ToxA-PTA2	38
ToxA-P5-7	39
ToxA-P5-6	40
ToxA-P9-10	41
ToxA-B2	42
ToxA-B3	43
ToxA-B5	44
ToxA-B6	45
ToxB-ED	46
ToxB-GT	47
ToxB-CP	48
ToxB-T	49
ToxB-B	50
ToxB-B2	51
ToxB-B7	52
B4 hybrid	53
Mutated sequences	
ToxA-ED (peptide)	54
ToxA-ED (encoding nucleic acid)	55
ToxA-GT (peptide)	56
ToxA-GT (encoding nucleic acid)	57

ToxB-ED (peptide)	58
ToxB-ED (encoding nucleic acid)	59
ToxB-GT (peptide)	60
ToxB-GT (encoding nucleic acid)	61
ToxA-CP (peptide)	62
ToxA-CP (encoding nucleic acid)	63
ToxB-CP (peptide)	64
ToxB-CP (encoding nucleic acid)	65
ToxA-PTA2 (encoding nucleic acid)	66
ToxA-P9-10 (encoding nucleic acid)	67
ToxB-B (encoding nucleic acid)	68
ToxB-B2 (encoding nucleic acid)	69
Additional useful sequences	
ToxA-PTA2 (nucleic acid)	70
ToxA-PTA2 (peptide)	71
ToxA-P9-10 (nucleic acid)	72
ToxA-P9-10 (peptide)	73
ToxA-P5-7 (nucleic acid)	74
ToxA-P5-7 (peptide)	75
ToxA-B3 (peptide)	76
ToxA-B3 (nucleic acid)	77
ToxA-B6 (peptide)	78
ToxA-B6 (nucleic acid)	79
ToxA-B5 (peptide)	80
ToxA-B5 (nucleic acid)	81
ToxA-B2 (nucleic acid)	82
ToxA-B2 (peptide)	83
ToxA-P5-6 (peptide)	84
ToxA-CP (nucleic acid)	85
ToxA-CP (peptide)	86
ToxA-T4 (nucleic acid)	87
ToxA-T4 (peptide)	88
ToxB-CP (nucleic acid)	89
ToxB-CP (peptide)	90
ToxB-ED (nucleic acid)	91
ToxB-ED (peptide)	92
ToxB-GT (nucleic acid)	93
ToxB-GT (peptide)	94
ToxB-B (nucleic acid)	95
ToxB-B (peptide)	96
ToxB-B2 (nucleic acid)	97
ToxB-B2 (peptide)	98

ToxB-B7 (nucleic acid)	99
ToxB-B7 (peptide)	100
ToxA-p5-6 H41D (peptide)	101
ToxA-P5-6 N42A (peptide)	102
ToxA-P5-6 H41D, N42A (peptide)	103
Optional N-terminal amino acid sequence	104
Optional C-terminal amino sequence	105
Hybrid polypeptide A-ToxA-P5-6wt	106
Hybrid polypeptide ToxA-P5-6wt-C	107
Hybrid polypeptide A-ToxA-P5-6wt-C	108
Hybrid polypeptide A-ToxA-P5-6 H41D, N42A	109
Hybrid polypeptide ToxA-P5-6 H41D, N42A -C	110
Hybrid polypeptide A-ToxA-P5-6 H41D, N42A -C)	111
Nucleic acid sequence encoding the hybrid polypeptide A-ToxA-P5-6 H41D, N42A-C	112

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CLAIMS

1. An immunogenic composition comprising a combination of *Clostridium difficile* antigens, said combination comprising

   a) a ToxB-GT antigen and a TcdA antigen; or

   b) a ToxA-GT antigen and a TcdB antigen.

2. The immunogenic composition of claim 1, wherein the ToxB-GT antigen and/or the ToxA-GT antigen are detoxified.

3. The immunogenic composition of claim 1 or claim 2 wherein,

   the ToxB-GT antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:18 or SEQ ID NO: 60; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:18 or SEQ ID NO: 60, or of a polypeptide having 80% or more identity to SEQ ID NO:18 or SEQ ID NO: 60 and that comprises an epitope of SEQ ID NO:18 or SEQ ID NO: 60;

   the ToxA-GT antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:4 or SEQ ID NO: 56; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:4 or SEQ ID NO: 56, or of a polypeptide having 80% or more identity to SEQ ID NO:18 or SEQ ID NO:56 and that comprises an epitope of SEQ ID NO:4 or SEQ ID NO:56;

   the TcdA antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:1; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:1 , or of a polypeptide having 80% or more identity to SEQ ID NO:1 and that comprises an epitope of SEQ ID NO:1; and

   the TcdB antigen is a polypeptide that comprises or consists of an amino acid sequence: (a) having 80% or more identity to SEQ ID NO:2; and/or b) that is a fragment of at least 7 consecutive amino acids of SEQ ID NO:2, or of a polypeptide having 80% or more identity to SEQ ID NO:2 and that comprises an epitope of SEQ ID NO:2.

4. The immunogenic composition of any one of claims 1 to 3 comprising a ToxB-GT antigen and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more TcdA antigens, optionally selected from (1) a ToxA-ED antigen (SEQ ID NO: 3), (2) a ToxA-GT antigen (SEQ ID NO: 4), (3) a ToxA-CP antigen (SEQ ID NO:5), (4) a ToxA-T antigen (SEQ ID NO: 6), (5) a ToxA-T4 antigen (SEQ ID NO: 7), (6) a ToxA-B antigen (SEQ ID NO: 8), (7) a ToxA-PTA2 antigen (SEQ ID NO: 9), (8) a

ToxA-P5-7 antigen (SEQ ID NO: 10), (9) a ToxA-P5-6 antigen (SEQ ID NO: 11), (10) a ToxA-P9-10 antigen (SEQ ID NO: 12), (11) a ToxA-B2 antigen (SEQ ID NO: 13), (12) a ToxA-B3 antigen (SEQ ID NO: 14), (13) a ToxA-B5 antigen (SEQ ID NO: 15), (14) a ToxA-B6 antigen (SEQ ID NO: 16) or a full-length TcdA antigen (SEQ ID NO:1).

5. The immunogenic composition of claim 4 further comprising 1, 2, 3, 4, 5, 6, 7, 8 or more additional TcdB antigens, optionally selected from (1) a ToxB-ED antigen (SEQ ID NO: 17), (2) a ToxB-GT antigen (SEQ ID NO: 18), (3) a ToxB-CP antigen (SEQ ID NO:19) (4) a ToxB-T antigen (SEQ ID NO: 20), (5) a ToxB-B antigen (SEQ ID NO: 21), (6) a ToxB-B2 antigen (SEQ ID NO: 22) (7) ToxB-B7 (SEQ ID NO: 23) or (8) a full-length TcdB antigen (SEQ ID NO:2).

6. The immunogenic composition of any one of claims 1 to 3 comprising a ToxA-GT antigen and 1, 2, 3, 4, 5, 6, 7, 8, 9 or more TcdB antigens, optionally selected from (1) a ToxB-ED antigen (SEQ ID NO: 17), (2) a ToxB-GT antigen (SEQ ID NO: 18), (3) a ToxB-CP antigen (SEQ ID NO:19) (4) a ToxB-T antigen (SEQ ID NO: 20), (5) a ToxB-B antigen (SEQ ID NO: 21), (6) a ToxB-B2 antigen (SEQ ID NO: 22) (7) ToxB-B7 (SEQ ID NO: 23) or (8) a full-length TcdB antigen (SEQ ID NO:2).

7. The immunogenic composition of claim 6 further comprising 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more additional TcdA antigens, optionally selected from (1) a ToxA-ED antigen (SEQ ID NO: 3), (2) a ToxA-GT antigen (SEQ ID NO: 4), (3) a ToxA-CP antigen (SEQ ID NO:5), (4) a ToxA-T antigen (SEQ ID NO: 6), (5) a ToxA-T4 antigen (SEQ ID NO: 7), (6) a ToxA-B antigen (SEQ ID NO: 8), (7) a ToxA-PTA2 antigen (SEQ ID NO: 9), (8) a ToxA-P5-7 antigen (SEQ ID NO: 10), (9) a ToxA-P5-6 antigen (SEQ ID NO: 11), (10) a ToxA-P9-10 antigen (SEQ ID NO: 12), (11) a ToxA-B2 antigen (SEQ ID NO: 13), (12) a ToxA-B3 antigen (SEQ ID NO: 14), (13) a ToxA-B5 antigen (SEQ ID NO: 15), (14) a ToxA-B6 antigen (SEQ ID NO: 16) or a full-length TcdA antigen (SEQ ID NO:1).

8. An immunogenic composition according to any preceding claim comprising a ToxB-GT antigen and a TcdA antigen, wherein the TcdA antigen is selected from the group consisting of ToxA-P5-6 and ToxA-B2.

9. An immunogenic composition comprising i) a ToxA-GT antigen or a ToxB-GT antigen; and ii) at least one TcdA antigen selected from ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6; and at least one TcdB antigen selected from ToxB-B, ToxB-B2 antigen, and/or ToxA-B7.

10. An immunogenic composition comprising i) a ToxA-GT antigen and a ToxB-GT antigen; and ii) at least one TcdA antigen selected from ToxA-B, ToxA-PTA2, ToxA-P5-7, ToxA-P5-6, ToxA-

P9-10, ToxA-B2, ToxA-B3, ToxA-B5 and/or ToxA-B6; and at least one TcdB antigen selected from ToxB-B, ToxB-B2 antigen, and/or ToxA-B7.

11. An immunogenic composition according to any preceding claim comprising a ToxB-GT antigen, a TcdA antigen and a further TcdB antigen, optionally wherein said composition comprises (a) ToxB-GT + ToxA-B2 + ToxB-B, or (b) ToxB-GT + ToxB-B + ToxA-P5-6.

12. An immunogenic composition according to any preceding claim comprising a ToxB-GT antigen, a ToxA-GT antigen, a further TcdA antigen and a further TcdB antigen, optionally wherein said combination comprises ToxB-GT + ToxA-GT + ToxA-B2 + ToxB-B.

13. The immunogenic composition according to any one of the preceding claims, wherein at least two of the antigens in the composition are in the form of a hybrid polypeptide.

14. The immunogenic composition according to any one of the claims 1-12, wherein none of the antigens are in the form of a hybrid polypeptide.

15. The immunogenic composition of any one of the preceding claims, wherein said composition induces neutralisation titers against *C. difficile* toxin A and toxin B.

16. The immunogenic composition of any one of the preceding claims, comprising at least one further *C. difficile* antigen, optionally wherein said further *C. difficile* antigen is a saccharide antigen.

17. The immunogenic composition of any one of the preceding claims being a vaccine composition.

18. The vaccine composition of claim 17 further comprising an adjuvant.

19. A vaccine composition according to claim 17 or claim 18 for use as a pharmaceutical.

20. The vaccine composition of any one of claims 17 to 19 for use in raising an immune response in a mammal.

21. The vaccine composition of claim 20, wherein said mammal is a human.

22. A vaccine composition according to any one of claims 18 to 21 for use in treating or preventing *C. difficile* associated disease.

23. A method for raising an immune response in a mammal comprising the step of administering to the mammal an effective amount of the immunogenic composition or vaccine of any one of the preceding claims.