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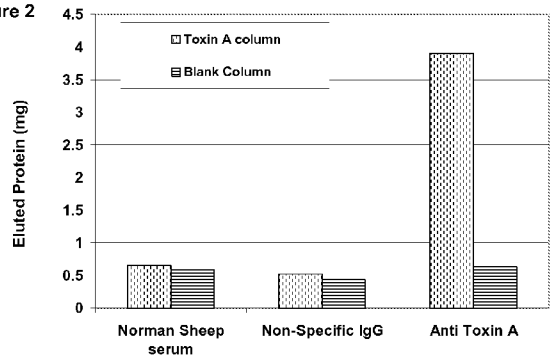
(54) Title: THERAPIES FOR PREVENTING OR SUPPRESSING CLOSTRIDIUM DIFFICILE INFECTION

Figure 2



(57) Abstract: The present invention provides an antibody composition comprising ovine antibodies, for use in the prevention or treatment of *C. difficile* infection wherein the antibodies bind to a *C. difficile* toxin, and wherein said prevention or treatment is by oral delivery of the antibody composition. Also provided is a pharmaceutical composition of ovine antibodies for oral delivery, which further comprises one or more means for protecting the antibodies from trypsin and/ or chymotrypsin and/ or stomach acid.

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Therapies for preventing or suppressing *Clostridium difficile* infection

The present invention relates to therapeutics and corresponding therapies for the treatment or suppression of *Clostridium difficile* infection (CDI).

Clostridium difficile infection (CDI) is now a major problem in hospitals worldwide. The bacterium causes nosocomial, antibiotic-associated disease which manifests itself in several forms ranging from mild self-limiting diarrhoea to potentially life-threatening, severe colitis. Elderly patients are most at risk from these potentially life-threatening diseases and incidents of CDI have increased dramatically over the last 10 years. In 2007 in the UK there were over 50,000 cases of CDI with over 8,000 associated deaths. CDI costs the NHS >£500M per annum.

The various strains of *C. difficile* may be classified by a number of methods. One of the most commonly used is polymerase chain reaction (PCR) ribotyping in which PCR is used to amplify the 16S-23S rRNA gene intergenic spacer region of *C. difficile*. Reaction products from this provide characteristic band patterns identifying the bacterial ribotype of isolates. Toxinotyping is another typing method in which the restriction patterns derived from DNA coding for the *C. difficile* toxins are used to identify strain toxinotype. The differences in restriction patterns observed between toxin genes of different strains are also indicative of sequence variation within the *C. difficile* toxin family. Toxin B shows sequence variation in some regions. For example, there's an approximate 13% sequence difference with the C-terminal 60kDa region of toxinotype 0 Toxin B compared to the same region in toxinotype III.

Strains of *C. difficile* produce a variety of virulence factors, notable among which are several protein toxins: Toxin A, Toxin B and, in some strains, a binary toxin which is similar to *Clostridium perfringens* iota toxin. Toxin A is a large protein cytotoxin/enterotoxin which plays a role in the pathology of infection and may influence the gut colonisation process. Outbreaks of CDI have been reported with Toxin A-negative/Toxin B-positive strains, which indicates that Toxin B is also capable of playing a key role in the disease pathology. Both Toxins A and B exert their mechanisms of action via multi-step mechanisms, which include binding to receptors on the cell surface, internalisation followed by translocation and release of the effector domain into the cell cytosol and finally intracellular action. For both Toxins A and B this involves the inactivation of small GTPases of the Rho family. For this inactivation, each toxin catalyses the transfer of a glucose moiety (from UDP-glucose) on to an amino residue of the Rho protein. Both Toxins A and B also contain

a second enzyme activity in the form of a cysteine protease which appears to play a role in the release of the effector domain into the cytosol after translocation. The *C. difficile* binary toxin modifies cell actin by a mechanism which involves the transfer of an ADP-ribose moiety from NAD onto its target protein.

Treatment of *C. difficile* infection currently relies on antibiotics of which metronidazole and vancomycin constitute those of choice. However, these antibiotics are not effective in all cases and 20-30% of patients suffer relapse of the disease. Of major concern is the appearance in the UK of more virulent strains which were first identified in Canada in 2002. These strains, which include those belonging to PCR ribotype 027, toxinotype III, cause CDI with a directly attributable mortality more than 3-fold that observed previously.

New therapeutics are therefore required especially urgently since the efficacy of current antibiotics appears to be decreasing.

Accordingly, there is a need in the art for new therapies/therapeutics capable of specifically addressing *C. difficile* infection (CDI). This need is addressed by the present invention, which solves one or more of the above-mentioned problems.

In more detail, a first aspect of the present invention provides ovine antibodies, for oral use in the prevention or treatment of CDI. Said oral therapy provides a simple treatment/ prevention/ suppression of CDI with unexpected efficacy and/ or with reduced side-effects. In another aspect, the invention provides an antibody composition comprising the ovine antibodies, in a form suitable for oral use in the prevention or treatment of CDI. In one embodiment, the ovine antibodies are polyclonal antibodies.

In use, the antibodies of the invention bind to a *C. difficile* toxin or a fragment thereof, preferably neutralising the biological activity of the toxin or fragment thereof. Accordingly, the antibodies of the present invention are capable of preventing or treating CDI, and/ or preferably also preventing a relapse in a patient.

The antibody therapy of the present invention provides a distinct advantage over other therapies in that it is able to inhibit the biological action of one or more of the toxins of *C. difficile*, whilst having a minimal or low immunogenic effect on a patient. Moreover, the antibodies of the present invention can be produced with very high toxin-neutralising titres. Thus, the ovine antibodies can be readily obtained and can protect

the patient against the pathological effects produced by *C. difficile* with minimal or no side-effects. The antibodies of the present invention may also be used prophylactically to prevent the onset of CDI.

The principal targets of the present invention are *C. difficile* toxins or fragments thereof. Suitable *C. difficile* toxins, to which the antibodies of the invention may bind to and/or neutralise, include any *C. difficile* toxins that cause or are associated with CDI or a symptom thereof. In a further embodiment, the antibodies of the invention bind to and/or neutralise one or more type of *C. difficile* toxin selected from the following: *C. difficile* Toxin A or a fragment thereof, *C. difficile* Toxin B or a fragment thereof, and *C. difficile* Binary Toxin or a fragment thereof.

Thus, in one embodiment, the antibody composition of the present invention comprises ovine antibodies that bind to and/or neutralise *C. difficile* Toxin A (or a fragment thereof). In another embodiment, the antibody composition of the present invention comprises ovine antibodies that bind to and/or neutralise *C. difficile* Toxin B (or a fragment thereof). In yet another embodiment, the antibody composition of the present invention comprises ovine antibodies that bind to and/or neutralise *C. difficile* Binary Toxin (or a fragment thereof).

In another embodiment, the antibody composition of the present invention comprises ovine antibodies that bind to and/or neutralise *C. difficile* Toxin A (or a fragment thereof) and to *C. difficile* Toxin B (or a fragment thereof). In another embodiment, the antibody composition of the present invention comprises ovine antibodies that bind to and/or neutralise *C. difficile* Toxin A (or a fragment thereof) and to *C. difficile* Binary Toxin (or a fragment thereof). In yet another embodiment, the antibody composition of the present invention comprises ovine antibodies that bind to and/or neutralise *C. difficile* Toxin B (or a fragment thereof) and to *C. difficile* Binary Toxin (or a fragment thereof).

The antibody composition of the present invention may also comprise ovine antibodies that bind to and/or neutralise *C. difficile* Toxin A (or a fragment thereof), to *C. difficile* Toxin B (or a fragment thereof) and to *C. difficile* Binary Toxin (or a fragment thereof).

The antibodies of the present invention interact with specific epitopes of the toxin. For example, an antibody can bind an epitope in the N-terminal domain (e.g. between amino acids 1-957) or the mid-region domains (e.g. between amino acids

958-1831) or the C-terminal repeat domains (e.g. between amino acids 1832-2710) of *C. difficile* Toxin A. For example, the antibody may bind to an epitope within amino acids 1832-2710 of *C. difficile* Toxin A. Similarly an antibody can bind an epitope in the N-terminal domain (e.g. between amino acids 1-955) or the mid-region domains (e.g. between amino acids 956-1831) or the C-terminal repeat domains (e.g. between amino acids 1832-2366) of Toxin B. For example, an antibody may bind to an epitope within amino acids 1832-2366 of Toxin B. In the case of the binary toxin antibodies may bind to the catalytic domain (Fragment A) or the receptor binding domain, which resides in the C-terminal portion of Fragment B (approx residues 400-870); and/ or to the N-terminal half of Fragment B (approx residues 1-400), which is involved in the binding and translocation of Fragment A into the cell.

In one embodiment, the *C. difficile* toxin is selected from one of toxinotypes 0 to XV. Preferred toxinotypes (plus example Ribotypes and Strains) are listed in the Table immediately below. The listed toxinotypes are purely illustrative and are not intended to be limiting to the present invention.

Toxinotype	Example Ribotypes	Example Strains	Reference
0	001, 106	VPI10463	Rupnik et al. (1998) J. Clinical Microbiol. 36: 2240-2247
I	003, 012, 102	EX623	
II	103	AC008	
III	027, 034, 075, 080	R20291, QCD-32g58	
IV	023, 034, 075, 080	55767	
V	066, 078	SE881	
VI	045, 063, 066	51377	
VII	063	57267	
VIII	017, 047	1470	
IX	019	51680	
X	036	8864	
XI	033	IS58, R11402	Rupnik et al. (2001) Microbiology 147: 439-447
XII	056	IS25	
XIII	070	R9367	
XIV	111	R10870	
XV	122	R9385	

Different antibodies of the present invention may bind to and/or neutralise a *C. difficile* toxin from the same or from different strains of *C. difficile*. For example, the antibodies may bind to and/or neutralise one or more of the following: *C. difficile*

Toxin A - Toxinotype 0; *C. difficile* Toxin B - Toxinotype 0; *C. difficile* Toxin A - Toxinotype III; *C. difficile* Toxin B - Toxinotype III; *C. difficile* Toxin A - Toxinotype V; and/or *C. difficile* Toxin B - Toxinotype V. Preferably, a mixture of antibodies is employed, which bind to and/ or neutralise Toxins A and B from all or most of these Toxinotypes. An antibody of the present invention may bind to an epitope in the N-terminal domain, the mid-region domain, and/or the C-terminal repeat domain of said strains of *C. difficile* Toxin A and/ or *C. difficile* Toxin B and/ or *C. difficile* Binary Toxin.

In certain embodiments, the antibodies of the present invention may bind to and/or neutralise at least one *C. difficile* toxin comprising an amino acid sequence at least 80%, 85%, 90%, 95%, 98%, 99%, or more identical to SEQ ID NOs: 1-6, or a fragment thereof.

The invention also embraces a corresponding method for prevention or treatment of CDI, said method comprising oral administration of the antibody composition of the present invention to a patient. The patient can be infected with *C. difficile*, or have a symptom of *C. difficile* (e.g. mild self-limiting diarrhoea, abdomenal pain, fever and loss of appetite to life-threatening conditions such as pseudomembranous colitis and cytotoxic megacolon) or have a predisposition towards *C. difficile* infection (e.g. undergoing treatment with antibiotics, having experienced *C. difficile* and at risk of relapse, or exposed to a second individual who has shown the clinical symptoms associated with *C. difficile* infection). The present invention thereby provides an effective means for preventing, suppressing or treating CDI (or a symptom thereof).

In one embodiment, said method of treating CDI comprises oral administration of the antibody composition of the present invention to a patient infected with *C. difficile*, or suffering from the symptoms of CDI. This can be accomplished using a therapeutically effective amount of the antibodies. Such administration may be effected by repeated administrations of antibody compositions of the present invention, for a prolonged period of time. The antibody components of said compositions may be the same or different (in terms of their toxinotype specificity and/ or targeted binding region or epitope on a *C. difficile* Toxin), and administration can be concurrent or sequential, and can be effected in any order.

In another embodiment, said method of preventing CDI comprises oral administration of the antibody composition of the present invention to a patient to provide passive immunity against CDI. This can be accomplished using a prophylactically effective

amount of the antibodies prior to the onset or in the very early stages of CDI. Such administration may be effected by repeated administrations of antibody compositions of the present invention, for a prolonged period of time. The antibody components of said compositions may be the same or different (in terms of their toxinotype specificity and/ or targeted binding region or epitope on a *C. difficile* Toxin), and administration can be concurrent or sequential, and can be effected in any order.

In another embodiment, said method of treating CDI comprises administering antibody systemically (eg. once or twice per day, or once or twice or 3- or 4-times per every 3-4 days; for a short period of typically 1-2 weeks) followed by a more prolonged period of oral administration (eg. once or twice or 3- or 4- or 5- or 6-times per day, or once or twice or 3- or 4- or 5- or 6-times per every 3-4 days, or once or twice or 3- or 4- or 5- or 6-times per week) of antibody. In this embodiment, the systemically administered antibody is provided as a formulation suitable for that route and the orally administered antibody is provided in the form of a composition of the present invention. Such administration may be effected by giving one or more administrations of antibody via the systemic route followed by repeated oral administrations of antibody compositions of the present invention, for a more prolonged period of time. The antibody components of said compositions may be the same or different (in terms of their toxinotype specificity and/ or targeted binding region or epitope on a *C. difficile* Toxin).

In another embodiment, the above oral administration may be performed prior to or simultaneously with a corresponding systemic administration of said antibodies. Naturally, when administered systemically, the antibodies are formulated accordingly (eg. such formulations are typically provided as isotoxic aqueous formulations and do not require means for protection against stomach acid or stomach enzymes such as trypsin and/ or chymotrypsin).

In one embodiment, the subject to be treated or protected is a subject in one or more or the following category: hospitalised; over 65 or 70 years' old; receiving broad-range spectrum antibiotics; having previous CDI history/ infection; having close proximity to symptomatic CDI patients; having mild-to-moderate disease severity; presenting as asymptomatic but considered at high risk of relapse (eg. because of one or more relapse episodes); having close proximity to CDI outbreak areas or patients.

Antibody preparation

The ovine antibodies are antibodies which have been raised in a sheep. Thus, the present invention includes a method of producing ovine antibodies for use in the antibody composition of the invention, said method generally involving (i) administering an immunogen comprising a *C. difficile* toxin or a fragment thereof to a sheep, (ii) allowing sufficient time for the generation of antibodies in the sheep, and (iii) obtaining the antibodies from the sheep. As used herein, sheep comprise any species that fall within the *Ovis* genus (e.g. *Ovis ammon, Ovis orientalis aries, Ovis orientalis orientalis, Ovis orientalis vignei, Ovis Canadensis, Ovis dalli, Ovis nivicola*).

The present invention also includes a method of producing ovine antibodies for use in the oral antibody composition of the invention, wherein the ovine antibodies are elicited by a sheep in response to an immunogen comprising a *C. difficile* toxin or a fragment thereof (preferably a fragment that possesses antigenic cross-reactivity with the full-length natural Toxin and/ or retains the toxin or toxin-like activity of the full-length natural Toxin).

The antibody may be obtained from the sheep serum. Thus, the procedures generate sheep antisera containing antibodies capable of binding and neutralising *C. difficile* toxins. In a further embodiment, the antibodies are isolated and/or purified. Thus, another aspect of the present invention involves purifying the antibodies from sheep antiserum.

In one embodiment, the immunogen used to generate the antibodies of the present invention is a *C. difficile* toxin or a fragment thereof, which has optionally been purified. Suitable *C. difficile* toxins include any *C. difficile* toxins that cause or are associated with CDI or a symptom thereof. In a further embodiment, the toxin is selected from at least one of the following toxins: *C. difficile* Toxin A or a fragment thereof, *C. difficile* Toxin B or a fragment thereof and *C. difficile* Binary Toxin or a fragment thereof. The *C. difficile* toxin may also be a toxin selected from one of the toxinotypes 0 to XV as defined hereinbefore.

Production of a purified *C. difficile* toxin is exemplified in the Examples. In certain embodiments, the immunogen is a *C. difficile* toxin variant. In another embodiment the immunogen comprises an amino acid sequence at least 80%, 85%, 90%, 95%, 98%, 99%, or more identical to SEQ ID NOs: 1-6, or a fragment thereof.

The immunogen used to generate the antibodies of the present invention may also be partially or completely inactivated, i.e. have reduced toxicity. Examples of

modification include: chemical treatment (e.g. treatment with UDP-dialdehyde, formaldehyde, glutaraldehyde, peroxide, or oxygen) and recombinant methods (e.g. deletions or mutations in the toxin). For example, the immunogen may be a *C. difficile* toxoid or a fragment thereof derived from the native toxin by treatment with formaldehyde. Alternatively, a recombinant toxoid may be generated by selectively inactivating the active site motif by site-directed mutagenesis. An example of site directed mutagenesis to reduce or ablate the toxin effects of Toxins A and B is modification of the DXD motif in the N-terminal domain of the toxin. The aspartates and/or other residues may be mutated to e.g. alanine in order to reduce the biological activity of either Toxin A and B. For example, for Toxin A one of more of the following amino acids may be mutated: Asp 269, Asp285, Asp 287, Asn383, Trp519, Tyr283, Arg272. For Toxin B one of more of the following amino acids may be mutated: Asp270, Asp286, Asp 288, Asn384, Trp520, Tyr284, Arg273.

Antigens may be formulated with an adjuvant. Suitable adjuvants may include alum (aluminium phosphate or aluminium hydroxide), which is used widely in humans, and other adjuvants such as saponin and its purified component Quil A, Freund's complete and incomplete adjuvant, RIBBI adjuvant, and other adjuvants used in research and veterinary applications.

The *C. difficile* toxins or toxoids may be used as immunogens separately or in combination, either concurrently or sequentially, in order to produce antibodies specific for individual *C. difficile* toxins or combinations. For example, two or more toxins or toxoids may be mixed together and used as a single immunogen. Alternatively a *C. difficile* toxin (e.g. *C. difficile* Toxin A) may be used separately as a first immunogen on a first sheep, and another *C. difficile* toxin (e.g. *C. difficile* Toxin B) may be used separately on a second sheep. The antibodies produced by separate immunisation may be combined to yield an antibody composition directed against *C. difficile* toxins.

Where the oral delivery aspect of the present invention includes a separate or additional therapeutic component (eg. a non-oral therapy/ therapeutic), the latter is formulated by conventional means – examples of non-oral therapies include administration of an antibody or antibodies of the present invention via any non-oral route, including subcutaneous, intramuscular, intraperitoneal, and intravenous.

The method comprises all modes of immunisation (ie. to generate the antibodies of the invention), including subcutaneous, intramuscular, intraperitoneal, and intravenous. The

invention also contemplates a wide variety of immunisation schedules. In one embodiment, a sheep or goat is administered toxin(s) on day zero and subsequently receives toxin(s) at intervals thereafter. It will be appreciated that the interval range and dosage range required depends on the precise nature of the immunogen, the route of administration, the nature of the formulation, and the judgement of the attending person. Variations in these dosage levels can be adjusted using standard empirical routines for optimisation. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is someday after day 56. Levels of the specific antibody, i.e. that which binds to the immunogen, should represent at least 3 g per litre of serum.

The antibodies of the invention may be modified as necessary after collection, so that, in certain instances, they are less immunogenic in the patient to whom they are administered. For example, if the patient is a human, the antibodies may be despeciated by methods well known in the art. One example as to how an antibody can be made less immunogenic is to prepare the $F(ab)_2$ fragment. The antibodies of the invention may be used to produce such antibody fragments for which various techniques have been developed. For example, the fragments may be derived by proteolytic digestion of intact antibodies. Other techniques for their production will be apparent to the skilled practitioner.

Antibody formulation and delivery

In use, the present invention employs a pharmaceutical composition, comprising the antibody composition of the present invention in a form suitable for oral administration. The purified intact antibodies, or their fragments, are formulated for such delivery. For example, antibody, or its fragment, at a concentration between 5-50 or 15-50 or 25-50 g/litre may be formulated in buffer. Examples of suitable buffer components include physiological salts such as sodium citrate and/ or citric acid. Preferred buffers contain 100-200 or 125-175 or approximately 150 (eg. 153) mM physiological salts such as sodium chloride...

Antibody compositions of the invention are formulated for oral delivery. A key problem with oral delivery is ensuring that sufficient antibody reaches the colon where it is required. In this regard, factors that may inhibit optimal amounts of antibody reaching the gut include the proteolytic enzymes present in the digestive secretions, which degrade the antibody molecule and also in some instances the effect of CDI itself which can cause paralytic ileus and other complications that prevent movement of fluids down the alimentary canal. Thus, in a preferred

embodiment of the present invention, the antibody composition is formulated by incorporation of means for countering/ reducing the undesirable effects of the alimentary enzymes (eg. stomach enzymes) and environment (eg. stomach acid). There now follows a non-limiting description of a variety of embodiments of said means. Each of said embodiments may be employed alone or in combination with each other. Additional means known to a skilled person are included within the context of the present invention, and may also be emplyed alone or in combination with any of the following embodiments.

The oral antibody formulations/ compositions of the present invention may include one or more inhibitor of trypsin (e.g. an inhibitor of trypsin-1 and/ or trypsin-2) and/ or chymotrypsin (e.g. an inhibitor of chymotrypsin B). In one embodiment, said inhibitor is a macromolecular inhibitor (eg. a macromolecular inhibitor having a molecular weight of at least 5kDa), such as a polypeptide-based inhibitor. By way of example, said inhibitor(s) may contain a polypeptide loop, which when cleaved by either trypsin or chymotrypsin causes the inhibitor to bind very strongly to the protease thus inhibiting the further action of trypsin and/ or chymotrypsin. One preferred inhibitor in this regard may be provided, for convenience, in the form of egg white (albumin). Alternatively (or in addition), the active component thereof (e.g. ovomucoid and ovostatin/ ovomacroglobulin) may be employed. Another example is soybean trypsin inhibitor.

In one embodiment, an inhibitor cocktail may be provided, for convenience, in the form of colostrum (e.g. bovine). Alternatively (or in addition), the active component(s) thereof may be employed. Colostrum is readily combinable with ovine antibodies to provide a suitable formulation of oral administration.

In one embodiment, the trypsin inhibitor is a small protein (eg. Mw 5-25 kDa) that is naturally synthesized in the exocrine pancreas which prevents conversion of trypsinogen to trypsin, so protecting itself against trypsin digestion. Pancreatic trypsin inhibitor competitively binds to the active site of trypsin and inactivates it at a very low concentration. Examples of trypsin inhibitors suitable for use in the present invention include both naturally produced and recombinantly produced molecules, such as:

Source	Mw	Additional information

Lima beans	8-10 kDa	There are six different lima bean inhibitors.
Bovine pancreas	6.5 kDa	*Kunitz inhibitor* is the best known pancreatic inhibitor. Chymotrypsin is also inhibited by this chemical, but less tightly. When extracted from lung tissue, this is known as aprotinin.
Ovomucoid	ca. 27 kDa	Ovomucoids are glycoprotein protease inhibitors found in raw avian egg white.
Ovostatin	ca. 175kDa	Ovostatins (ovomacroglobulins) are protease inhibitors found in raw avian egg white.
Soybeans	20.7-22.3 kDa	Soybeans contain several trypsin inhibitors. All also bind to and inactivate chymotrypsin.

Natural pancreatic trypsin inhibitors are produced by the acinar cells and provide security against accidental trypsinogen activation and consequential unbridled proteolysis. By way of example, the intracellular basic trypsin inhibitor (BPTI) was first crystallized by Kunitz and Northrop in 1936. Basic pancreatic trypsin inhibitor (BPTI) forms a very stable 1:1 complex with bovine trypsin between pH 3 and 10, and also human trypsins. Chymotrypsin is also inhibited by BPTI. Soybean trypsin inhibitor (SBTI) first crystallized by Kunitz (1945) is one of several trypsin inhibitors found in soybeans. The best known preparation is that of Kunitz (Mw 21,500 ± 800; isoelectric point: 4.5). The Kunitz soybean inhibitor consists of a single polypeptide chain crosslinked by two disulfide bridges, and inhibits trypsin mole-for-mole and to a lesser extent chymotrypsin. Ovomucoids (Mw 28,500 ± 3,500) are the glycoprotein protease-inhibitors of avian egg white, and act upon bovine trypsin and chymotrypsin. Lima bean trypsin inhibitor (LBI) acts upon both trypsin and chymotrypsin by forming equimolar complexes. The trypsin susceptible binding site is a lys-ser peptide bond, whereas the site of chymotrypsin action is a leu-ser bond (Krahn and Stevens 1970). Lima bean trypsin inhibitors (Mw 8,000-10,000) may be chromatographically separated into as many as six variants. All have similar but not identical amino acid composition, contain six or seven disulfide bonds and lack methionine and tryptophan.

By way of further example, Bowman Birk protease inhibitors are a group of chymotrypsin and trypsin inhibitors produced by Soybeans and a range of

leguminous plants. They are small disulphide rich proteins of 7-10kda which are non-toxic to humans and well tolerated. Chymotrypsin peptide inhibitors which are extremely stable to extremes of pH occur in turtle egg whites. These small peptide inhibitors (approx 13 kDa) form stable complexes with chymotrypsin (Guha et al (1984) J. Bioscience 6: 155-163).

In one embodiment, the trypsin and/ or chymotrypsin inhibitor(s) component may be an antibody (including a fragment thereof) that binds to (eg. specifically binds to) and inactivates the enzymatic activity of trypsin and/ or chymotrypsin. Such antibody-based inhibitors may be used as an alternative or in addition to the above non-antibody-based inhibitors. Thus, an inhibitor combination of an antibody-based inhibitor and a non-antibody inhibitor may be employed. By way of example, a non-antibody inhibitor (eg. ovomucoid) may be used in combination with an antibody inhibitor where the antibody inhibits chymotrypsin (and/ or trypsin). Similarly, a non-antibody chymotrypsin inhibitor may be used in combination with an antibody inhibitor where the antibody inhibits trypsin (and/ or chymotrypsin). Such antibodies may be prepared routinely (eg. see Example 10).

The above-described trypsin and/ or chymotrypsin inhibitor(s) may be orally administered prior to, simultaneously with, or subsequent to the antibody component. In one embodiment, the inhibitor(s) are administered prior to or simultaneously with the antibody component.

In one embodiment, the oral antibody formulations of the present invention may include an antacid component. In use, said antacid component helps protect the antibody component from the highly acid gastric environment that exists within a patient.

An antacid is any substance, generally a base or basic salt, which counteracts stomach acidity. In other words, antacids are stomach acid neutralizers that raise the stomach pH, ideally above pH 4.0, for a limited time period Antacids perform a neutralization reaction, i.e. they buffer gastric acid, raising the pH to reduce acidity in the stomach.

Examples of suitable antacids for use in the present invention include: aluminium hydroxide (eg. Amphojel, AlternaGEL); magnesium hydroxide (e.g. Phillips' Milk of Magnesia); aluminum hydroxide with magnesium hydroxide (e.g. Maalox, Mylanta, Diovol); Aluminum carbonate gel (eg. Basaljel); calcium carbonate (eg. Alcalak,

TUMS, Quick-Eze, Rennie, Titralac, Rolaids); sodium bicarbonate (eg. bicarbonate of soda, Alka-Seltzer); magnesium carbonate; magnesium trisilicate; hydrotalcite (eg. $Mg_6Al_2(CO_3)(OH)_{16} \cdot 4(H_2O)$; Talcid); bismuth subsalicylate (e.g. Pepto-Bismol); alginates (e.g. sodium alginate, alginic acid); magaldrate with simethicone (eg. Pepsil); any of the above in combination with simethicone for example Asilone, which has three active ingredients, aluminium hydroxide and magnesium oxide neutralise the acid removing the cause of the pain, and dimethicone.

In addition (or alternatively) to the above-described formulation components, the composition may include a physical and/ or chemical means for protecting the antibody from the acidic environment of the stomach so that an active antibody is ultimately delivered to the intestine site of action (eg. the colon).

By way of example, the antibodies may be encapsulated (eg. pellets, granular matrices, beads, microspheres, nanoparticles, or liposomes) and/ or may be chemically protected (eg. by PEGylation).

Conventional encapsulation techniques suitable for use in the present invention include:

Technique employed	Polymer(s) used
pH dependent	Eudragit L100 and S100
	Eudragit L100 and S100
	Eudragit L100 and S100
	Eudragit S, Eudragit FS, Eudragit P4135 F
	Eudragit L 30 D-55 and Eudragit FS 30 D
Time dependent	Hydroxy propyl methyl cellulose
	Hydroxyethyl cellulose, ethyl cellulose, microcrystalline cellulose
	Lactose/ behinic acid
	Hydroxy propyl methyl cellulose
	Hydroxy propyl methyl cellulose acetate succinate
Bacteria dependent/ Polysaccharide based	Chitosan
	Pectin
	Guar gum
	Chondroitin sulphate
	Amylose
	Alginates

The pH in the terminal ileum and colon (except ascending colon) is higher than in any other region of the GI tract. Thus a dosage form that disintegrates preferentially at high pH levels is optimal for site-specific delivery into this region. One of the simplest approaches for designing a pH-dependent multiparticulate colon-specific delivery system is enteric coated granules. Enteric coating has traditionally been used to

prevent drug release in the upper GI tract. Enteric coating polymers may be used as both binders and as coating materials for granules. The incorporation of citric acid into the coating and/ or the tablet matrix helps to retard *in vitro* release and *in vivo* absorption because of the prolongation in disintegration time of the core system due to the presence of the acid. Most commonly used pH-dependent coating polymers for peroral delivery are methacrylic acid copolymers, Eudragit L100 and Eudragit S100, which dissolve at pH 6.0 and 7.0 respectively. The combination of these two polymers in various ratios makes it possible to manipulate drug release within 6.0-7.0 pH range. Capsules comprising these polymers may be further coated with solutions of polymethacrylates.

Similarly, excipients such as aqueous hydroxypropyl methyl cellulose acetate succinate as a coating material and citric acid as a pH regulating agent may be added. Glyceryl palmitostearate may be used as a retardant material to formulate controlled release matrices.

Coating formulations (e.g. Eudragit S100) may be further covered with a layer of chitosan HCl. Upon hydration, the capsule shell dissolves and the chitosan layer forms a gel (internal pH of 4.5), which generates an acidic environment around the Eudragit film so that it does not dissolve in the ascending colon. In the ascending colon, the chitosan HCl gel is degraded by the colonic micro flora, thereby exposing the Eudragit film to the colonic environment. But since the ascending colon is weakly acidic with a pH is less than 7.0, the film coat still remains intact. However, on arrival in the descending colon where pH is greater than 7, the Eudragit film coat dissolves and the drug is released in a controlled fashion from the matrices. Multi-layer coats may be employed based on, for example, an inner coat (a combination of Eudragit RL/RS), and an outer coat (Eudragit FS 30D). Eudragit FS 30D is an-ionic co-polymer of methyl acrylate, methyl methacrylate and methacrylic acid and is pH sensitive and dissolves at pH above 6.5.

Microbially-controlled delivery systems may also be employed, which rely on the unique enzymatic ability of the colonic micro flora. Delivery systems of this type enable a more specific targeting, independent of pH variations along the GI tract. Many natural polysaccharides such as chondroitin sulphate, pectin, dextran, guar gum etc. may be employed. Multiparticulate systems comprising hydrogel beads (chitosan and tripolyphosphate (TPP)) are one option - TPP acts as a counter ion to positively charged chitosan to form gel beads. The beads are loaded with bovine serum albumin (BSA), a protein that is liable to degradation in the upper parts of GI

tract, and the cross-linking of chitosan with TPP results in reduced solubility of chitosan, thereby resulting in lesser protein (antibody) release during upper GI transit. Amylose is a particularly good film-forming polymer (via gelation), and may also be mixed with Eudragit RS/RL 30D aqueous dispersions. Similarly, amidated low methoxy pectin which forms rigid gels with divalent cations (eg. calcium or zinc) may be employed to produce calcium pectinate gel beads for colonic delivery. Pectin may be combined with calcium salts - calcium pectinate (the insoluble salt of pectin) is not degraded by gastric or intestinal enzymes but is capable of degradation by colonic pectinolytic enzymes. As an alternative to crosslinking of soluble polysaccharides to form insoluble salts, the polysaccharide based system may be coated with pH sensitive polymers. By way of example, chitosan microcores may be prepared and coated with acrylic polymers, such as Eudragit L100 and Eudragit S100 respectively. Eudragit P-4135 F represents a further example of a suitable pH-sensitive polymer, which may be employed to prepare microparticles for colonic delivery.

Multiparticulate systems may be employed, which combine pH sensitive delivery and biodegradation in the colonic environment. By way of example, an inner entrapment matrix of chitosan microcores may be prepared using a technique such as spray drying, followed by application of chitosan microcores microencapsulated within Eudragit polymers by a technique such as oil-in oil solvent evaporation. Upon dissolution of the outer Eudragit coat at appropriate pH the exposed chitosan microcores swell and form a gel barrier in alkaline pH, and, in the colonic region, the chitosan undergoes degradation thereby enhancing release. Similar colonic delivery multiparticulate systems may be based on chitosan microspheres coated with Eudragit L100 or S100. Suitable preparation techniques include emulsion solvent evaporation. The chitosan may be cross-linked with glutaraldehyde.

Polyacrylates represent a further example of a suitable delivery vehicle for use in the present invention. By way of example, a terpolymer of styrene and hydroxyethyl methacrylate cross-linked with a difunctional azo-compound may be employed. The system depends on cleavage of the azo bond by colonic microflora resulting in degradation of polymer. Similarly, a pH responsive poly (methacrylic-*g*-ethylene glycol) hydrogel may be employed as an oral delivery vehicle. Once inside the basic and neutral environment of the small intestine, the gels rapidly swell and dissociate.

In another embodiment, a microcapsule formulation may be employed for peroral colon-specific delivery. In more detail, aqueous colloidal terpolymers of ethylacrylate/

methyl methacrylate/ 2-hydroxyl ethyl methacrylate (poly (EA/MME/ HEMA), for example as synthesized by emulsion polymerization technique(s) may be employed. These polymers exhibit delayed release profiles which were characterized by a long lag time and subsequent rapid release of the entrapped moiety.

In another embodiment, orally administered nanoparticles may serve as suitable delivery vehicles. By way of example, loaded nanoparticles may be entrapped into pH sensitive microspheres, which serve to deliver the incorporated nanoparticle to the desired colonic site of action. Nanoparticles have a large specific surface, which is indicative of high interactive potential with biological surfaces. Thus, bioadhesion can be induced by binding nanoparticles with different molecules. By way of example, nanoparticles may be prepared from gliadin protein isolate from wheat gluten and then conjugated with lectins (glycoproteins of non-immune origin which provide specific bioadhesion). Accordingly, nanoparticles are provided, which have a high capacity for non-specific interaction with intestine and the binding of lectin provided greater specificity for colonic mucosa.

In one embodiment, a delivery vehicle based on an albumin–chitosan mixed matrix microsphere-filled coated capsule formulation may be employed. In this regard, an antibody preparation of the invention is filled into hard gelatin capsules and enteric coated.

In one embodiment, albumin microspheres may be employed as the oral delivery system.

In one embodiment, squalane oil-containing multiple emulsions may be employed

In one embodiment, poly(lactide-co-glycolide) microspheres may be employed as the oral delivery vehicle.

In one embodiment, a colonic delivery coating comprising a mixture of pH-responsive enteric polymer (Eudragit S) and biodegradable polysaccharide (resistant starch) in a single layer matrix film may be employed. Examples of these delivery vehicles are available commercially, such as from Encap Drug Delivery (Livingston, UK) – particular embodiments include PHLORAL™ and ENCODE™.

In addition (or alternatively) to the above delivery vehicle embodiment, the antibodies/ antibody fragments of the present invention may be protected from acid

erosion by PEGylation with polyethylene glycol (PEG). PEG of various molecular weights (500 – 40000Da) may be coupled to IgG, for example, in a ratio of 2-20 PEG molecules per antibody molecule. We refer to Greenwald, R.B *et al* (2003) "Effective drug delivery by PEGylated drug conjugates", Advanced Drug Delivery Reviews 55, pp.217-250. This publication is incorporated in its entirety by reference thereto.

In one embodiment, delivery capsules such as liposomes, micro- or nanocapsules (eg. chitosan nanocapsules) may be chemically modified with poly(ethylene glycol) (PEG). The typical degree of PEGylation is in the range of 0.1% to 5%, such as 0.5% to 2%, for example 0.5% or 1%. The presence of PEG, whether alone or grafted to chitosan, improves the stability of the delivery capsules in the gastrointestinal fluids.

In one embodiment, the antibodies of the present invention may be treated with monomethoxypoly(ethylene) glycols activated by cyanuric chloride, succinimidyl succinate, and tresyl chloride.

PEGylated delivery vehicles such as liposomes, micro- or nanocapsules have an intrinsic ability to accumulate at disease sites and facilitate transfection of target cells. Unlike many viral vectors, PEGylated liposomes are generally considered to be non-immunogenic.

In one embodiment, a branched PEGylating reagent is employed as branched PEG protecting groups are more effective than linear PEG molecules.

Since the antibody formulations of the present invention are for oral delivery, said formulations may include a sweetener, such as vanilla essence, a sugar (eg. glucose, sucrose, etc), sugar alcohols, honey, fruit, syrups (eg. maple syrup, rice syrup, birch syrup, pine syrup, hickory syrup, poplar syrup, palm syrup, sugar beet syrup, sorghum syrup, corn syrup, cane syrup, golden syrup, barley malt syrup, molasses (treacle), brown rice syrup, agave syrup, yacon syrup), acesulfame potassium (also known as Sunett), alitame (also known as aclame), aspartame (also known as Equal or Nutrasweet), anethole, cyclamate, glycyrrhizin, lo han guo, neotame, perillartine, saccharin (also known as Sweet 'n' Low), stevioside, sucralose (also known as SucraPlus and Splenda), or inulin.

Compositions suitable for oral delivery may be in the form of solutions, suspensions or dry powders which are dissolved or suspended in a suitable vehicle prior to use.

In preparing pharmaceutical formulations, the antibodies and/ or fragments thereof can be dissolved in the vehicle, and sterilised for example by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Advantageously additives such as buffering, solubilising, stabilising, preservative or bactericidal or suspending and/or local anaesthetic agents may be dissolved in the vehicle.

Dry powders, which are dissolved or suspended in a suitable vehicle prior to use, may be prepared by filling pre-sterilised ingredients into a sterile container using aseptic technique in a sterile area. Alternatively the ingredients may be dissolved into suitable containers using aseptic technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

The dosage ranges for administration of the antibodies of the present invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the antibody or composition, the nature of the formulation, the age of the patient, the nature, extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician. Variations in these dosage levels can be adjusted using standard empirical routines for optimisation.

In one embodiment, typical daily dosages are in the range of 5-20mg (e.g. 8-15mg or approximately 10mg) per kg of body weight. The unit dosage can vary from less than 100mg, but typically will be in the region of 250-500 mg per dose, which may be administered daily (eg. 1x, 2x, 3x or 4x per day) or less frequently (e.g. on alternative days, or say once per week).

It is also within the scope of the invention to use the antibodies of the invention in oral therapeutic methods for the prevention or treatment of CDI in combination with one another, or as an adjunct to, or in conjunction with, other established therapies normally used in the treatment in CDI. For example, the antibodies of the present invention may be administered in conjunction with a suitable antibiotic (e.g. metronidazole and/or vancomycin)

The combination treatment may be carried out in any way as deemed necessary or convenient by the person skilled in the art and for the purpose of this specification, no limitations with regard to the order, amount, repetition or relative amount of the compounds to be used in combination is contemplated.

Definitions Section

Clostridium difficile is a species of Gram-positive bacteria of the genus Clostridium.

Clostridium difficile infection (CDI) means a bacterial infection which affects humans and animals and which results in a range of symptoms from mild self-limiting diarrhoea to life-threatening conditions such as pseudomembranous colitis and cytotoxic megacolon. In this disease, *C. difficile* replaces the normal gut flora and produces cytotoxins which attack and damage the gut epithelium. Primary risk factors for human CDI include: receiving broad-spectrum antibiotics, over 65 years old and hospitalised.

Clostridium difficile Toxin A is a family of protein cytotoxins/enterotoxins of approximately 300 kDa in size. Toxin A has an enzyme activity within the N-terminal region which acts to disrupt the cytoskeleton of the mammalian cell causing cell death. There are a number of naturally occurring variants of Toxin A within the strains of *Clostridium difficile* which are call 'toxinotypes'. The various toxinotypes of Toxin A have variations within their primary sequence of usually <10% overall. Examples of suitable Toxin A sequences include SEQ ID Nos: 1 and 3.

Clostridium difficile Toxin B is a family of protein cytotoxins of approximately 270 kDa in size which are similar to Toxin A but significantly more cytotoxic. Like Toxin A, Toxin B has an enzyme activity within the N-terminal region which acts to disrupt the cytoskeleton of the mammalian cell causing cell death. There are a number of naturally occurring variants of Toxin B within the strains of *C. difficile* which are call 'toxinotypes'. The various toxinotypes of Toxin B have variations within their primary sequence of usually <15% overall. Examples of suitable Toxin A sequences include SEQ ID Nos: 2 and 4.

Binary Toxin is a two component cytotoxin produced by some but not all strains of *C. difficile*. The binary toxins are similar in action to *Clostridium botulinum* C2 and *Clostridium perfringens* iota toxins, which like *C. difficile* binary toxin, consist of a cell binding fragment of approximately 100 kDa and an enzymically active 'effector' fragment of approx. 50 kDa. Examples of suitable Binary Toxin sequences include SEQ ID Nos: 5 and 6.

As used herein, the term "toxin" encompasses said toxin fragments. The fragment may range from any number of amino acids between 10 and 2700 (e.g. at least 50,

100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, 1500, 2000 or 2500) of the reference toxin. The fragment preferably includes at least one epitope of the gene product in question. The "fragment" may also have a common antigenic cross-reactivity and/or substantially the same *in vivo* biological activity as the toxin from which it is derived. For example, an antibody capable of binding to a fragment would be also capable of binding to the toxin from which it is derived. Alternatively, the fragment may share a common ability to induce a "recall response" of a T-lymphocyte which has been previously exposed to an antigenic component of a *C. difficile* toxin.

Reference to the term Toxin embraces "variants" thereof – for example, a peptide or peptide fragment having at least 80 or 85 or 90 or 95 or 96 or 97 or 98 or 99 percent amino acid sequence homology with a *C. difficile* Toxin. In a further embodiment, a "variant" may be a mimic of the peptide or peptide fragment, which mimic reproduces at least one epitope of the peptide or peptide fragment.

Reference to the Toxin embraces Toxin "toxoid", which is discussed in more detail below.

Toxinotypes are often used to classify strains of *C. difficile*. Toxinotypes are based on a method which characterises the restriction patterns obtained with the toxin genes. As described above, toxinotypes of Toxins A and B represent variants, by primary amino acid sequence, of these protein toxins.

Clostridium difficile Toxoid is used to describe a *C. difficile* toxin (Toxin A, Toxin B or Binary Toxin) or a mixture of *C. difficile* toxins that has been partially or completely inactivated. A toxin is considered inactivated if it has less toxicity (e.g. 100%, 99%, 95% or 90% less toxicity) than untreated toxin as measured by an *in vitro* cytotoxicity assay or by animal toxicity.

An antibody that binds to a toxin of interest is one capable of binding that toxin with sufficient affinity such that the antibody is useful as a therapeutic agent. An antibody that binds to a toxin of interest is one that binds to a toxin of *C. difficile* with an affinity (K_a) of at least 10^4 M.

Toxin neutralising means the action of a substance (e.g. an antibody) which blocks the biological action of one or more of the cytotoxins (Toxin A and/or Toxin B and/or binary toxin) of *C. difficile*. The cytotoxin's biological action being defined as its

ability to kill or impair the function of mammalian cells, in particular cells of the mammalian gut epithelium. Toxin neutralising activity of a substance may be measured by its ability to prevent the death of mammalian cells grown in culture.

A therapeutically effective amount refers to the amount of the antibody, which when administered alone or in combination to a patient for treating CDI, or at least one of the clinical symptoms of CDI, is sufficient to affect such treatment of the disease, or symptom. The therapeutically effective amount can vary depending, for example, on the antibody, the infection, and/or symptoms of the infection, severity of the infection, and/or symptoms of the infection, the age, weight, and/or health of the patient to be treated, and the judgment of the prescribing physician. An appropriate therapeutically effective amount in any given instance may be ascertained by those skilled in the art or capable of determination by routine experimentation. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody are outweighed by the beneficial effects.

A "prophylactically effective amount" is any amount of the antibody that, when administered alone or in combination to a patient, inhibits or delays the onset or reoccurence of the CDI, or at least one of the clinical symptoms of CDI. In some embodiments, the prophylactically effective amount prevents the onset or reoccurence of the *Clostridium difficile* infection entirely. "Inhibiting" the onset means either lessening the likelihood of the infection's onset, or preventing the onset entirely.

An oral antibody formulation is one which allows a prophylactically effective amount of antibody, when administered orally, to reach the gut and inhibit or delay the onset or reoccurrence of the CDI. Oral formulations prevent or reduce the degradation of antibodies in the gut environment by various mechanisms including the use of protease inhibitors, physical and chemical barriers

Sheep means any species that falls within the Ovis genus (e.g. Ovis ammon, Ovis orientalis aries, Ovis orientalis orientalis, Ovis orientalis vignei, Ovis Canadensis, Ovis dalli, Ovis nivicola).

An ovine antibody is an antibody that has at least 100%, 99%, 95%, 90%, 80%, 75%, 60%, 50%, 25% or 10% amino acid sequence identity to an antibody that has been raised in a sheep.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences may be compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequent coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percentage sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison may be conducted, for example, by the local homology alignment algorithm of Smith and Waterman [Adv. Appl. Math. 2: 484 (1981)], by the algorithm of Needleman & Wunsch [J. Mol. Biol. 48: 443 (1970)] by the search for similarity method of Pearson & Lipman [Proc. Nat'l. Acad. Sci. USA 85: 2444 (1988)], by computer implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA - Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705), or by visual inspection [see Current Protocols in Molecular Biology, F.M. Ausbel et al, eds, Current Protocols, a joint venture between Greene Publishing Associates, In. And John Wiley & Sons, Inc. (1995 Supplement) Ausbubel].

Examples of algorithms suitable for determining percent sequence similarity are the BLAST and BLAST 2.0 algorithms [see Altschul (1990) J. Mol. Biol. 215: pp. 403-410; and "http://www.ncbi.nlm.nih.gov/" of the National Center for Biotechnology Information].

In one homology comparison, the identity exists over a region of the sequences that is at least 10 or 20 or 30 or 40 or 50 amino acid residues in length. In another homology comparison, the identity exists over a region of the sequences that is at least 60 or 70 or 80 or 90 or 100 amino acid residues in length.

An "antibody" is used in the broadest sense and specifically covers polyclonal antibodies and antibody fragments so long as they exhibit the desired biological activity. In particular, an antibody is a protein including at least one or two, heavy (H) chain variable regions (abbreviated herein as VHC), and at least one or two light (L) chain variable regions (abbreviated herein as VLC). The VHC and VLC regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). The extent of the framework region and CDRs has

been precisely defined (see, Kabat, E.A., et al. Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, 1991, and Chothia, C. et al, J. Mol. Biol. 196:901-917, 1987, which are incorporated herein by reference). Preferably, each VHC and VLC is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FRI, CDRI, FR2, CDR2, FR3, CDR3, FR4.

The VHC or VLC chain of the antibody can further include all or part of a heavy or light chain constant region. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are inter-connected by, e.g., disulfide bonds. The heavy chain constant region includes three domains, CHI, CH2 and CH3. The light chain constant region is comprised of one domain, CL. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (Clq) of the classical complement system. The term "antibody" includes intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or lambda.

The term antibody, as used herein, also refers to a portion of an antibody that binds to a toxin of *C. difficile* (e.g. Toxin B), e.g., a molecule in which one or more immunoglobulin chains is not full length, but which binds to a toxin. Examples of binding portions encompassed within the term antibody include (i) a Fab fragment, a monovalent fragment consisting of the VLC, VHC, CL and CHI domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fc fragment consisting of the VHC and CHI domains; (iv) a Fv fragment consisting of the VLC and VHC domains of a single arm of an antibody, (v) a dAb fragment (Ward et al, Nature 341:544-546, 1989), which consists of a VHC domain; and (vi) an isolated complementarity determining region (CDR) having sufficient framework to bind, e.g. an antigen binding portion of a variable region. An antigen binding portion of a light chain variable region and an antigen binding portion of a heavy chain variable region, e.g., the two domains of the Fv fragment, VLC and VHC, can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VLC and VHC regions pair to form monovalent molecules (known as single chain Fv

(scFv); see e.g., Bird et al. (1988) Science IAI-ATi-AIβ; and Huston et al. (1988) Proc. Natl. Acad. ScL USA 85:5879-5883). Such single chain antibodies are also encompassed within the term antibody. These are obtained using conventional techniques known to those with skill in the art, and the portions are screened for utility in the same manner as are intact antibodies.

There now follows a brief description of the Figures, which illustrate aspects and/ or embodiments of the present invention.

Figure 1 Measurement of antibodies to Toxin A in serum by affinity chromatography. Antibody binding to Toxin A immobilised onto Sepharose gel which was subsequently eluted. The Figure shows the linear relationship between serum load and eluted Toxin A-specific antibody. Experimental details are provided in Example 9.

Figure 2 Measurement of antibodies to Toxin A in serum by affinity chromatography. Antibody binding to Toxin A immobilised onto Sepharose gel which was subsequently eluted. The Figure demonstrates specific antibody in sheep immunised with a toxoid of Toxin A. Antibodies to Toxin A were present in the sheep serum at >3 mg/ml (3 g/litre). Experimental details are provided in Example 9.

Figure 3 Inhibition of trypsin activity by egg whites colostrum and normal ovine sheep serum. The data show that trypsin can be inhibited by a variety of natutally occurring inhibitors such as those found in chichen and turkey egg white, colostrum and ovine serum.

Figure 4 Trypsin activity in casein and colostrum agar radial protease diffusion plates. Shows the inhition of trypsin by colostrum using a radial protease diffusion method

Figure 5 *In vivo* experiment 1 - Protection of hamsters from CDI by oral delivery of an antibody in the presence of an antacid. These data show that antibodies to *C. difficile* Toxins A and B orally delivered in the presence of an antacid afford protection of hamsters from CDI

Figure 6 *In vivo* experiment 2 - Protection of hamsters from CDI by oral delivery of an antibody in the presence of an antacid. These data show that antibodies to *C. difficile* Toxins A and B orally delivered in the presence of an antacid afford protection of hamsters from CDI

Figure 7 *In vivo* experiment 3 - Protection of hamsters from CDI by oral delivery of an antibody in an encapsulated form. These data show that antibodies to *C. difficile* Toxins A and B orally delivered in enteric costed capsules afford some protection of hamsters from CDI

Summary of Examples

Example 1 Purification of *C. difficile* Toxins A and B of Toxinotype 0

Example 2 Purification of *C. difficile* Toxins A and B of other Toxinotypes

Example 3 Purification of recombinant *C. difficile* Toxins A and B

Example 4 Purification of *C. difficile* binary toxin

Example 5 Preparation of Toxoids of *C. difficile* Toxins A and B

Example 6 Preparation of antiserum

Example 7 Preparation of antiserum to Toxins A and B of Toxinotype 0

Example 8 Assessment of the neutralising efficacy for antisera to toxins using the in vitro cell assay

Example 9 Quantifying the amount of specific antibody to *C. difficile* toxins in serum using immunoaffinity columns

Example 10 Preparation of antibody inhibitors against trypsin and/ or chymotrypsin

Example 11 Preparation of trypsin and/ or chymotrypsin inhibitors from egg white (albumin)

Example 12 Demonstration of inhibition of proteolytic activity of trypsin

Example 13 Demonstration of inhibition of proteolytic activity of chymotrypsin

Example 14 Preparation of formulation containing antibody-based inhibitors of trypsin and/ or chymotrypsin

Example 15 Preparation of formulation containing drug substance-based inhibitors of trypsin and/ or chymotrypsin

Example 16 Preparation of bovine colostrum – ovine antibody formulations

Example 17 Preparation of antibodies modified with polyethylene glycol (PEG)

Example 18 Coating of antibodies with copolymers of methyl acrylate, methyl methacrylate and methacrylic acid (Eudragit)

Example 19 Coating of antibodies with copolymers of alginate/chitosan

Example 20 Coating of antibodies with pectin

Example 21 Assessing the stability of antibody formulations to digestive enzymes using simulated gastric and intestinal conditions

Example 22 Assessment of the *in vivo* efficacy of ovine antibodies for

preventing CDI
Example 23 Assessment of the *in vivo* efficacy of ovine antiserum for treating CDI
Example 24 Clinical uses of antibody formulations (drug substance)
Example 25 Clinical use of a combination of orally delivered antibody and antibiotics
Example 26 Clinical uses of a combination of systemically and orally delivered antibody formulations

Summary of SEQ ID NOs

Where an initial Met amino acid residue or a corresponding initial codon is indicated in any of the following SEQ ID NOs, said residue/ codon is optional.

1. Protein sequence of *Clostridium difficile* Toxin A – Toxinotype 0
2. Protein sequence of *Clostridium difficile* Toxin B – Toxinotype 0
3. Protein sequence of *Clostridium difficile* Toxin A – Toxinotype III
4. Protein sequence of *Clostridium difficile* Toxin B – Toxinotype III
5. Protein sequence of *Clostridium difficile* Binary toxin fragment A
6. Protein sequence of *Clostridium difficile* Binary toxin fragment B
7. Protein sequence of human trypsin-1 (Swiss Prot Accession P07477)
8. Protein sequence of human trypsin-2 (Swiss Prot Accession P07478)
9. Protein sequence of chymotrypsin-2 (Swiss Prot Accession P17538)

Examples

Example 1 Purification of Clostridium difficile Toxins A and B of Toxinotype 0

A *C. difficile* strain producing Toxinotype 0 Toxins A and B (e.g. VPI 10463) was grown in dialysis sac culture as described (Roberts and Shone (2001) Toxicon 39: 325-333). After growth, the cell slurry was collected from the dialysis sacs and then centrifuged for 10000 x g for 30 min and the pH of the resulting supernatant fluid adjusted to pH 7.5 and made 70% saturated with respect to ammonium sulphate. The precipitate containing the toxins was collected by centrifugation then resuspended in 50mM bistris pH 6.5 buffer and dialysed against the same buffer at 4°C. After dialysis, the solution of crude Toxins A and B was purified by chromatography on Q Sepharose, anion exchange chromatography and the protein peaks containing the toxins eluted with a gradient of NaCl. The peak containing Toxin A was dialysed against 50mM Hepes pH 7.4 buffer containing 0.5 M NaCl and purified on a Zn chelating column (Zn Sepharose). After loading the toxin and

washing the contaminating proteins from the column, the purified Toxin A was eluted with a buffer containing 50 mM Hepes pH 7.4, 20 mM EDTA and 0.1M NaCl. The purified Toxin A was dialysed against 50mM Hepes pH 7.4 buffer containing 0.15 M NaCl and stored at 4°C or frozen until use. The peak containing the Toxin B from the initial Q Sepharose column was further purified by chromatography on a column of high resolution Mono Q anion exchange resin. After loading the toxin onto the column in 50mM bistris pH 6.5 buffer, the purified Toxin B was eluted with a NaCl gradient and the fractions containing the toxin pooled. The purified Toxin B was dialysed against 50mM Hepes pH 7.4 buffer containing 0.15 M NaCl and stored at 4oC or frozen until use.

Example 2 Purification of *C. difficile* Toxins A and B of other Toxinotypes

Toxins A and B representing any of the known Toxinotypes are purified as described in Example 1. Known *C. difficile* strains producing Toxins A and B of various toxinotypes are given in Table 1 and by selecting the required strain for purification, Toxins A and B of the required Toxinotype are purified. Alternatively, *C. difficile* may be toxinotyped as described previously (Rupnik et al. (1998) J. Clinical Microbiol. 36: 2240-2247; Rupnik et al. (2001) Microbiology 147: 439-447) until a *C. difficile* strain producing toxin of the desired toxinotype is obtained. Each of these references is incorporated in its entirety by reference.

To produce Toxinotype III Toxins A and B, *C. difficile* strain R20291 (also known as NCTC 13366) was grown in dialysis sac culture as described (Roberts and Shone (2001) Toxicon 39: 325-333, which is incorporated in its entirety by reference) and the toxins purified as described in Example 1.

Example 3 Purification of recombinant *C. difficile* Toxins A and B

Amino acid sequences of examples of the *C. difficile* Toxins A and B are shown Seq IDs 1 to 4. Genes encoding these peptides are available commercially with codon bias for any desired expression host (e.g. *E. coli*, *Pichia pastoris*). Peptides are expressed from these genes using standard molecular biology methods (e.g. Sambrook et al. 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and the resulting soluble expressed polypeptides are purified by a combination of hydrophobic interaction chromatography, ion exchange chromatography and ceramic hydroxyl apatite chromatography. Alternative chromatographic techniques well known to the art of protein purification, such as size exclusion chromatography and/or affinity chromatography, may be used. For the latter, recombinant fragments may be

expressed with affinity purification tags (e.g. Histidine-6, streptag) such as described in the pET vector Expression System Manual, 11th Edition published by Merck KGaA, Darmstadt, Germany.

To produce a recombinant toxin from a *C. difficile* toxinotype for which the sequence is unknown, DNA is extracted and the toxin sequence(s) derived by standard molecular biology methods. The recombinant toxin is then expressed from a synthetic gene as above.

Example 4 Purification of recombinant *C. difficile* binary toxin

Amino acid sequences of the *C. difficile* binary toxin fragments A and B are shown Seq IDs 5 and 6, respectively. Genes encoding these peptides are available commercially with codon bias for any desired expression host (e.g. *E. coli, Pichia pastoris*). Peptides are expressed from these genes using standard molecular biology methods (e.g. Sambrook et al. 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and the resulting soluble expressed peptides are purified by a combination of hydrophobic interaction chromatography, ion exchange chromatography and ceramic hydroxyl apatite chromatography. Alternative chromatographic techniques well known to the art of protein purification, such as size exclusion chromatography and/or affinity chromatography, may be used.

Recombinant fragments are expressed with affinity purification tags (e.g Histidine-6, streptag) such as described in the pET vector Expression System Manual, 11th Edition published by Merck KGaA, Darmstadt, Germany (herein incorporated in its entirety). Details of the purification of the binary toxin components are described in Sundriyal *et al.* 2010 (Protein Expression & Purification 74: 42-48), which is herein incorporated in its entirety.

The peptides may be expressed with a histidine-6 purification tag to improve solubility using a commercially available expression vector such as pET52b and refolded by on-column refolding techniques as described by the review of Lia et al. and references contained therein (Lia M et al (2004) Protein Expression & Purification 33, 1-10), which is hereby incorporated by reference thereto.

Example 5 Preparation of Toxoids of *C. difficile* Toxins A and B

Purified *C. difficile* toxins at a concentration of between 0.2 – 2 mg/ml are dialysed against a suitable buffer (e.g. 10mM Hepes buffer pH 7.4 containing 150mM NaCl)

and then formaldehyde added at a final concentration of between 0.05 and 0.5% and incubated for between 1 and 25 days at 35°C. After incubation, the formaldehyde is removed by dialysis. Conditions for the treatment with formaldehyde may vary slightly between peptides and final conditions are fine-tuned accordingly on the basis of outcome of protective efficacy evaluations.

Example 6 Preparation of antiserum

A number of conventional factors are taken into consideration during the preparation of antiserum in order to achieve the optimal humoral antibody response. These include: breed of animal; choice of adjuvant; number and location of immunisation sites; quantity of immunogen; and number of and interval between doses

Conventional optimisation of these parameters it is routine to obtain specific antibody levels in excess of 6 g/litre of serum.

For sheep, 2 ml of buffer solution containing between 10 and 500 µg of *C. difficile* antigen is mixed with 2.6 ml of Freund's adjuvant. The complete form of the adjuvant is used for the primary immunisation and incomplete Freund's adjuvant for all subsequent boosts. Mixing of the adjuvant is carried out for several minutes to ensure a stable emulsion. About 4.2 ml of the antigen/adjuvant mixture is used to immunise each sheep by im injection and spread across 6 sites including the neck and all the upper limbs. This is repeated every 28 days. Blood samples are taken 14 days after each immunisation. Once adequate antibody levels are achieved, larger volumes are taken (10 ml /kg body weight) into sterile bags. The bags are rotated slowly to accelerate clotting, centrifuged for 30 min at 4500 x g and the serum removed under aseptic conditions and pooled. Any animal showing low titres to the desired *C. difficile* antigen is removed from the flock.

Example 7 Preparation of antiserum to Toxins A and B of toxinotype 0

Toxins A and B from a toxinotype 0 strain (e.g. VPI 10463) were prepared as described in Example 1. Alternatively, Toxin A or B may be made by recombinant methods as described by Yang *et al.* (Yang G, Zhou B, Wang J, He X, Sun X, Nie W, Tzipori S, Feng H (2008) Expression of recombinant Clostridium difficile toxin A and B in *Bacillus megaterium*. BMC Microbiol. 8: 192). Purified Toxins may be toxoided as described in Example 5.

For immunisation of sheep with Toxoids A or B, 2 ml of buffer solution containing between 10 and 500 µg of either *C. difficile* Toxoids A or B was mixed with 2.6 ml of

Freund's adjuvant. The complete form of the adjuvant was used for the primary immunisation and incomplete Freund's adjuvant used for all subsequent boosts. Mixing of the adjuvant was carried out for several minutes to ensure a stable emulsion. After mixing, approx 4.2 ml of the antigen/adjuvant mixture was used to immunise each sheep by im injection and spread across 6 sites including the neck and all the upper limbs. This was repeated every 28 days and serum samples collected 14 days after each immunisation. Once adequate antibody levels were achieved, larger production sample were taken (10 ml /kg body weight) into sterile bags. The bags were rotated slowly to accelerate clotting, centrifuged for 30 min at 4500 x g and the serum removed under aseptic conditions and pooled. Any animal showing low titres to either Toxins A or B was omitted from the flock.

Example 8 Assessment of the neutralising efficacy for antisera to toxins using the *in vitro* cell assay

The toxin neutralizing activity of the antisera against *C. difficile* Toxins is measured by cytotoxicity assays using Vero cells. A fixed amount of either purified *C. difficile* Toxin A or Toxin B is mixed with various dilutions of the antibodies, incubated for 1h at 37°C and then applied to Vero cells growing on 24-well tissue culture plates. Both Toxin A and B possess cytotoxic activity which results in a characteristic rounding of the Vero cells over a period of 24 - 72 h. In the presence of neutralising antibodies this activity is inhibited and the neutralising strength of an antibody preparation is assessed by the dilution required to neutralise the effect of a designated quantity of either Toxin A or B.

Data demonstrating the neutralising activity of ovine antibody to *C. difficile* Toxin A are shown in Table 2. In this experiment, various dilutions of ovine antibody were mixed with Toxin A at a final concentration of 50 ng/ml and incubated for 1 h at 37°C and then applied to Vero cells as above and incubated at 37° and monitored over a period of 24 -72 h. The antibody dilutions which protect the cells against the cytotoxic effects of the Toxin A were calculated. Table 2 shows that sheep immunised for a period of 14 weeks had a neutralising titre of 16000 (i.e. a 1/16000 dilution of the serum protected the cells from the cytotoxic effects of Toxin A).

Table 2 Neutralisation Titres of Ovine Antibodies Raised Against Formaldehyde-Treated Toxin A

Number of vaccinations	Immunisation period (weeks)	Antibody Neutralising Titre¶

0	0	<10
1	0	<10
2	6	2000
3	10	4000
4	14	16000

¶ Dilution of serum required to neutralise 50 ng/ml of Toxin A in cell neutralisation assays

For antiserum produced by Toxin B (Toxoid), a 14 week schedule with one immunisation of 250 µg/dose given to each animal every 4 weeks resulted in antiserum with an antiserum titre of >1/10000 (using a fixed concentration of Toxin B at 0.5 ng/ml).

Table 3 Neutralisation Titres of Ovine Antibodies Raised Against Formaldehyde-Treated Toxin B

The data show that higher immunising doses of Toxoid B antigen results in a better ovine toxin-neutralising immune response as measured by Vero cell cytotoxicity assays. These assays are described in Example 8.

Immunising dose (µg) of C. difficile Toxoid B	**Neutralisation titre against Toxin B in cell assays***
Sheep anti toxoid B (10ug)	1/1280
Sheep anti toxoid B (50ug)	1/2560
Sheep anti anti toxoid B (250ug)	1/10240

All animals were given 2 doses of formaldehyde-treated Toxin B

* Dilution of serum required to completely neutralise 0.5 ng/ml of Toxin B in cell neutralisation assays

Tables 4, 5 and 6 demonstrate that very high toxin-neutralising tires (>20,000) units per ml of serum can be obtained in sheep by immunisation with toxoids derived from

Toxin A and B. These titres are significantly higher than that previously reported in other species.

Table 4 Neutralisation titres of ovine antibodies raised using different doses formaldehyde-treated Toxin A

The data show that after an extended immunisation period, very high titres can be obtained with various toxoid doses in sheep. These assays are described in Example 8.

Immunising dose (µg) of *C. difficile* Toxoid A	**Dosing**	**Neutralisation titre/ml serum against Toxin A (50 ng/ml)¶**
Sheep anti toxoid A (25µg)	5 doses over 22 weeks	25,600
Sheep anti toxoid A (100µg)	5 doses over 22 weeks	25,600
Sheep anti toxoid A (250µg)	5 doses over 22 weeks	25,600

¶ Dilution of serum required to neutralise 50 ng/ml of Toxin A in cell neutralisation assays

Table 5 Neutralisation titres of ovine Antibodies raised against formaldehyde-treated toxin B

The data show that a 250µg immunising dose of Toxoid B antigen over an extended immunisation period results in very high toxin neutralising titres. These assays are described in Example 8.

Immunising dose (µg) of C. difficile Toxoid B	**Dosing**	**Neutralisation titre/ml serum against Toxin B (0.5 ng/ml)***
Sheep anti toxoid B (10µg)	5 doses over 18 weeks	1,280
Sheep anti toxoid B (50µg)	5 doses over 18 weeks	5,120
Sheep anti toxoid B (250µg)	5 doses over 18 weeks	20,480

* Dilution of serum required to completely neutralise 0.5 ng/ml of Toxin B in cell neutralisation assays

Table 6 Neutralisation titres of purified IgG obtained against by immunisation of formaldehyde-treated Toxins A and B

The table show the high toxin-neutralisation titres of purified IgG derived from ovine antiserum by precipitation of other proteins with caprylic acid

Purified sheep IgG	Neutralisation titre against Toxin A (50 ng/ml)	Neutralisation titre against Toxin B (0.5 ng/ml)
IgG (50 mg/ml) from Toxoid A immunised sheep	40,000	N/A
IgG (50 mg/ml) from Toxoid B immunised sheep	N/A	20,000

Example 9 Quantifying the amount of specific antibody to *C. difficile* toxins in serum using immunoaffinity columns

Column Preparation

CNBr-activated Sepharose 4 Fast Flow (0.5 g dry weight) is weighed into a suitable clean container (glass or plastic). About 10 ml of diluted hydrochloric acid (1mM) is added to swell the gel and, after 20-30 min, the gel is transferred to a 10-mL glass column and washed with a further 20mL of HCl (1mM), followed by 20mL of coupling buffer (sodium bicarbonate, 100mM, pH 8.3, containing 500mM sodium chloride). Toxin (Toxin A, Toxin B or a binary toxin fragment solution (1mL) at a concentration of 1 mg/mL is diluted to 5mL with coupling buffer and added to the column containing the activated gel and the contents mixed gently until the gel is re-suspended and rotated at room temperature overnight (16-18hr). The column is then drained and 5ml of blocking reagent (ethanolamine solution, 1M) added, mixed gently and rotated for 2hr at room temperature. Next, the column is washed with 20mL coupling buffer followed by 20mL of elution buffer (glycine solution 100mM, pH 2.5). This step is repeated twice. The column is finally washed with 20mL of assay buffer (sodium phosphate buffer, 10mM, pH 7.4 containing 500mM sodium chloride and sodium azide at a final concentration of 1g/L) and stored in 3-5mL of assay buffer at 2-8°C until used.

Column Assessment

The specific binding and non-specific capacity of the column is typically assessed prior to use. The column is removed from the refrigerator and allowed to equilibrate to room temperature and then washed with 25mL of assay buffer. Increasing volumes of the product (whole antisera, purified IgG, Fab or $F(ab')_2$) are individually loaded onto the column and mixed end-over-end gently for 1hr at room temperate.

The unbound fraction is washed off with 25mL of assay buffer and the bound fraction then eluted from the column with 20 ml of elution buffer (glycine buffer 100mM, pH 2.5). The protein content of the eluted fraction is determined spectrophotometrically at 280nm using an extinction coefficient relevant to the product namely 1.5 for sheep IgG (Curd et al., 1971) or 1.4 for sheep Fab and $F(ab')_2$ (Allen, 1996). A saturation curve is obtained by plotting the amount of eluted protein against the volume loaded.

Non-specific binding (NSB) is assessed using normal sheep serum (NSS) prior to immunisation. Thus it is necessary to differentiate between this and binding due to some specific antibodies in normal serum (since all animals will have been exposed to C. difficile). Figure 1 demonstrates the typical binding capacity curve showing an increase in specific binding as a result of increasing the antiserum loading volume. There is a little change in non-specific binding (NSB) with the increase of loading volume. The 0.5 g (1.5-2.0 ml swelled gel) contain 1mg of toxin (coupling ratio of 2mg/g) is sufficient for the volume of specific antisera (0.5-4mL) loaded. In this regard, 1ml is the recommended loading volume for easy and convenient calculations.

The coefficient of variation for 10 replicates (between assay CV) is approximately 6%. There is no decline in the column capacity with time (estimated when used 80-100 times). This indicates that there is no leaching of the toxin from the column.

Affinity Column for Product Assessment

The column is used for GMP/GLP assessment of in-process and final product viz whole antisera, purified IgG, Fab and $F(ab')_2$. It is also used to assess and monitor the immune response of the immunised animals and to detect antitoxin antibodies in human samples.

The column is removed from the refrigerator and allowed to equilibrate to room temperature when it is washed with 25mL of assay buffer. Product (1mL) is added to the column and mixed end-over-end gently for 1hr at room temperature following which the unbound fraction is washed off with 25mL of assay buffer (sodium phosphate buffer, 10mM, pH 7.4 containing 500mM sodium chloride and sodium azide at a final concentration of 1g/L). The bound fraction is then eluted with 20 ml of elution buffer (glycine buffer 100mM, pH 2.5) and its protein content determined spectrophotometrically at 280nm using an extinction coefficient relevant to the product. Figure 2 shows the analysis of serum from sheep immunised with a toxoid of Toxin A.

Example 10 Preparation of antibody inhibitors against trypsin/ chymotrypsin

Suitable antigens may be prepared, for example, by protocol (a) or (b) below.

(a) Immunisation with native and/or toxoided trypsin and chymotrypsin

Human trypsin-1, trypsin-2 and chymotrypsin are obtained commercially. These enzymes are dialysed into a suitable buffer such as MES (50 mM, pH 6.0) containing 150mm NaCl and toxoided by addition of 0.2% formaldehyde followed by incubation for between 1 and 14 days at between 4 and 37°C.

(b) Immunisation with recombinant trypsin and chymotrypsin

Amino acid sequences of the principal human trypsin and chymotrypsin are shown SEQ IDs 7, 8 and 9, respectively. Catalytically inactive antigens are provided, for example, by changing one or more of the underlined residues (e.g. histidine, aspartate, serine) to an amino acid residue such as analine (or a conservative substitution thereof). Genes encoding these modified trypsin and chymotrypsin peptides are commercially available with codon bias for any desired expression host (e.g. *E. coli, Pichia pastoris*). Peptides are expressed from these genes using standard molecular biology methods (e.g. Sambrook et al. 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and the resulting soluble expressed peptides are purified by a combination of hydrophobic interaction chromatography, ion exchange chromatography and ceramic hydroxyl apatite chromatography. Alternative chromatographic techniques well known to the art of protein purification, such as size exclusion chromatography and/ or affinity chromatography, may be used.

Recombinant fragments may also be expressed with affinity purification tags (e.g. Histidine-6, streptag) such as described in the pET vector Expression System Manual, 11th Edition published by Merck KGaA, Darmstadt, Germany.

The peptides may be expressed with a histidine-6 purification tag to improve solubility using a commercially available expression vector such as pET52b and refolded by on-column refolding techniques as described by the review of Lia et al. and references contained therein (Lia M et al (2004) Protein Expression & Purification 33, 1-10), which is hereby incorporated by reference thereto.

The above antigens, either singularly or in various combinations, are used to generate antibodies by the following method. For preparation of antibodies in sheep,

2 ml of buffer solution containing between 10 and 500 µg of trypsin and/or chymotrypsin antigen(s) is mixed with 2.6 ml of Freund's adjuvant. The complete form of the adjuvant is used for the primary immunisation and incomplete Freund's adjuvant for all subsequent boosts. Mixing of the adjuvant is carried out for several minutes to ensure a stable emulsion. About 4.2 ml of the antigen/adjuvant mixture is used to immunise each sheep by im or ic injection and spread across 6 sites including the neck and all the upper limbs. This is repeated every 28 days. Blood samples are taken 14 days after each immunisation. Once adequate antibody levels are achieved, larger volumes are taken (10 ml /kg body weight) into sterile bags. The bags are rotated slowly to accelerate clotting, centrifuged for 30 min at 4500 x g and the serum removed under aseptic conditions and pooled. Any animal showing low titres to the desired *C. difficile* antigen is removed from the flock.

Example 11 Preparation of trypsin/chymotrypsin inhibitors from egg white

Egg white contains only traces of lipids and carbohydrates and consists largely of protein. Protein fractions containing the predominant protease inhibitors, ovomucoid and ovostatin can be readily obtained by precipitation or by standard protein purification methods such as ion exchange chromatography and size exclusion. In one such method, the white of eggs are separated from the yolk and suspended in 2 volumes of 1 % NaCl in 50 mM Tris-HCl (pH 7.5) containing 1mM EDTA and homogenized with an ultrasonic disruptor before being centrifuged at 15,000 x *g* for 20 min. The supernatant fluid is then dialyzed against 10 mM Tris-HCl buffer, pH 7.5, and applied to a Q-Sepharose column. The column is eluted with 10 mM Tris-HCl buffer containing NaCl with a linear gradient from 0 M to 0.5 M to obtain various peaks of the protease inhibitors. Crude or purified protein fractions containing protease inhibitor activity against trypsin and chymotrypsin (as assessed in Examples 12 and 13) may be optionally combined to produce an enriched protease inhibitor protein mixture.

In other methods, concentrated mixtures of the egg white protease inhibitors are readily obtained by precipitation of the egg white proteins with various agents such e.g. acetone (Lineweaver & Murray (1947) J. Biol. Chem. 171, 565-581) or by precipitation with up to 70% ammonium sulphate. Alternatively, egg white protease is commercially available.

Example 12 Demonstration of inhibition of proteolytic activity of trypsin

Trypsin activity is measured using the L-BAPNA assay: This method is based on the spectrophotometric determination of the breakdown products of benzoyl-DL-arginine-

p-nitroanilide (DL-BAPNA) by a given concentration of trypsin, in the presence and absence of the inhibitor (Kakade *et al.* 1974), which is incorporated in its entirety by reference.

Materials

Assay buffer: Tris-buffer (0.05M, pH 8.2) containing 0.02M $CaCl_2$

Substrate solution: Benzoyl-DL-arginine-p-nitroanilide hydrochloride (DL-BAPNA) (10mg) was dissolved in 0.2mL of dimethyl sulphoxide (DMS) and diluted to 20mL with assay buffer. The solution was prepared daily and stored at 37˚C while in use.

Trypsin solution (0.2mg/mL: Trypsin 40mg was dissolved into 200mL of diluted HCl (0.001M). The solution can be stored at 2-8˚C for 2 weeks.

Stopping solution: acetic acid solution (30% v/v) was prepared by mixing a glacial acetic acid (30mL) with distilled water (70mL).

Procedure

Various volumes of trypsin inhibitor (e.g. antibodies, or egg white, or egg white derivatives or colostrum) were pipetted into duplicate sets of test tubes and adjusted to 1mL with assay buffer. Control samples contained equivalent protein concentrations of non-specific protein or antibody. Trypsin solution was added to each tube followed by 1mL of DL-BAPNA solution. After incubation for 5 minutes at room temperature, the reaction was terminated by adding 0.5mL of stopping solution and the absorbance of each tube measured spectrophotometrically at 410nm. Blank samples were prepared by adding the stopping solution prior to the substrate solution.

Test sample containing effective trypsin inhibitors were found to inhibit the cleavage of DL-BAPNA and hence the increase in absorbance at 410nm compared to the control samples.

Demonstration of trypsin-neutralising activity in various inhibitor preparations (e.g. egg white and colostrum)

Reagents

Chicken egg white was separated manually from the yolk and diluted at a ratio of 1:1 with assay buffer (Tris-buffer, 0.05M, pH 8.2, containing 0.02M $CaCl_2$). A highly

purified Type II trypsin inhibitor from turkey egg white (Sigma UK) and Bovine colostrum (Colostrum UK ltd) reagent were prepared at concentrations of 0.4 g/L and 100g/L in assay buffer, respectively.

Procedure

Various volumes of trypsin inhibitors (chicken egg white, turkey egg white Type II trypsin inhibitor, normal ovine serum; bovine colostrum) were pipetted into duplicate sets of test tubes and adjusted to 1 mL with Tris-buffer, (0.05M, pH 8.2) containing 0.02M $CaCl_2$. Trypsin solution (0.2 mg/ml) was prepared in 1mM hydrochloric acid and 1 ml was added to each tube followed by 1 ml of DL-BAPNA solution (0.5 mg/ml). After incubation for 5 min at room temperature, the reaction was terminated by adding 0.5 ml of Acetic acid solution (30%v/v) and the absorbance of each tube measured spectrophotometrically at 410nm. Blank samples were prepared by adding the stopping solution prior to the substrate solution.

For the tubes containing colostrum, sodium sulphate solution (360g/l) was added in an equal volume and centrifuged for 45min at 3500rpm to precipitate out the casine protein. The supernatant of each tube was collected and the absorbance measured as above.

Result

Inhibition of trypsin activity was demonstrated in all preparations tested including ovine serum, bovine colostrum and chicken and turkey egg whites (Figure 3). Trypsin inhibition is due to the intrinsic inhibitors present in these preparations.

<u>Demonstration of inhibition of trypsin by colostrum by radial protease diffusion</u>

This technique was used to measure the trypsin inhibition activity of colostrum. Diffusion plates were prepared by dissolving 0.5 g of agar (Bio-Rad) in 50 ml of assay buffer (Tris-buffer, 0.05M, pH 8.2; containing 0.02M $CaCl_2$) in a boiling water bath, cooling to 60°C and adding 50 ml of a casein or colostrum suspension (20 g/l) in assay buffer to give a final concentration of 10 g/L. The warm suspension was poured in 90 cm plastic plates to yield a layer 2.5 mm thick which was allowed to solidify in a humid chamber at room temperature for 2 hours. Wells of 5 mm in diameter were punched out and 20µL of various porcine trypsin concentrations (6.25, 12.5, 25 and 50mg/L) were loaded to each well. The plates were incubated at room temperature (22°C) in a humid chamber for 24hr. The diameters (d^2 mm^2) of the transparent circles resulting from trypsin diffusion and digestion of the casein were measured and plotted against the trypsin concentration (Figure 4).

The results showed that colostrum at a concentration of 10g/L inhibited the proteolytic activity of the trypsin at the tested concentrations.

Protection of ovine immunoglobulin from digestion by trypsin by in presence of chicken egg white

Ovine IgG was purified by caprylic and formulated at 25g/L into sodium citrate saline buffer pH 6.0. Porcine trypsin was dissolved in 1mM hydrochloric acid to a concentration of 2 g/L and added to the ovine IgG at a concentration of 5% w/w of the total protein. An equal volume of chicken egg white diluted in a ratio of 1:1 with Tris-buffer, 0.05M, pH 8.2; containing 0.02M $CaCl_2$, was added and the mixture incubated at 37°C for 20 h. The digestion was monitored by size exclusion gel filtration (FPLC).

The results showed that chick egg white completely protected the IgG from digestion with trypsin. The control experiment has demonstrated that under these experimental conditions and in the absence of chicken egg white, trypsin completely digested the IgG to Fab and small fragments.

Example 13 Demonstration of inhibition of proteolytic activity of chymotrypsin

The protease reaction velocity is determined by measuring an increase in absorbance at 256 nm resulting from the hydrolysis of benzoyl-L-tyrosine ethyl ester. One unit hydrolyzes one micromole of benzoyl-L-tyrosine ethyl ester (BTEE) per minute at pH 7.8 and 25°C under the specified conditions.

Reagents

0.08 M Tris·HCl buffer, pH 7.8 containing 0.1 M calcium chloride

0.00107 M Benzoyl-L-tyrosine ethyl ester (BTEE) in 50% w/w methanol (63 ml absolute methanol added to 50 ml reagent grade water)

Dissolve enzyme at one mg/ml in 0.001 N HCl. Dilute in 0.001 N HCl to 10-30 µg/ml for assay.

Procedure

Adjust the spectrophotometer to 256 nm and 25°C.

Various volumes of chymotrypsin inhibitor (e.g. antibodies, egg white derivatives, or colostrum) were pipetted into duplicate sets of test tubes and adjusted to 1.5 ml with

assay buffer (0.08 M Tris·HCl buffer, pH 7.8 with 0.1 M CaCl2). Control samples contained equivalent protein concentrations of non-specific protein or antibody.

Add to the above 1.4 ml of 0.00107 M BTEE

Incubate in spectrophotometer at 25°C for 4-5 minutes to achieve temperature equilibrium and record blank rate, if any. Add 0.1 ml of appropriately diluted enzyme and record increase in absorbance at 256 nm for 4-5 minutes. Calculate ΔA256/min from the initial linear portion of the curve.

Test sample containing effective chymotrypsin inhibitors were found to inhibit the cleavage of DL-BAPNA and hence the increase in absorbance at 256 nm compared to the control samples

Example 14 Preparation of a formulation containing antibody inhibitors to trypsin and chymotrypsin

Formulation of antibodies effective at preventing and treating CDI when delivered orally may contain the following components:

- Ovine antibodies against difficile Toxins A and/ or B
- optionally, ovine antibodies against difficile binary toxin
- ovine antibodies that have inhibitory activity against human trypsin(s) and/ or ovine antibodies that have inhibitory activity against human chymotrypsin(s)
- optionally, an antacid component to assist in the neutralisation of stomach acid
- optionally, a flavouring such as a sweetener to make the mixture more palatable

In detail, a typical formulation contains:

- ovine antibodies to Toxin A and/ or B at 5 - 50 mg/ml
- optionally, ovine antibodies to binary toxin at 5 - 50 mg/ml
- antibody inhibitors of trypsin and/ or chymotrypsin at 5 - 50 mg/ml
- optionally, an antacid component (e.g. magnesium hydroxide or sodium bicarbonate) at, for example, a concentration of 0.05 to 0.5 M
- optionally, a flavouring agent (e.g. a sweetener) such as vanilla essence

Example 15 Preparation of a formulation based on drug substance (e.g. egg-derived) inhibitors of trypsin and/ or chymotrypsin

Formulation of antibodies effective at preventing and treating CDI when delivered orally contain the following components:

- ovine antibodies against difficile Toxins A and/ or B
- optionally, ovine antibodies against difficile binary toxin
- crude or purified protein fractions from hen eggs containing the protease inhibitor activity against trypsin and chymotrypsin as described in Example 11
- optionally, an antacid component to assist in the neutralisation of stomach acid
- optionally, a flavouring such as a sweetener to make the mixture more palatable

In detail, a typical formulation contains:

- ovine antibodies to Toxin A and/ or B at 5 - 50 mg/ml
- optionally, ovine antibodies to binary toxin at 5 - 50 mg/ml
- hen egg white derived protease inhibitors (as purified as described in Example 11) at a concentration of 5 - 50 mg/ml
- optionally, an antacid component (e.g. magnesium hydroxide or sodium bicarbonate) at, for example, a concentration of 0.05 to 0.5 M
- optionally, a flavouring agent (e.g. a sweetener) such as vanilla essence

Example 16 Preparation of bovine colostrum – ovine antibody formulations

Ovine antibody formulations with bovine colostrum may be prepared in several ways:

By mixing liquid bovine colostrum with a solution of ovine IgG.

By mixing lyophilised or dried bovine colostrum with liquid IgG.

By mixing liquid bovine colostrum with lyophilised ovine IgG.

By mixing lyophilised or dried bovine colostrum with lyophilised ovine IgG and reconstituting with water or buffer saline to the desired concentration.

In the above formulations, the colostrum component has a final concentration of between 10% and 90% of its initial concentration. The final concentration of IgG is ideally between 10-50 mg/ml.

Formulation of antibodies effective at preventing and treating CDI when delivered orally contain the following components:

- ovine antibodies against difficile Toxins A and/ or B
- optionally, ovine antibodies against difficile binary toxin
- a colostrum component has a final concentration of between 10% and 90% of its initial concentration

- optionally, an antacid component to assist in the neutralisation of stomach acid
- optionally, a flavouring such as a sweetener to make the mixture more palatable

In detail, a typical formulation contains:

- ovine antibodies to Toxin A and/ or B at 5 - 50 mg/ml
- ovine antibodies to binary toxin at 5 - 50 mg/ml
- a colostrum component has a final concentration of between 10% and 90% of its initial concentration
- optionally, an antacid component (e.g. magnesium hydroxide or sodium bicarbonate) at, for example, a concentration of 0.05 to 0.5 M
- optionally, a flavouring agent (e.g. a sweetener) such as vanilla essence

Example 17 Preparation of antibodies modified with polyethylene glycol or dextran

Molecules of polyethylene glycol (PEG) or dextran are attached to the antibody in a variety of ways, which may be used singularly or in combination.

N-hydroxysuccinimide PEG derivatives allow attachment to ovine IgG via amino groups. For these reactions, freshly prepared N-hydroxysuccinimide PEG in aqueous buffer (e.g. HEPES 50mM, between pH6.5 and 8.0) is mixed with IgG solution (5 - 100 mg/ml for up to 3 h at 37°C or 24 h at 4°C.

Carboxyl PEGylation: After being activated by EDEC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide, HCl salt) at mild acidic pH, the carboxyl group of antibodies readily react with PEG-hydrazide, while the amino groups present in all reagents remain inactive under these particular conditions.

Via carbohydrate (PEG hydrazide to react with aldehyde groups formed by periodate oxidation of oligosaccharide residues to form a hydrazone.

Using the above coupling methods PEG of various molecular weights (500 – 40000Da) is coupled to IgG in a ratio of 2- 20 PEG molecules per antibody molecule.

Dextrans offer an alternative to PEG as a derivatising agent and they are available in a range of molecular sizes (500 – 40000Da) which can be covalently attached to IgG in a ratio of 2- 20 dextran molecules per antibody molecule using sodium periodate

creating polyaldehyde derivatives of the dextran. Unlike PEG chemistry, each dextran moiety can attach at more than one site on the antibody. A very similar strategy for modification with dextran can be used as described for PEGylation above and these are described by Hermanson (Hernamson, GT (1996) Bioconjugate Techniques, Academic Press)

The biological activity of pegylated or dextran-derivatised IgG preparations is measured by their capacity to neutralise either Toxin A, B or binary toxin in cell assays as described in Example 8. The stability of the pegylated or dextran-derivatised IgG is be assessed using simulated gastric and intestinal conditions as described in Example 21. These assessments, combined with *in vivo* efficacy studies, are used to optimise the above pegylation conditions to provide an IgG formulation with the desired stability to the digestive environment.

Retention of biological activity (toxin-neutralising activity) of the antibodies will be a trade off against their protection from the digestive environment. Pegylation or dextan derivatisation conditions preferably result in the highest overall delivery of active IgG to the gut.

Example 18 Coating of antibodies with copolymers of methyl acrylate, methyl methacrylate and methacrylic acid (Eudragit)

In one method to formulate ovine antibodies for oral delivery, antibodies are first made into small granules by mixing with corn starch powder. In this method, purified ovine IgG is mixed with corn starch in the ratio of approximately 1 part IgG to 4 parts starch. This mixture is then granulated in granulator (e.g. Yokomizo Granular model FR160 x 60) at a temperature between 20 – 37°C, humidity between 60 - 90% for between 2 -15 min in order to produce granules of between 1 - 4 mm in diameter. In stage two of the process, the IgG granules are sealed by immersing in PEG aqueous solution (3 – 10%) of PEG (which is chosen from the molecular weight range 3000-10000) and then air drying at 37°C. In the final stage of the process, the sealed IgG granules are coated with a solution of Eudragit (e.g. Eudragit L100-55; Rohm GmbH, Germany). For this, an aqueous solution of Eudragit L100-55 (between 10-20%) is made by slowly adding the polymer to water and adding NaOH to partially neutralise 5-15% of the carboxyl groups. The PEG coated IgG granules are then coated in polymer solution by repeated immersion and air drying at 37°C to give a final polymer coating of between 5-40% (w/w).

The stability of the coated IgG is assessed using simulated gastric and intestinal conditions as described in Example 21. These assessments, combined with *in vivo* efficacy studies, can be used to optimise the above coatings conditions to provide an IgG formulation with the desired stability to the digestive environment.

Example 19 Coating of antibodies with copolymers of alginate/chitosan

Alginate/chitosan microcapsules are prepared by methods similar to that described by Esquisabel et al. (J. Microencapsul, 14:627-638; 1997), which is incorporated in its entirety by reference. In this method 2% (w/v) alginate containing 0.2% (w/v) calcium chloride in water is added to purified ovine IgG (final concentration 0.1 – 20 mg/ml). After mixing, this aqueous phase is mixed with an oil phase (e.g. soybean oil containing 0.2% Tween 80) in a ratio of 1 part aqueous to 10 parts oil and emulsified for 5-10 min. After adjusting the pH to approx pH 5, the mixture is agitated for 15 min until the gelation reaction is complete. To this suspension, a water/n-hexane (80:20) mixture is added and the antibody microcapsules allowed to partition into the aqueous phase. This aqueous phase is separated and added to a chitosan solution 0.1 to 10% (w/v) in 1% catic acid solution in various proportions and allowed to react for 30 min before being filtered and dried.

The stability of the coated IgG is then assessed using simulated gastric and intestinal conditions as described in Example 21. These assessments, combined with *in vivo* efficacy studies, are used to optimise the above coatings conditions to provide an IgG formulation with the desired stability to the digestive environment.

Example 20 Coating of antibodies with pectin

Pectin beads are formed as described by Munjeri et al. (Drug Delivery 5: 239-241; 1998). Solutions (4% w/v) of amidated low methoxyl pectin in water are prepared by high speed mixing. Amidated pectin-antibody beads are prepared by combining the pectin and antibody in various ratios from 200:1 to 10:1 (pectin to antibody) and adding the solution drop-wise to a solution of calcium chloride (approx 2% w/v). The solution is pumped through tubes of diameter ranging from 1 - 5 mm and the resulting beads air dried and stored at 4°C.

Example 21 Assessing the stability of antibody formulations to digestive enzymes using simulated gastric and intestinal conditions

Formulations are assessed for gastric stability by exposure to simulated gastric conditions. These are prepared as described in the United Stated Pharmacopeia (United Stated Pharmacopeial Convention Council of Experts (2004) 27, volume 22 p

2728), which is incorporated in its entirety, and consist of 3.2 mg/ml pepsin in 30mM NaCl at pH 1.2. Antibody formulation is mixed with this solution in the ratio of 1 part pepsin solution to 250 parts antibody solution and incubated for various times (e.g. 0 - 360 min) at 37°C. At the end of the this time the integrity of the antibody is assessed for Toxin A/B neutralising efficacy as described in Example 8. The degradation of the 150 kDa antibody molecule is also assessed on SDS PAGE gels on which the amount of the intact 150kDa can be qualified relative to untreated control samples.

Formulations are assessed for intestinal stability by exposure to simulated intestinal conditions. These are prepared as described in the United Stated Pharmacopeia (United Stated Pharmacopeial Convention Council of Experts (2004) 27, volume 22 p 2728), which is incorporated in its entirety, and consist of 10 mg/ml pancreatin in 50mM potassium phosphate buffer at pH 6.8 Antibody formulation is mixed with this solution in the ratio of 1 part pancreatin solution to 50 parts antibody solution and incubated for various times (e.g. 0 - 360 min) at 37°C. At the end of the this time the integrity of the antibody is be assessed for Toxin A/B neutralising efficacy as described in Example 8. The degradation of the 150 kDa antibody molecule is also be assessed on SDS PAGE gels on which the amount of the intact 150kDa is qualified relative to untreated control samples.

A combination of the above simulated gastric and intestinal conditions is also used to assess the stability of antibody formulations. In this case, after treatment with the pepsin solution the pH of the mixture is raised to 6.8 by adding e.g. 0.1 M sodium bicarbonate solution or 0.1M Tris-HCl before adding the pancreatin solution as described above.

Example 22 Assessment of the *in vivo* efficacy of ovine antibodies for preventing CDI

To demonstrate the efficacy of antibodies to prevent CDI *in vivo*, Syrian hamsters are given an antibody formulation orally. For assessing the efficacy of a prophylactic formulation, hamsters are given antibody orally (up to 0.5ml) at various times from 96 hours pre-challenge to 240 hours post challenge with *C. difficile*

During the administration of formulation, CDI is induced in hamsters by giving a broad spectrum antibiotic (e.g. clindamycin) and then 12-72 h later by challenge with *C. difficile* spores by mouth. Animals are then monitored for up to 15 days for symptoms of *C. difficile*-associated disease. Control, untreated animals develop

signs of the disease (e.g. diarrhoea, swollen abdomen, lethargy, ruffled fur) while those treated with ovine antibody formulation either appear normal or develop only mild disease symptoms.

Example 23 Assessment of the in vivo efficacy of ovine antiserum for treating CDI

To demonstrate the efficacy of antibodies to treat CDI *in vivo*, Syrian hamsters are given antibody formulation (as described in Example 15 and 16) orally. For assessing the efficacy of a treatment formulation, hamsters will be given antibody orally (up to 0.5ml) at various times from 6 hours post-challenge to 240 hours post challenge with *C. difficile.*

Prior to the administration of formulation, CDI is induced in hamsters by giving a broad spectrum antibiotic (e.g. clindamycin) and then 12-72 h later by challenge with *C. difficile* spores by mouth. Animals are then monitored for up to 15 days for symptoms of *C. difficile*-associated disease. Control, untreated animals develop signs of the disease (e.g. diarrhoea, swollen abdomen, lethargy, ruffled fur) while those treated with ovine antibody formulation either appear normal or develop only mild disease symptoms.

In vivo experiment 1 – Oral delivery of an antibody in the presence of an antacid

Aim:

To assess the efficacy of an orally administered mixture of ovine antibodies to Toxins A and B to protect from CDI induced by challenge with *C. difficile* spores (strain VPI 10463). Two different dose levels were assessed which were given once on day 0 and then 2 times daily over 4 days

Methodology

Three groups of animals were used:

Group 1 and 2 – which were divided 'Test Sub-Group' of 10 animals and a 'Control Sub-Group' of 4 animals (which did not receive a spore challenge).

Group 3 - 'Test Sub-Group' of 10 animals

Group 1 – Test and control groups received PBS containing 0.1M sodium bicarbonate (The sodium bicarbonate was added to 0.1M immediately before dosing)

Group 2 – Test and control groups received ovine antibody A+B mixture in a 1:1 ratio containing 0.1M sodium bicarbonate (Sodium bicarbonate was added to 0.1M immediately before dosing). Ovine anti-Toxin A - batch CDA000185 and ovine anti-Toxin B - batch CDB000229 were used in a 1:1 ratio. Final antibody concentration was 45 mg per ml for the mixture. Hamsters received 45 mg antibody per day.

Group 3 – Test and control groups received ovine antibody A+B mixture in a 1:1 ratio containing 0.1M sodium bicarbonate (Sodium bicarbonate was added to 0.1M immediately before dosing). Ovine anti-Toxin A - batch CDA000185 and ovine anti-Toxin B - batch CDB000229 were used in a 1:1 ratio and diluted 5-fold with PBS. Final antibody concentration was 9 mg per ml for the mixture. Hamsters received 9 mg antibody per day

Clindamycin (0.2 ml of 10mg/ml solution) and *C. difficile* VPI 10463 spores (250 cfu in a 0.2 ml dose) were given orogastrically.

Dosing timetable for *in vivo* experiment 1 (all doses given orogastrically)

Day (from challenge)	**AM (9-10 am)**	**PM (3-4 pm)**
Day -3	Clindamycin	-
Day 0	Spore challenge	0.5 ml Antibody (or PBS)
Day 1	0.5 ml Antibody (or PBS)	0.5 ml Antibody (or PBS)
Day 2	0.5 ml Antibody (or PBS)	0.5 ml Antibody (or PBS)
Day 3	0.5 ml Antibody (or PBS)	0.5 ml Antibody (or PBS)
Day 4	0.5 ml Antibody (or PBS)	0.5 ml Antibody (or PBS)

Results & Conclusions

Survival data are shown in Figure 5. The control Group showed rapid onset of severe CDI with all animals succumbing to severe disease within 3 days post challenge.

The orally administered antibody mixture offered protection from rate of disease onset. On Day 4 post challenge, while a 100% of animal had succumbed to disease in the PBS control (Group1), 30% and 60% were surviving in the low (Group 3) and high (Group 2) antibody dose groups, respectively.

At the termination of experiment 20% of animal survived in both antibody groups compared to 0% in the PBS control group. Orally administered antibody in the

presence of an antacid doses administered offer some protection against the onset of CDI

In vivo experiment 2 – Oral delivery of a high antibody dose in the presence of an antacid

Aim:

To assess the efficacy of an orally administered mixture of ovine antibodies to Toxins A and B to protect from CDI induced by challenge with *C. difficile* spores (strain VPI 10463). The antibody was given once on day 0 and then 3 times daily over 4 days.

Methodology

Two groups of animals were used:

Group 1 – was divided 'Test Sub-Group' of 10 animals and a 'Control Sub-Group' of 4 animals (which did not receive a spore challenge).

Group 2 – 'Test Group' of 10 animals

Group 1 – Test and control sub-groups received no treatment

Group 2 – Test group received ovine antibody A+B mixture in a 1:1 ratio containing 0.1M sodium bicarbonate. (Sodium bicarbonate was added to 0.1M immediately before dosing). Ovine anti-Toxin A - batch CDA000264 and ovine anti-Toxin B - batch CDB000229 were used in a 1:1 ratio. Final antibody concentration was 45 mg per ml for the mixture. Hamsters received 68 mg antibody per day

Clindamycin (0.2 ml of 10mg/ml solution) and *C. difficile* VPI 10463 spores (500 cfu in a 0.2 ml dose) were given orogastrically.

Results & Conclusions

The survival data are shown in Figure 6. The control animals (Group 1) showed a relatively slow onset of severe CDI with 60% of animals succumbing to severe disease within 9 days post challenge. Group 2 animals treated with buffered liquid antibody (68 mg total per day) showed no symptoms of CDI over a 16 day period of the experiment and were completely protected. One animal succumbed to disease on Day 17. Overall, there was significant protection from CDI of animals in Group 2 compared to the control group (Group 1).

The data show that orally administered ovine antibody can both prevent and treat CDI.

Dosing timetable for *in vivo* experiment 2 (all doses given orogastrically)

Day (from challenge)	AM (9-10 am)	Midday	PM (3-4 pm)
Day -3	Clindamycin (Gp1 and 2)	-	-
Day 0	Spore challenge (Gp1 and 2)	-	0.5 ml Antibody (Gp2)
Day 1	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)
Day 2	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)
Day 3	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)
Day 4	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)	0.5 ml Antibody (Gp2)

In vivo experiment 3 – Oral delivery of antibody in the from of enteric-coated capsules

Aim:

To assess the efficacy of an orally administered mixture of ovine antibodies to Toxins A and B in an encapsulated form to protect from CDI induced by challenge with *C. difficile* spores (strain VPI 10463).

Methodology

Two groups of animals were used:

Group 1 – was divided 'Test Sub-Group' of 10 animals and a 'Control Sub-Group' of 4 animals (which did not receive a spore challenge). Group 2 - 'Test Group' of 10 animals

Group 1 – Test and control groups received no treatment

Group 2 – Test groups received enteric-coate capsules (Encap: Batch 244/15/1). Capsules received 2 enteric coats (Eudragit + PEG400) to maintain their integrity through the stomach and small intestine, These contained approximately 10 µl of a mixture of ovine anti-Toxin A - batch CDA000264 and ovine anti-Toxin B - batch CDB000229 in a 1:1 ratio. Hamsters received approximately 1.5 mg of the antibody mixture per day

Results & Conclusions

The survival data are shown in Figure 7. Group 2 animals were given a small dose of antibody in capsule form (approx 1.5 mg per day). Some protection from this antibody dose was observed. On Day 5 post challenge, while 40% of the Group 1 control animals had succumbed to severe disease, 90% were surviving in Group 2. At the end of the experiment 60% of the Group 2 capsule-treated animal survived compared to 40% in the Group 1 controls.

Dosing timetable for *in vivo* experiment 3 (all doses given orogastrically)

Day (from challenge)	**AM (9-10 am)**	**Midday**	**PM (3-4 pm)**
Day -3	Clindamycin (Gp1 and 2)	-	-
Day 0	Spore challenge (Gp1 and 2)	-	1 x capsule (Gp2)
Day 1	1 x capsule (Gp2)	1 x capsule (Gp2)	1 x capsule (Gp2)
Day 2	1 x capsule (Gp3)	1 x capsule (Gp2)	1 x capsule (Gp2)
Day 3	1 x capsule (Gp3)	1 x capsule (Gp2)	1 x capsule (Gp2)
Day 4	1 x capsule (Gp3)	1 x capsule (Gp2)	1 x capsule (Gp2)

Example 24 Clinical uses of antibody formulations (drug substance)

Prophylactic treatment of 'at risk' patient groups

As a prophylactic for CDI, patients identified as 'at risk' will the treated with the drug substance. Parameters for defining such groups of patients include:

- hospitalised
- over 65 yr
- receiving broad-spectrum antibiotics
- previous history of CDI or close proximity to symptomatic cases

Patients groups which are particularly appropriate for oral antibody therapy include:

- those with mild to moderate disease severity
- those who are asymptomatic but are considered at high risk of relapse (perhaps because of one or more relapse episodes)
- those in close proximity to outbreak cases

Patients falling into this category will be administered orally, formulations of the drug substance 10 – 50 ml up to 6 times daily over a period of up to 2 weeks. None of the patient will develop symptoms of CDI whilst being treated.

Example 25 Clinical use of a combination of orally delivered antibody and antibiotics

The oral administration of antibodies to treat CDI is performed in conjunction with standard antibiotic therapy as detailed in the example below.

A patient with recurrent *Clostridium difficile* infections, Mrs CL, develops diarrhoea while in a residential care home following a course of antibiotics prescribed to treat a urinary tract infection. Some days later she develops watery diarrhoea and is transferred to hospital where Toxin A and Toxin B are detected in a stool sample. The 84 year old patient is given a course of metronidazole and appears to make a full recovery. However a few days later her diarrhoea reoccurs and, again, CDI is diagnosed by the appropriate procedure. A course of vancomycin results in a transient cessation of her diarrhoea but, within days, her CDI recurrent for a third time. Finally, a complete cure is obtained by a combination of a tapering dose of vancomycin followed by a four week course of the orally administered ovine polyclonal antibodies (500mg bd).

Example 26 Clinical uses of a combination of systemically and orally delivered antibody formulations

Where patients suffer severe CDI, which can result in bowel obstruction, a combination of systemically and orally delivered antibody is employed. An example of such usage is given below.

A patient with a refractory *Clostridium difficile* infection, Mrs MN, a 72 years old pensioner, is admitted to hospital following a mild stroke. She is making an uneventful recovery when she develops a chest infection requiring antibiotics. Ten days later she experiences severe diarrhoea associated with mild abdominal pain. Both Toxin A and Toxin B are detected in a stool samples from which *C. difficile* is cultured and subsequently and shown to be ribotype 027. Immediately her *C. difficile* infection (CDI) is diagnosed, Mrs MN is given a course of metronidazole. However, although her symptoms improve, she continues to pass watery stools. A course of vancomycin also fails to resolve completely her CDI. *C. difficile* toxins are still present in her faeces so she receives three intravenous injections of ovine anti-*C. difficile* toxin antibodies (250mg on alternate days). In combination, she receives orally (via a

three week course) ovine antibodies (500mg bd) as an outpatient, and makes a full recovery.

SEQ ID NOs

SEQ ID NO: 1 ***Clostridium difficile*** **Toxin A – Toxinotype 0**

MSLISKEELIKLAYSIRPRENEYKTILTNLDEYNKLTTNNNENKYLQLKKLNESIDVFMNKYKTS
SRNRALSNLKKDILKEVILIKNSNTSPVEKNLHFVWIGGEVSDIALEYIKQWADINAEYNIKLWY
DSEAFLVNTLKKAIVESSTTEALQLLEEEIQNPQFDNMKFYKKRMEFIYDRQKRFINYYKSQIN
KPTVPTIDDIIKSHLVSEYNRDETVLESYRTNSLRKINSNHGIDIRANSLFTEQELLNIYSQELLN
RGNLAAASDIVRLLALKNFGGVYLDVDMLPGIHSDLFKTISRPSSIGLDRWEMIKLEAIMKYKK
YINNYTSENFDKLDQQLKDNFKLIIESKSEKSEIFSKLENLNVSDLEIKIAFALGSVINQALISKQ
GSYLTNLVIEQVKNRYQFLNQHLNPAIESDNNFTDTTKIFHDSLFNSATAENSMFLTKIAPYLQ
VGFMPEARSTISLSGPGAYASAYYDFINLQENTIEKTLKASDLIEFKFPENNLSQLTEQEINSL
WSFDQASAKYQFEKYVRDYTGGSLSEDNGVDFNKNTALDKNYLLNNKIPSNNVEEAGSKNY
VHYIIQLQGDDISYEATCNLFSKNPKNSIIIQRNMNESAKSYFLSDDGESILELNKYRIPERLKN
KEKVKVTFIGHGKDEFNTSEFARLSVDSLSNEISSFLDTIKLDISPKNVEVNLLGCNMFSYDFN
VEETYPGKLLLSIMDKITSTLPDVNKNSITIGANQYEVRINSEGRKELLAHSGKWINKEEAIMSD
LSSKEYIFFDSIDNKLKAKSKNIPGLASISEDIKTLLLDASVSPDTKFILNNLKLNIESSIGDYIYYE
KLEPVKNIIHNSIDDLIDEFNLLENVSDELYELKKLNNLDEKYLISFEDISKNNSTYSVRFINKSN
GESVYVETEKEIFSKYSEHITKEISTIKNSIITDVNGNLLDNIQLDHTSQVNTLNAAFFIQSLIDYS
SNKDVLNDLSTSVKVQLYAQLFSTGLNTIYDSIQLVNLISNAVNDTINVLPTITEGIPIVSTILDGI
NLGAAIKELLDEHDPLLKKELEAKVGVLAINMSLSIAATVASIVGIGAEVTIFLLPIAGISAGIPSL
VNNELILHDKATSVVNYFNHLSESKKYGPLKTEDDKILVPIDDLVISEIDFNNNSIKLGTCNILAM
EGGSGHTVTGNIDHFFSSPSISSHIPSLSIYSAIGIETENLDFSKKIMMLPNAPSRVFWWETGA
VPGLRSLENDGTRLLDSIRDLYPGKFYWRFYAFFDYAITTLKPVYEDTNIKIKLDKDTRNFIMP
TITTNEIRNKLSYSFDGAGGTYSLLLSSYPISTNINLSKDDLWIFNIDNEVREISIENGTIKKGKLI
KDVLSKIDINKNKLIIGNQTIDFSGDIDNKDRYIFLTCELDDKISLIIEINLVAKSYSLLLSGDKNYLI
SNLSNTIEKINTLGLDSKNIAYNYTDESNNKYFGAISKTSQKSIIHYKKDSKNILEFYNDSTLEFN
SKDFIAEDINVFMKDDINTITGKYYVDNNTDKSIDFSISLVSKNQVKVNGLYLNESVYSSYLDFV
KNSDGHHNTSNFMNLFLDNISFWKLFGFENINFVIDKYFTLVGKTNLGYVEFICDNNKNIDIYF
GEWKTSSSKSTIFSGNGRNVVVEPIYNPDTGEDISTSLDFSYEPLYGIDRYINKVLIAPDLYTSL
ININTNYYSNEYYPEIIVLNPNTFHKKVNINLDSSSFEYKWSTEGSDFILVRYLEESNKKILQKIR
IKGILSNTQSFNKMSIDFKDIKKLSLGYIMSNFKSFNSENELDRDHLGFKIIDNKTYYYDEDSKL
VKGLININNSLFYFDPIEFNLVTGWQTINGKKYYFDINTGAALTSYKIINGKHFYFNNDGVMQL
GVFKGPDGFEYFAPANTQNNNIEGQAIVYQSKFLTLNGKKYYFDNNSKAVTGWRIINNEKYY
FNPNNAIAAVGLQVIDNNKYYFNPDTAIISKGWQTVNGSRYYFDTDTAIAFNGYKTIDGKHFYF
DSDCVVKIGVFSTSNGFEYFAPANTYNNNIEGQAIVYQSKFLTLNGKKYYFDNNSKAVTGLQT
IDSKKYYFNTNTAEAATGWQTIDGKKYYFNTNTAEAATGWQTIDGKKYYFNTNTAIASTGYTII
NGKHFYFNTDGIMQIGVFKGPNGFEYFAPANTDANNIEGQAILYQNEFLTLNGKKYYFGSDS
KAVTGWRIINNKKYYFNPNNAIAAIHLCTINNDKYYFSYDGILQNGYITIERNNFYFDANNESK
MVTGVFKGPNGFEYFAPANTHNNNIEGQAIVYQNKFLTLNGKKYYFDNDSKAVTGWQTIDG
KKYYFNLNTAEAATGWQTIDGKKYYFNLNTAEAATGWQTIDGKKYYFNTNTFIASTGYTSING

KHFYFNTDGIMQIGVFKGPNGFEYFAPANTDANNIEGQAILYQNKFLTLNGKKYYFGSDSKAV
TGLRTIDGKKYYFNTNTAVAVTGWQTINGKKYYFNTNTSIASTGYTIISGKHFYFNTDGIMQIG
VFKGPDGFEYFAPANTDANNIEGQAIRYQNRFLYLHDNIYYFGNNSKAATGWVTIDGNRYYF
EPNTAMGANGYKTIDNKNFYFRNGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIRYQNRFL
HLLGKIYYFGNNSKAVTGWQTINGKVYYFMPDTAMAAAGGLFEIDGVIYFFGVDGVKAPGIY
G

SEQ ID NO: 2 Protein Sequence of *C. difficile* Toxin B – Toxinotype 0

MSLVNRKQLEKMANVRFRTQEDEYVAILDALEEYHNMSENTVVEKYLKLKDINSLTDIYIDTY
KKSGRNKALKKFKEYLVTEVLELKNNNLTPVEKNLHFVWIGGQINDTAINYINQWKDVNSDYN
VNVFYDSNAFLINTLKKTVVESAINDTLESFRENLNDPRFDYNKFFRKRMEIIYDKQKNFINYY
KAQREENPELIIDDIVKTYLSNEYSKEIDELNTYIEESLNKITQNSGNDVRNFEEFKNGESFNLY
EQELVERWNLAAASDILRISALKEIGGMYLDVDMLPGIQPDLFESIEKPSSVTVDFWEMTKLE
AIMKYKEYIPEYTSEHFDMLDEEVQSSFESVLASKSDKSEIFSSLGDMEASPLEVKIAFNSKGII
NQGLISVKDSYCSNLIVKQIENRYKILNNSLNPAISEDNDFNTTTNTFIDSIMAEANADNGRFM
MELGKYLRVGFFPDVKTTINLSGPEAYAAAYQDLLMFKEGSMNIHLIEADLRNFEISKTNISQS
TEQEMASLWSFDDARAKAQFEEYKRNYFEGSLGEDDNLDFSQNIVVDKEYLLEKISSLARSS
ERGYIHYIVQLQGDKISYEAACNLFAKTPYDSVLFQKNIEDSEIAYYYNPGDGEIQEIDKYKIPSI
ISDRPKIKLTFIGHGKDEFNTDIFAGFDVDSLSTEIEAAIDLAKEDISPKSIEINLLGCNMFSYSIN
VEETYPGKLLLKVKDKISELMPSISQDSIIVSANQYEVRINSEGRRELLDHSGEWINKEESIIKDI
SSKEYISFNPKENKITVKSKNLPELSTLLQEIRNNSNSSDIELEEKVMLTECEINVISNIDTQIVE
ERIEEAKNLTSDSINYIKDEFKLIESISDALCDLKQQNELEDSHFISFEDISETDEGFSIRFINKET
GESIFVETEKTIFSEYANHITEEISKIKGTIFDTVNGKLVKKVNLDTTHEVNTLNAAFFIQSLIEYN
SSKESLSNLSVAMKVQVYAQLFSTGLNTITDAAKVVELVSTALDETIDLLPTLSEGLPIIATIIDG
VSLGAAIKELSETSDPLLRQEIEAKIGIMAVNLTTATTAIITSSLGIASGFSILLVPLAGISAGIPSL
VNNELVLRDKATKVVDYFKHVSLVETEGVFTLLDDKIMMPQDDLVISEIDFNNNSIVLGKCEIW
RMEGGSGHTVTDDIDHFFSAPSITYREPHLSIYDVLEVQKEELDLSKDLMVLPNAPNRVFAW
ETGWTPGLRSLENDGTKLLDRIRDNYEGEFYWRYFAFIADALITTLKPRYEDTNIRINLDSNTR
SFIVPIITTEYIREKLSYSFYGSGGTYALSLSQYNMGINIELSESDVWIIDVDNVVRDVTIESDKI
KKGDLIEGILSTLSIEENKIILNSHEINFSGEVNGSNGFVSLTFSILEGINAIIEVDLLSKSYKLLIS
GELKILMLNSNHIQQKIDYIGFNSELQKNIPYSFVDSEGKENGFINGSTKEGLFVSELPDVVLIS
KVYMDDSKPSFGYYSNNLKDVKVITKDNVNILTGYYLKDDIKISLSLTLQDEKTIKLNSVHLDES
GVAEILKFMNRKGNTNTSDSLMSFLESMNIKSIFVNFLQSNIKFILDANFIISGTTSIGQFEFICD
ENDNIQPYFIKFNTLETNYTLYVGNRQNMIVEPNYDLDDSGDISSTVINFSQKYLYGIDSCVNK
VVISPNIYTDEINITPVYETNNTYPEVIVLDANYINEKINVNINDLSIRYVWSNDGNDFILMSTSE
ENKVSQVKIRFVNVFKDKTLANKLSFNFSDKQDVPVSEIILSFTPSYYEDGLIGYDLGLVSLYN
EKFYINNFGMMVSGLIYINDSLYYFKPPVNNLITGFVTVGDDKYYFNPINGGAASIGETIIDDKN
YYFNQSGVLQTGVFSTEDGFKYFAPANTLDENLEGEAIDFTGKLIIDENIYYFDDNYRGAVEW
KELDGEMHYFSPETGKAFKGLNQIGDYKYYFNSDGVMQKGFVSINDNKHYFDDSGVMKVG
YTEIDGKHFYFAENGEMQIGVFNTEDGFKYFAHHNEDLGNEEGEEISYSGILNFNNKIYYFDD
SFTAVVGWKDLEDGSKYYFDEDTAEAYIGLSLINDGQYYFNDDGIMQVGFVTINDKVFYFSD
SGIIESGVQNIDDNYFYIDDNGIVQIGVFDTSDGYKYFAPANTVNDNIYGQAVEYSGLVRVGE

DVYYFGETYTIETGWIYDMENESDKYYFNPETKKACKGINLIDDIKYYFDEKGIMRTGLISFEN
NNYYFNENGEMQFGYINIEDKMFYFGEDGVMQIGVFNTPDGFKYFAHQNTLDENFEGESINY
TGWLDLDEKRYYFTDEYIAATGSVIIDGEEYYFDPDTAQLVISE

SEQ ID NO:3 Protein Sequence of *C. difficile* Toxin A – Toxinotype III

MSLISKEELIKLAYSIRPRENEYKTILTNLDEYNKLTTNNNENKYLQLKKLNESIDVFMNKYKNS
SRNRALSNLKKDILKEVILIKNSNTSPVEKNLHFVWIGGEVSDIALEYIKQWADINAEYNIKLWY
DSEAFLVNTLKKAIVESSTTEALQLLEEEIQNPQFDNMKFYKKRMEFIYDRQKRFINYYKSQIN
KPTVPTIDDIIKSHLVSEYNRDETLLESYRTNSLRKINSNHGIDIRANSLFTEQELLNIYSQELLN
RGNLAAASDIVRLLALKNFGGVYLDVDMLPGIHSDLFKTIPRPSSIGLDRWEMIKLEAIMKYKK
YINNYTSENFDKLDQQLKDNFKLIIESKSEKSEIFSKLENLNVSDLEIKIAFALGSVINQALISKQ
GSYLTNLVIEQVKNRYQFLNQHLNPAIESDNNFTDTTKIFHDSLFNSATAENSMFLTKIAPYLQ
VGFMPEARSTISLSGPGAYASAYYDFINLQENTIEKTLKASDLIEFKFPENNLSQLTEQEINSL
WSFDQASAKYQFEKYVRDYTGGSLSEDNGVDFNKNTALDKNYLLNNKIPSNNVEEAGSKNY
VHYIIQLQGDDISYEATCNLFSKNPKNSIIIQRNMNESAKSYFLSDDGESILELNKYRIPERLKN
KEKVKVTFIGHGKDEFNTSEFARLSVDSLSNEISSFLDTIKLDISPKNVEVNLLGCNMFSYDFN
VEETYPGKLLLSIMDKITSTLPDVNKDSITIGANQYEVRINSEGRKELLAHSGKWINKEEAIMSD
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KLEPVKNIIHNSIDDLIDEFNLLENVSDELYELKKLNNLDEKYLISFEDISKNNSTYSVRFINKSN
GESVYVETEKEIFSKYSEHITKEISTIKNSIITDVNGNLLDNIQLDHTSQVNTLNAAFFIQSLIDYS
SNKDVLNDLSTSVKVQLYAQLFSTGLNTIYDSIQLVNLISNAVNDTINVLPTITEGIPIVSTILDGI
NLGAAIKELLDEHDPLLKKELEAKVGVLAINMSLSIAATVASIVGIGAEVTIFLLPIAGISAGIPSL
VNNELILHDKATSVVNYFNHLSESKEYGPLKTEDDKILVPIDDLVISEIDFNNNSIKLGTCNILAM
EGGSGHTVTGNIDHFFSSPYISSHIPSLSVYSAIGIKTENLDFSKKIMMLPNAPSRVFWWETG
AVPGLRSLENNGTKLLDSIRDLYPGKFYWRFYAFFDYAITTLKPVYEDTNTKIKLDKDTRNFIM
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LIEDVLSKIDINKNKLIIGNQTIDFSGDIDNKDRYIFLTCELDDKISLIIEINLVAKSYSLLLSGDKNY
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TIINGKHFYFNTDGIMQIGVFKGPDGFEYFAPANTHNNNIEGQAILYQNKFLTLNGKKYYFGSD
SKAVTGLRTIDGKKYYFNTNTAVAVTGWQTINGKKYYFNTNTYIASTGYTIISGKHFYFNTDGI
MQIGVFKGPDGFEYFAPANTDANNIEGQAIRYQNRFLYLHDNIYYFGNDSKAATGWATIDGN
RYYFEPNTAMGANGYKTIDNKNFYFRNGLPQIGVFKGPNGFEYFAPANTDANNIDGQAIRYQ
NRFLHLLGKIYYFGNNSKAVTGWQTINSKVYYFMPDTAMAAAGGLFEIDGVIYFFGVDGVKA
PGIYG

SEQ ID NO: 4 Protein Sequence of *C. difficile* Toxin B – Toxinotype III

MSLVNRKQLEKMANVRFRVQEDEYVAILDALEEYHNMSENTVVEKYLKLKDINSLTDIYIDTY
KKSGRNKALKKFKEYLVTEVLELKNNNLTPVEKNLHFVWIGGQINDTAINYINQWKDVNSDYN
VNVFYDSNAFLINTLKKTIVESATNDTLESFRENLNDPRFDYNKFYRKRMEIIYDKQKNFINYY
KTQREENPDLIIDDIVKIYLSNEYSKDIDELNSYIEESLNKVTENSGNDVRNFEEFKGGESFKLY
EQELVERWNLAAASDILRISALKEVGGVYLDVDMLPGIQPDLFESIEKPSSVTVDFWEMVKLE
AIMKYKEYIPGYTSEHFDMLDEEVQSSFESVLASKSDKSEIFSSLGDMEASPLEVKIAFNSKGI
INQGLISVKDSYCSNLIVKQIENRYKILNNSLNPAISEDNDFNTTTNAFIDSIMAEANADNGRFM
MELGKYLRVGFFPDVKTTINLSGPEAYAAAYQDLLMFKEGSMNIHLIEADLRNFEISKTNISQS
TEQEMASLWSFDDARAKAQFEEYKKNYFEGSLGEDDNLDFSQNTVVDKEYLLEKISSLARS
SERGYIHYIVQLQGDKISYEAACNLFAKTPYDSVLFQKNIEDSEIAYYYNPGDGEIQEIDKYKIP
SIISDRPKIKLTFIGHGKDEFNTDIFAGLDVDSLSTEIETAIDLAKEDISPKSIEINLLGCNMFSYS
VNVEETYPGKLLLRVKDKVSELMPSISQDSIIVSANQYEVRINSEGRRELLDHSGEWINKEESI
IKDISSKEYISFNPKENKIIVKSKNLPELSTLLQEIRNNSNSSDIELEEKVMLAECEINVISNIDTQ
YVEGRIEEAKSLTSDSINYIKNEFKLIESISDALYDLKQQNELEESHFISFEDILETDEGFSIRFID
KETGESIFVETEKAIFSEYANHITEEISKIKGTIFDTVNGKLVKKVNLDATHEVNTLNAAFFIQSLI
EYNSSKESLSNLSVAMKVQVYAQLFSTGLNTITDAAKVVELVSTALDETIDLLPTLSEGLPVIA
TIIDGVSLGAAIKELSETSDPLLRQEIEAKIGIMAVNLTAATTAIITSSLGIASGFSILLVPLAGISA
GIPSLVNNELILRDKATKVVDYFSHISLAESEGAFTSLDDKIMMPQDDLVISEIDFNNNSITLGK
CEIWRMEGGSGHTVTDDIDHFFSAPSITYREPHLSIYDVLEVQKEELDLSKDLMVLPNAPNRV
FAWETGWTPGLRSLENDGTKLLDRIRDNYEGEFYWRYFAFIADALITTLKPRYEDTNIRINLD
SNTRSFIVPVITTEYIREKLSYSFYGSGGTYALSLSQYNMNINIELNENDTWVIDVDNVVRDVTI
ESDKIKKGDLIENILSKLSIEDNKIILDNHEINFSGTLNGGNGFVSLTFSILEGINAVIEVDLLSKS
YKVLISGELKTLMANSNSVQQKIDYIGLNSELQKNIPYSFMDDKGKENGFINCSTKEGLFVSEL
SDVVLISKVYMDNSKPLFGYCSNDLKDVKVITKDDVIILTGYYLKDDIKISLSFTIQDENTIKLNG
VYLDENGVAEILKFMNKKGSTNTSDSLMSFLESMNIKSIFINSLQSNTKLILDTNFIISGTTSIGQ
FEFICDKDNNIQPYFIKFNTLETKYTLYVGNRQNMIVEPNYDLDDSGDISSTVINFSQKYLYGID
SCVNKVIISPNIYTDEINITPIYEANNTYPEVIVLDTNYISEKININDLSIRYVWSNDGSDFILMS
TDEENKVSQVKIRFTNVFKGNTISDKISFNFSDKQDVSINKVISTFTPSYYVEGLLNYDLGLISL
YNEKFYINNFGMMVSGLVYINDSLYYFKPPIKNLITGFTTIGDDKYYFNPDNGGAASVGETIID
GKNYYFSQNGVLQTGVFSTEDGFKYFAPADTLDENLEGEAIDFTGKLTIDENVYYFGDNYRA
AIEWQTLDDEVYYFSTDTGRAFKGLNQIGDDKFYFNSDGIMQKGFVNINDKTFYFDDSGVMK
SGYTEIDGKYFYFAENGEMQIGVFNTADGFKYFAHHDEDLGNEEGEALSYSGILNFNNKIYYF
DDSFTAVVGWKDLEDGSKYYFDEDTAEAYIGISIINDGKYYFNDSGIMQIGFVTINNEVFYFSD
SGIVESGMQNIDDNYFYIDENGLVQIGVFDTSDGYKYFAPANTVNDNIYGQAVEYSGLVRVG

EDVYYFGETYTIETGWIYDMENESDKYYFDPETKKAYKGINVIDDIKYYFDENGIMRTGLITFE
DNHYYFNEDGIMQYGYLNIEDKTFYFSEDGIMQIGVFNTPDGFKYFAHQNTLDENFEGESINY
TGWLDLDEKRYYFTDEYIAATGSVIIDGEEYYFDPDTAQLVISE

SEQ ID NO: 5 Protein Sequence of *C. difficile* Binary toxin fragment A

MKKFRKHKRISNCISILLILYLTLGGLLPNNIYAQDLQSYSEKVCNTTYKAPIESFLKDKEKAKE
WERKEAERIEQKLERSEKEALESYKKDSVEISKYSQTRNYFYDYQIEANSREKEYKELRNAIS
KNKIDKPMYVYYFESPEKFAFNKVIRTENQNEISLEKFNEFKETIQNKLFKQDGFKDISLYEPG
KGDEKPTPLLMHLKLPRNTGMLPYTNTNNVSTLIEQGYSIKIDKIVRIVIDGKHYIKAEASVVNS
LDFKDDVSKGDSWGKANYNDWSNKLTPNELADVNDYMRGGYTAINNYLISNGPVNNPNPEL
DSKITNIENALKREPIPTNLTVYRRSGPQEFGLTLTSPEYDFNKLENIDAFKSKWEGQALSYPN
FISTSIGSVNMSAFAKRKIVLRITIPKGSPGAYLSAIPGYAGEYEVLLNHGSKFKINKIDSYKDGT
ITKLIVDATLIP

SEQ ID NO: 6 Protein Sequence of *C. difficile* Binary toxin fragment B

MKIQMRNKKVLSFLTLTAIVSQALVYPVYAQTSTSNHSNKKKEIVNEDILPNNGLMGYYFSDE
HFKDLKLMAPIKDGNLKFEEKKVDKLLDKDKSDVKSIRWTGRIIPSKDGEYTLSTDRDDVLMQ
VNTESTISNTLKVNMKKGKEYKVRIELQDKNLGSIDNLSSPNLYWELDGMKKIIPEENLFLRDY
SNIEKDDPFIPNNNFFDPKLMSDWEDEDLDTDNDNIPDSYERNGYTIKDLIAVKWEDSFAEQ
GYKKYVSNYLESNTAGDPYTDYEKASGSFDKAIKTEARDPLVAAYPIVGVGMEKLIISTNEHA
STDQGKTVSRATTNSKTESNTAGVSVNVGYQNGFTANVTTNYSHTTDNSTAVQDSNGESW
NTGLSINKGESAYINANVRYYNTGTAPMYKVTPTTNLVLDGDTLSTIKAQENQIGNNLSPGDT
YPKKGLSPLALNTMDQFSSRLIPINYDQLKKLDAGKQIKLETTQVSGNFGTKNSSGQIVTEGN
SWSDYISQIDSISASIILDTENESYERRVTAKNLQDPEDKTPELTIGEAIEKAFGATKKDGLLYF
NDIPIDESCVELIFDDNTANKIKDSLKTLSDKKIYNVKLERGMNILIKTPTYFTNFDDYNNYPST
WSNVNTTNQDGLQGSANKLNGETKIKIPMSELKPYKRYVFSGYSKDPLTSNSIIVKIKAKEEK
TDYLVPEQGYTKFSYEFETTEKDSSNIEITLIGSGTTYLDNLSITELNSTPEILDEPEVKIPTDQE
IMDAHKIYFADLNFNPSTGNTYINGMYFAPTQTNKEALDYIQKYRVEATLQYSGFKDIGTKDK
EMRNYLGDPNQPKTNYVNLRSYFTGGENIMTYKKLRIYAITPDDRELLVLSVD

SEQ ID NO: 7 HumanTrypsin-1 (Swiss Prot accession P07477)

IVGGYNCEENSVPYQVSLNSGYHFCGGSLINEQWVVSAG**H**CYKSRIQVRLGEHNIEVLEGN
EQFINAAKIIRHPQYDRKTLNN**D**IMLIKLSSRAVINARVSTISLPTAPPATGTKCLISGWGNTAS
SGADYPDELQCLDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSCQGD**S**GGPVVCNGQ
LQGVVSWGDGCAQKNKPGVYTKVYNYVKWIKNTIAANS

SEQ ID NO: 8 Human Trypsin-2 (Swiss Prot accession P07478)

IVGGYICEENSVPYQVSLNSGYHFCGGSLISEQWVVSAG**H**CYKSRIQVRLGEHNIEVLEGNE
QFINAAKIIRHPKYNSRTLDN**D**ILLIKLSSPAVINSRVSAISLPTAPPAAGTESLISGWGNTLSSG
ADYPDELQCLDAPVLSQAECEASYPGKITNNMFCVGFLEGGKDSCQGD**S**GGPVVSNGELQ
GIVSWGYGCAQKNRPGVYTKVYNYVDWIKDTIAANS

SEQ ID NO: 9 Chymotrypsin-B (Swiss Prot accession P17538)

GCGVPAIHPVLSGLSRIVNGEDAVPGSWPWQVSLQDKTGFHFCGGSLISEDWVVTAA**H**CGV
RTSDVVVAGEFDQGSDEENIQVLKIAKVFKNPKFSILTVNN**D**ITLLKLATPARFSQTVSAVCLP
SADDDFPAGTLCATTGWGKTKYNANKTPDKLQQAALPLLSNAECKKSWGRRITDVMICAGA
SGVSSCMGD**S**GGPLVCQKDGAWTLVGIVSWGSDTCSTSSPGVYARVTKLIPWVQKILAAN

CLAIMS

1. An antibody composition comprising ovine antibodies, for use in the prevention or treatment of *C. difficile* infection wherein the antibodies bind to a *C. difficile* toxin, and wherein said prevention or treatment is by oral delivery of the antibody composition.

2. The antibody composition for use according to Claim 1, wherein the antibodies are polyclonal antibodies.

3. The antibody composition for use according to any preceding claim, wherein the antibodies bind to a *C. difficile* toxin selected from the group consisting of: *C. difficile* Toxin A, *C. difficile* Toxin B, and *C. difficile* Binary Toxin.

4. The antibody composition for use according to any Claim 3, wherein the antibodies bind to *C. difficile* Toxin A and to *C. difficile* Toxin B.

5. The antibody composition for use according to any Claim 3, wherein the antibodies bind to *C. difficile* Toxin A and to *C. difficile* Binary Toxin.

6. The antibody composition for use according to any Claim 3, wherein the antibodies bind to *C. difficile* Toxin B and to *C. difficile* Binary Toxin.

7. The antibody composition for use according to any of Claims 1-5, wherein the *C. difficile* toxin is a Toxin A selected from the following group: Toxinotype 0, Toxinotype III and Toxinotype V.

8. The antibody composition for use according to any of Claims 1-4 or Claim 6, wherein the *C. difficile* toxin is a Toxin B selected from the following group: Toxinotype 0, Toxinotype III, Toxinotype V, and Toxinotype VIII (eg. from *Clostridium difficile* ribotype 017).

9. A pharmaceutical composition for oral delivery, comprising the antibody composition as defined in any of Claims 1 to 8, together with one or more means for protecting said antibody composition from trypsin and/ or chymotrypsin and/ or stomach acid.

10. A pharmaceutical composition for oral delivery according to Claim 9, wherein the one or more means for protecting said antibody composition from trypsin and/ or chymotrypsin and/ or stomach acid is selected from:

(a) a polypeptide which binds specifically to and suppresses or inactivates the proteolytic activity of trypsin and/ or chymotrypsin; and/ or

(b) an antibody that binds to trypsin and/ or chymotrypsin and suppresses or inactivates the protease activity of said trypsin and/ or chymotrypsin; and/ or

(c) a delivery vehicle selected from a liposome, a microsome, a nanosome, a pellet, a granular matrix, a bead, a microsphere, a nanoparticle formulation, or an aqueous solution; and/ or

(d) an antacid molecule; and/ or

(e) a PEGylation moiety covalent attached to one of more of the antibodies.

11. A pharmaceutical composition for oral delivery according to Claim 9 or Claim 10, wherein the composition includes an antacid molecule and:

(a) a polypeptide which binds specifically to and suppresses or inactivates the proteolytic activity of trypsin and/ or chymotrypsin; or

(b) an antibody that binds to trypsin and/ or chymotrypsin and inactivates the protease activity of said trypsin and/ or chymotrypsin.

12. A method for prevention or treatment of *C. difficile* infection, said method comprising oral administration of the antibody composition according to any of Claims 1-8 or a pharmaceutical composition according to any of Claims 9-11.

13. A pharmaceutical composition according to any of Claims 9-11, for use in the prevention or treatment of *C. difficile* infection.

14. A method of producing ovine antibodies for use in the oral antibody composition according to any of Claims 1-8, or for use in a pharmaceutical composition according to any of Claims 9-11, wherein the ovine antibodies are elicited by a sheep in response to an immunogen comprising a *C. difficile* toxin or a fragment thereof.

15 A method of producing ovine antibodies for use in the method of Claim 12, said method comprising (i) administering an immunogen comprising a *C. difficile* toxin or a fragment thereof to a sheep, (ii) allowing sufficient time for the

generation of antibodies in the sheep, and (iii) obtaining the antibodies from the sheep.

16. The method according to Claim 14 or Claim 15, wherein the immunogen is a *C. difficile* toxoid.

17. A method according to Claim 12 or a pharmaceutical composition for use according to Claim 13, wherein said one or more trypsin and/ or chymotrypsin inhibitor and/ or antacid molecule is administered prior to, simultaneously with, or subsequent to administration of the antibody composition.

18. An antibody composition for use according to any of Claims 1-8 or a pharmaceutical composition for use according to Claim 13, wherein said oral administration is performed prior to, simultaneously with, or subsequent to non-oral administration of a second ovine antibody composition wherein said second ovine antibody composition comprises ovine antibodies as defined in any of Claims 1-8;
preferably wherein said non-oral (eg. systemic) administration is performed prior to said oral administration

20. A method according to Claim 12 wherein said oral administration is performed prior to, simultaneously with, or subsequent to non-oral administration of a second ovine antibody composition wherein said second ovine antibody composition comprises ovine antibodies as defined in any of Claims 1-8;
preferably wherein said non-oral (eg. systemic) administration is performed prior to said oral administration.

21. An antibody composition for use according to any of Claims 1-8 or 18 or a pharmaceutical composition for use according to Claim 13 or Claim 18, or a method according to Claim 12 or Claim 20, wherein the subject to be treated or protected is a subject in one or more or the following categories: hospitalised; over 65 or 70 years' old; receiving broad-range spectrum antibiotics; having previous CDI history/ infection; having close proximity to symptomatic CDI patients; having mild to moderate disease severity; presenting as asymptomatic but considered at high risk of relapse (eg. because of one or more relapse episodes); having close proximity to CDI outbreak areas or patients.

Figure 1

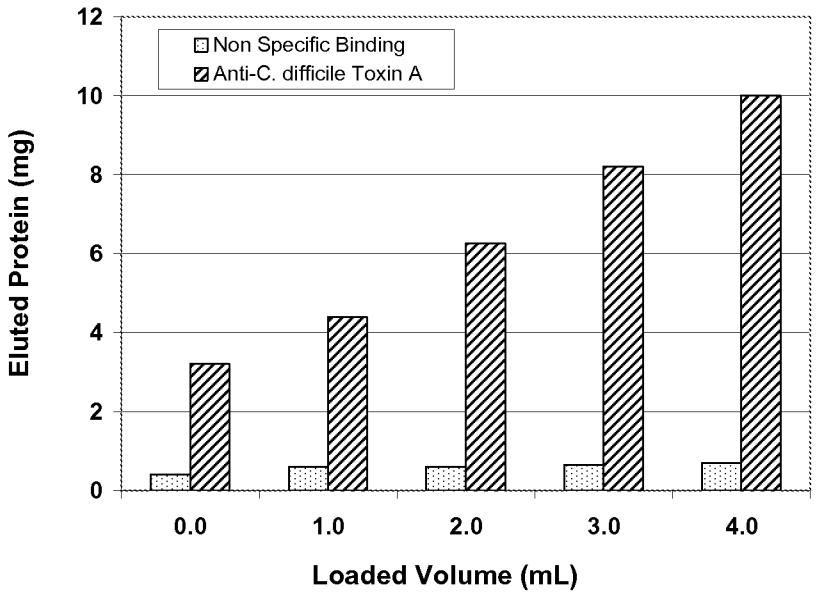


Figure 2

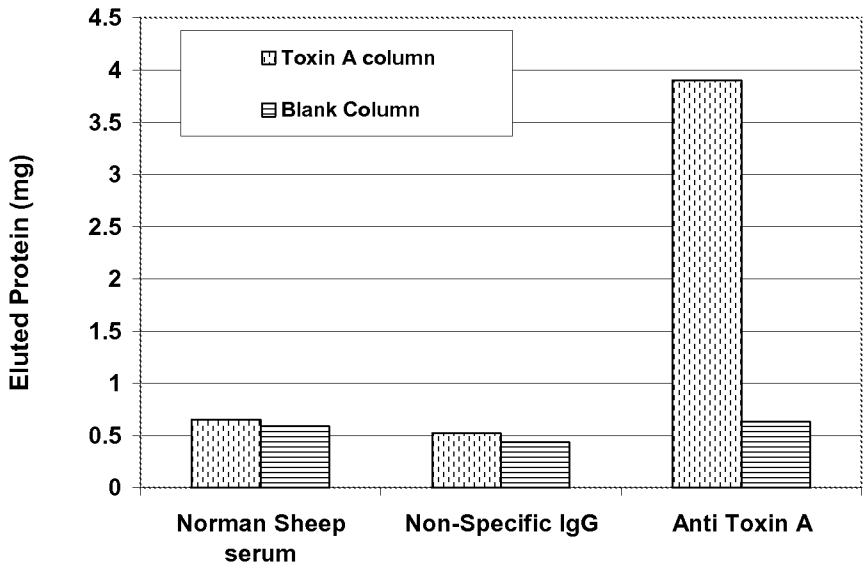


Figure 3

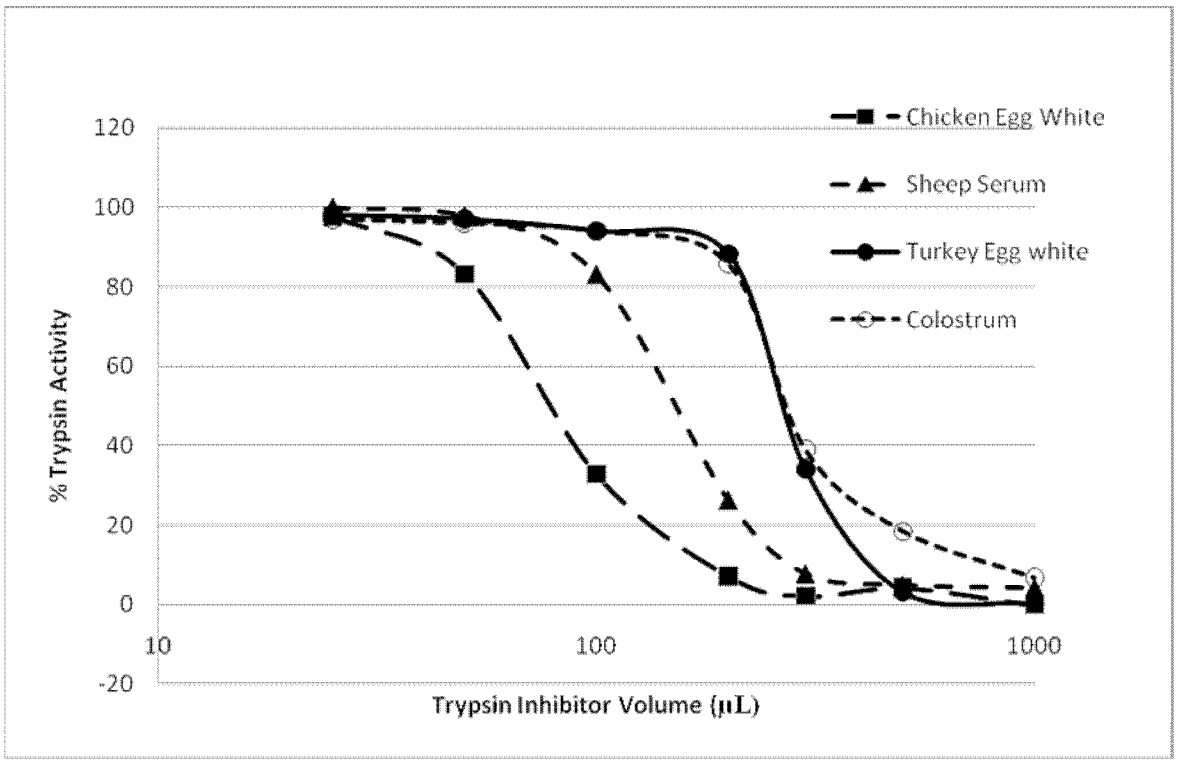


Figure 4

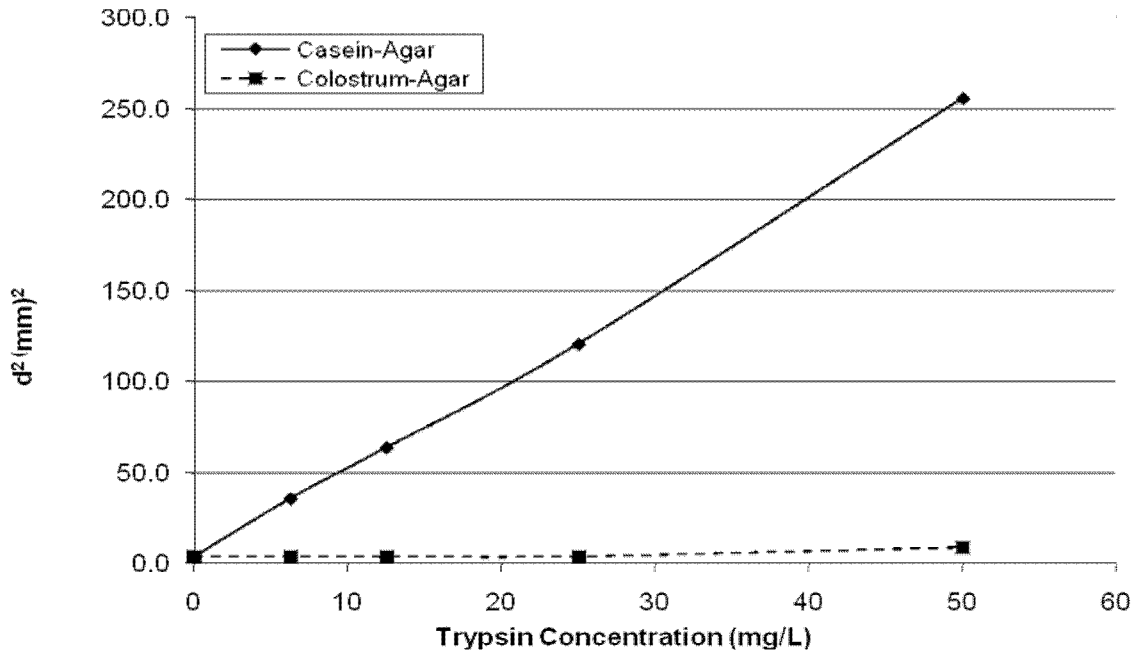


Figure 5

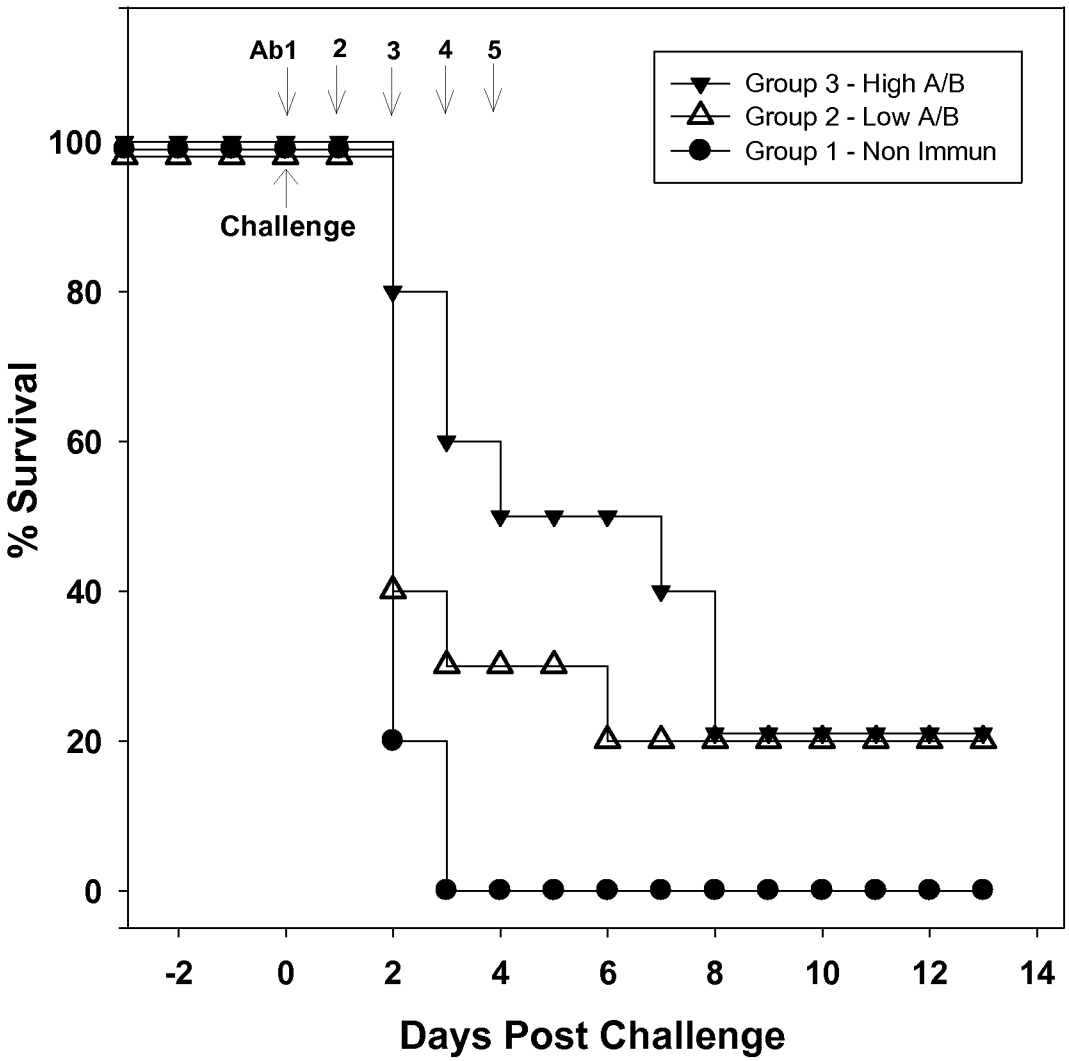

Ab1
2
3
4
5
Challenge
Group 3 - High A/B
Group 2 - Low A/B
Group 1 - Non Immun
% Survival
100
80
60
40
20
0
-2
0
2
4
6
8
10
12
14
Days Post Challenge

Figure 6

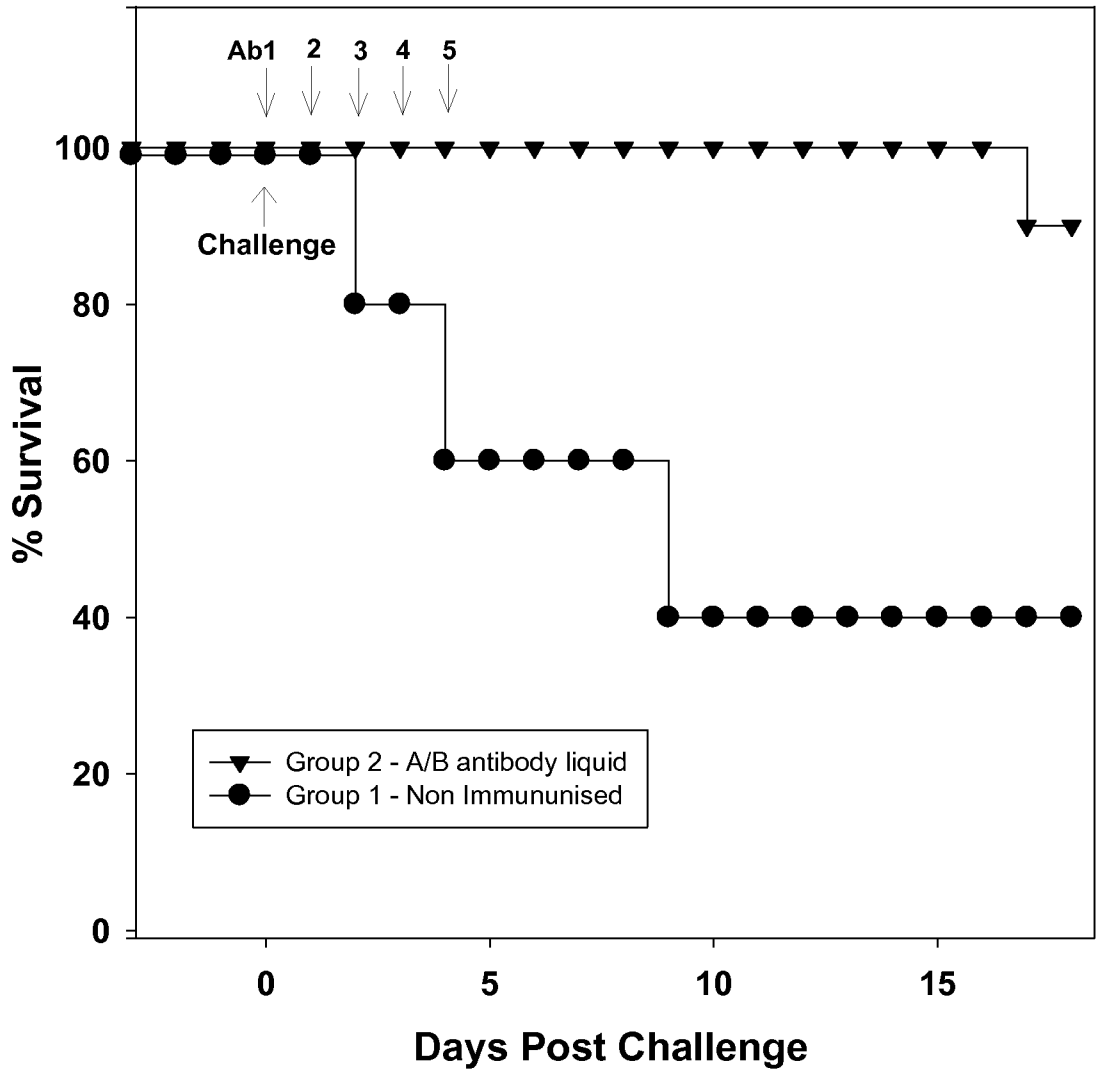
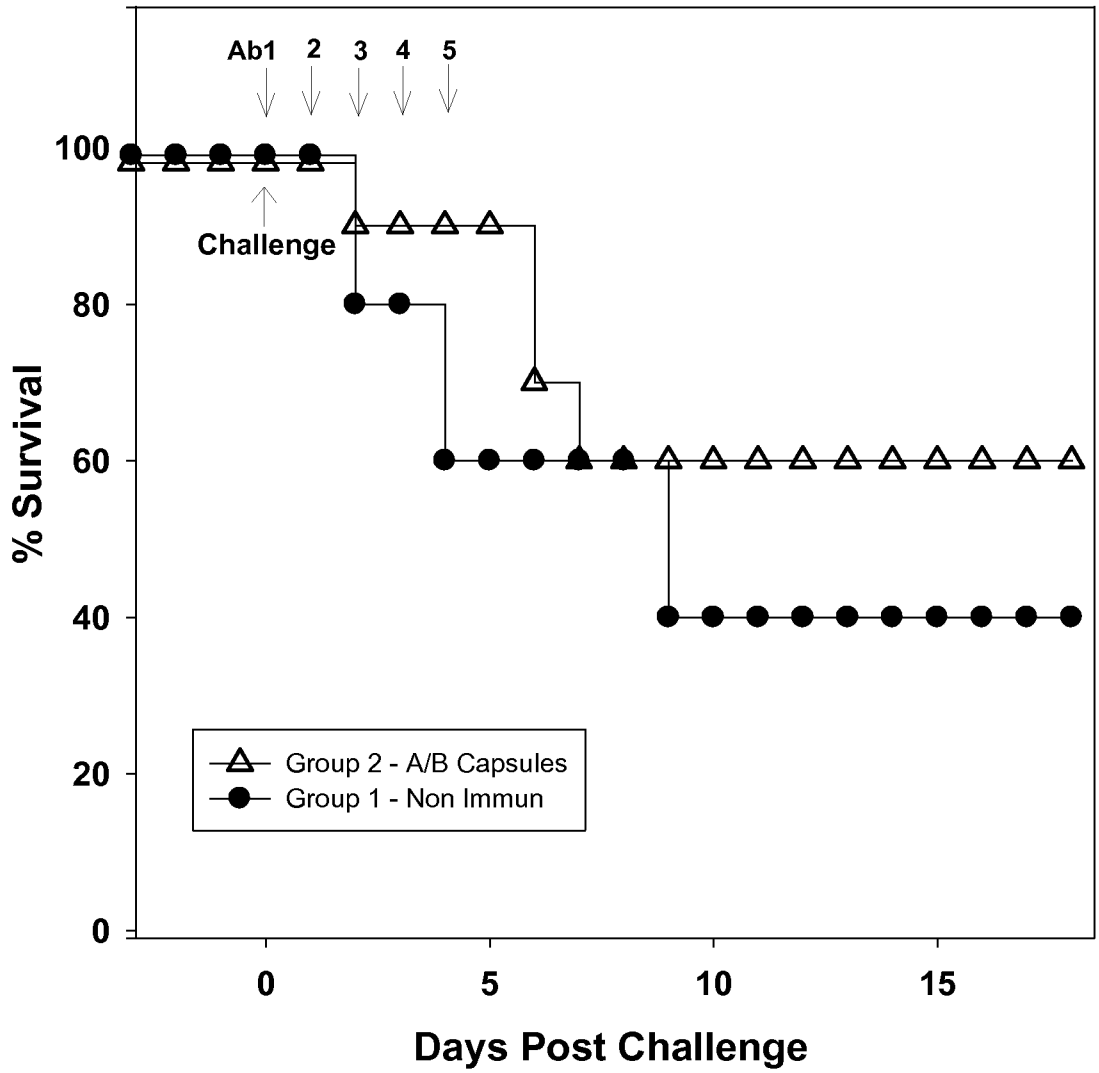

Ab1
2
3
4
5
Challenge
100
80
60
40
20
0
% Survival
0
5
10
15
Days Post Challenge
Group 2 - A/B antibody liquid
Group 1 - Non Immununised

Figure 7


Ab1
2
3
4
5
Challenge
100
80
60
40
20
0
% Survival
0
5
10
15
Days Post Challenge
Group 2 - A/B Capsules
Group 1 - Non Immun

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/052035

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/40 C07K16/12
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 601 823 A (WILLIAMS JAMES A [US] ET AL) 11 February 1997 (1997-02-11) the whole document	1-20
Y	KINK J A ET AL: "ANTIBODIES TO RECOMBINANT CLOSTRIDIUM DIFFICILE TOXINS A AND B ARE EFFECTIVE TREATMENT AND PREVENT RELAPSE OF C. DIFFICILE-ASSOCIATED DISEASE IN A HAMSTER MODEL OF INFECTION", INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 66, no. 5, 1 May 1998 (1998-05-01), pages 2018-2025, XP002912254, ISSN: 0019-9567 the whole document	1-20

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2011	18/03/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kalsner, Inge

3

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/052035

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	REDWAN E R M ET AL: "Production and purification of ovine anti-tetanus antibody", COMPARATIVE IMMUNOLOGY, MICROBIOLOGY AND INFECTIOUS DISEASES, PERGAMON PRESS, OXFORD, GB, vol. 28, no. 3, 1 May 2005 (2005-05-01), pages 167-176, XP004862864, ISSN: 0147-9571, DOI: DOI:10.1016/J.CIMID.2005.01.001 the whole document ----	1-20
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