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(71) Demandeurs/Applicants:
HEALTH PROTECTION AGENCY, GB;
MICROPHARM LIMITED, GB

(72) Inventeurs/Inventors:
SHONE, CLIFFORD, GB;
LANDON, JOHN, GB

(74) Agent: SIM & MCBURNEY

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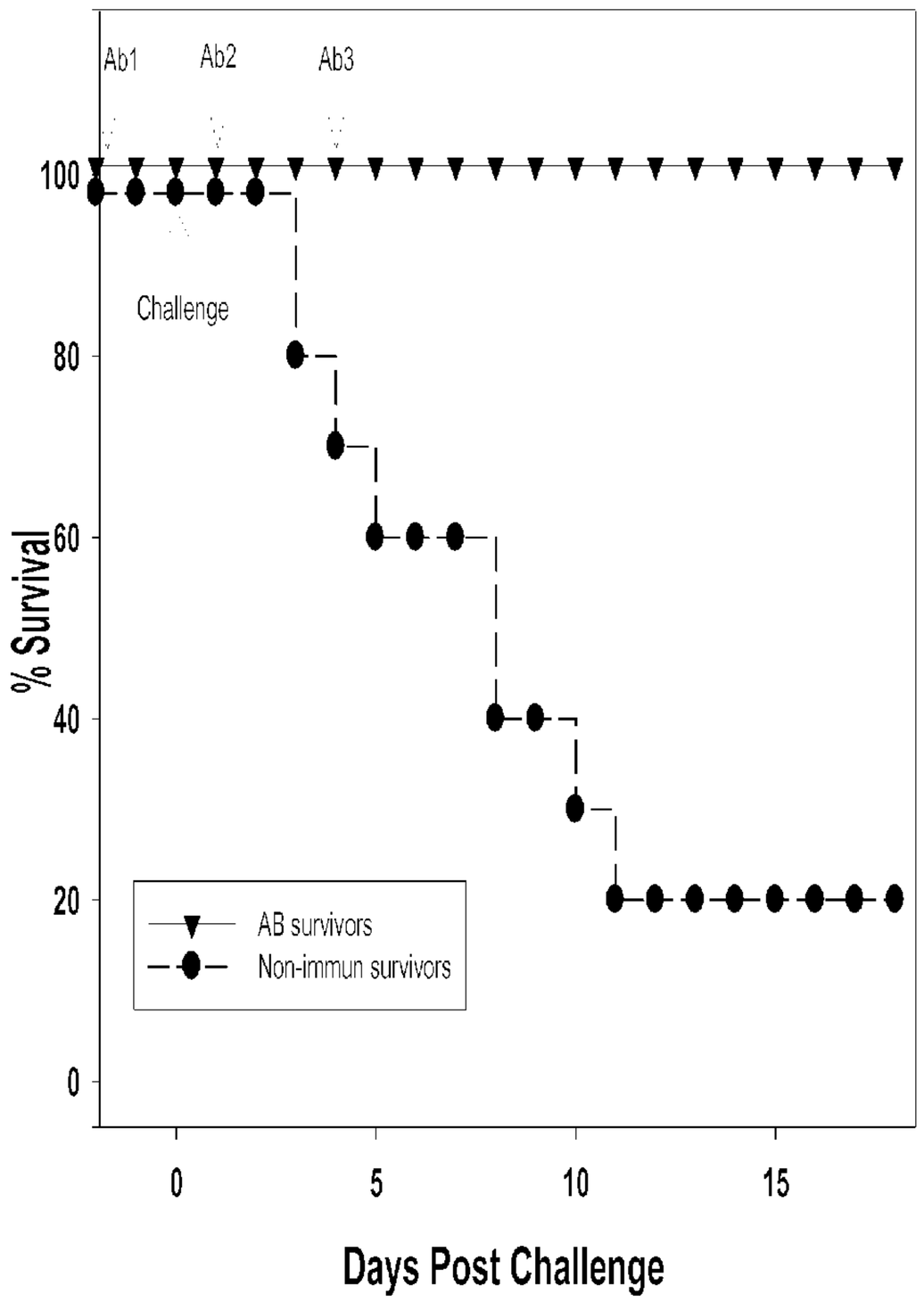
Figure 6



(57) Abrégé/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.

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(71) Applicants *(for all designated States except US)*: **HEALTH PROTECTION AGENCY** [GB/GB]; Porton Down, Salisbury, Wiltshire SP4 0JG (GB). **MICROPHARM LIMITED** [GB/GB]; Station Road Industrial Estate, Newcastle Emlyn, Carmarthenshire SA38 9BX (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHONE, Clifford** [GB/GB]; Health Protection Agency, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). **LANDON, John** [GB/GB]; MicroPharm Limited, Station Road Industrial Estate, Newcastle Emlyn, Carmarthenshire SA38 9BX (GB).

(74) Agents: **MACLEAN, Martin** et al.; Mathys & Squire LLP, 120 Holborn, London Greater London EC1N 2SQ (GB).

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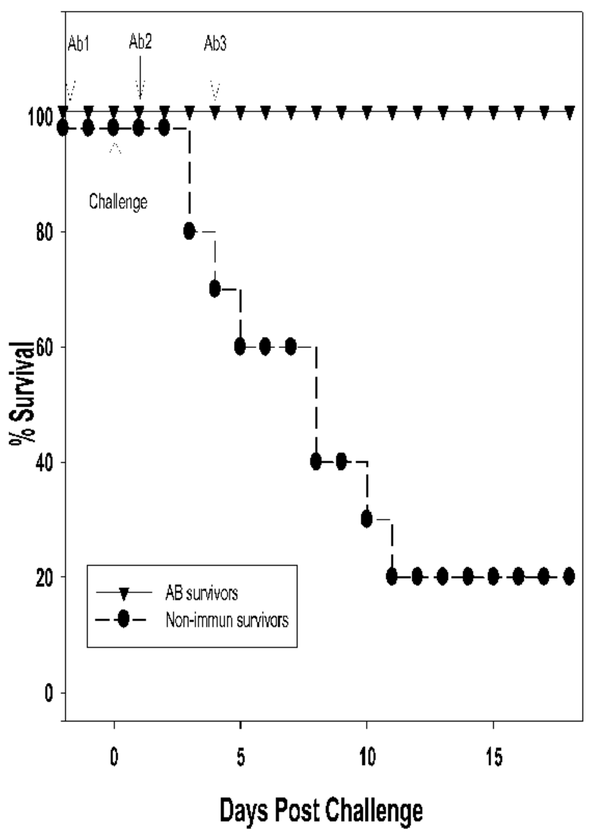
Figure 6



(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.

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Antibodies to *Clostridium difficile* Toxins

The present invention relates to ovine antibodies and their use in the prevention or treatment 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 difference 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. Although such sequence differences are relatively small, they can be extremely important with respect to the antigenic properties of the molecule and can have a profound impact on antibody binding and hence the toxin-neutralisation properties by antibodies.

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 in the gut colonisation process. Outbreaks of CDI have been reported with Toxin A-negative/Toxin B-positive strains which suggests 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) onto 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 works in a different way, modifying 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 the treatment 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 belong 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 use in the prevention or treatment of CDI. In another aspect, the invention provides an antibody composition comprising the ovine

antibodies, for 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 preferably also preventing a relapse in a patient.

The antibodies of the present invention provide a distinct advantage over other therapeutics in that they are able to inhibit the biological action of one or more of the toxins of *C. difficile*, whilst having a low immunogenic effect on a patient. Moreover, the antibodies of the present invention can be produced in high titres. Thus, the ovine antibodies can be readily obtained and can protect and/ or the patient against the pathological effects produced by *C. difficile* with minimal side-effects. The antibodies of the present invention may also be utilised in the development of a vaccine for passive immunization against 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 at least one 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).

In one embodiment, the antibody composition comprises a first antibody that binds to and/ or neutralises *C. difficile* Toxin A (or a fragment thereof), and a second antibody selected from an antibody that binds to and/ or neutralises *C. difficile* Toxin B (or a fragment thereof) or an antibody that bind to and/or neutralise *C. difficile* Binary Toxin (or a fragment thereof). In this embodiment, the second antibody may bind to and/ or neutralise *C. difficile* Toxin B (or a fragment thereof), and the composition optionally includes a third antibody that binds to and/ or neutralises *C. difficile* Binary Toxin (or a fragment thereof).

In another embodiment, the antibody composition comprises a first antibody that binds to and/ or neutralises *C. difficile* Toxin B (or a fragment thereof), and a second antibody selected from an antibody that binds to and/ or neutralises *C. difficile* Toxin A (or a fragment thereof) or an antibody that bind to and/or neutralise *C. difficile* Binary Toxin (or a fragment thereof). In this embodiment, the second antibody may bind to and/ or neutralise *C. difficile* Toxin A (or a fragment thereof), and the composition optionally includes a third antibody that binds to and/ or neutralises *C. difficile* Binary Toxin (or a fragment thereof).

In another embodiment, the antibody composition comprises a first antibody that binds to and/ or neutralises *C. difficile* Binary Toxin (or a fragment thereof), and a second antibody selected from an antibody that binds to and/ or neutralises *C. difficile* Toxin A (or a fragment thereof) or an antibody that bind to and/or neutralise *C. difficile* Toxin B (or a fragment thereof). In this embodiment, the second antibody may bind to and/ or neutralise *C. difficile* Toxin A (or a fragment thereof), and the composition optionally includes a third antibody that binds to and/ or neutralises *C. difficile* Toxin B (or a fragment thereof). Alternatively, the second antibody may bind to and/ or neutralise *C. difficile* Toxin B (or a fragment thereof), and the composition optionally includes a third antibody that binds to and/ or neutralises *C. difficile* Toxin A (or a fragment thereof).

An antibody of the invention may (specifically) bind and/ or neutralise one of *C. difficile* Toxin A (or a fragment thereof) or *C. difficile* Toxin B (or a fragment thereof) or *C. difficile* Binary Toxin (or a fragment thereof). Alternatively, an antibody of the invention may bind two or more of *C. difficile* Toxin A (or a fragment thereof) or *C. difficile* Toxin B (or a fragment thereof) or *C. difficile* Binary Toxin (or a fragment thereof). When an antibody binds and/ or neutralises two or more Toxin types, said antibody preferably binds and/ or neutralises *C. difficile* Toxin B (or a fragment thereof) plus one or both of *C. difficile* Toxin A (or a fragment thereof) and/or *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 Table 1 immediately below. The listed toxinotypes are purely illustrative and are not intended to be limiting to the present invention.

Table 1

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 the same or different strains of *C. difficile* toxin. 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 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.

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 administering 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, abdomen 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 or treating CDI.

In one embodiment, said method of treating CDI comprises administering 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 administering 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.

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 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 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 or goat, and another *C. difficile* toxin (e.g. *C. difficile* Toxin B) may be used separately on a second sheep or goat. The antibodies produced by separate immunisation may be combined to yield an antibody composition directed against *C. difficile* toxins.

The method comprises all modes of immunisation, 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 $(Fab)_2$ 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 delivery

In use, the present invention employs a pharmaceutical composition, comprising the antibody composition of the present invention in a form suitable for parenteral, usually intravenous 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. Preferred buffers maintain the pharmaceutical composition at a pH that is close to the physiological pH of the patient – for example, at a pH of 5.5-6.4, or at a pH of 5.6-6.3, or at a pH of 5.7-6.2, or at a pH of 5.8-6.2. The antibody-containing compositions of the present invention preferably exclude adjuvant(s) as it is undesirable to stimulate an immune response against said antibodies in the patient.

Antibodies of the invention may be formulated for, but not limited to intramuscular, subcutaneous or intravenous delivery. Compositions suitable for intramuscular, subcutaneous or intravenous injection include sterile aqueous solutions. Such aqueous solutions should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. The antibody compositions of the present invention are not oral formulations, and this mode of administration is not employed. In this regard, a key problem with oral delivery is ensuring that sufficient antibody reaches the colon where it is required. Factors which prevent 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.

Compositions suitable for injection 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 solutions of the antibodies or their fragments can be dissolved in the vehicle, the solution being made isotonic if necessary by addition of sodium chloride and sterilised 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 route of administration, 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.

Suitable daily dosages are in the range of 5-20mg per kg of body weight. The unit dosage can vary from less than 100mg, but typically will be in the region of 250-2000 mg per dose, which may be administered daily or less frequently (e.g. on alternative days for up to 1 week)

It is also within the scope of the invention to use the antibodies of the invention in 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.

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).

Goat means any species that falls within the *Capra* genus (e.g. e.g. *Capra pyrenaicais, Capra ibex, Capra nubiana, Capra sibirica, Capra walie, Capra caucasica, Capra cylindricornis Capra aegagrus, Capra falconeri*).

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 a preferred homology comparison, the identity exists over a region of the sequences that is at least 10 amino acid, preferably at least 20 amino acid, more preferably at least 30 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-Alβ; 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 Protection from CDI by passive immunisation with ovine anti *Clostridium difficile* Toxins A and B mixture. Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A and B at 10 mg/dose (▼), 2 mg/dose (■) or with a non-specific control antibody (●) at the times indicated. Animals received clindamycin at Day -2 and at Day 0 were challenged with *C. difficile* spores (2 $x10^2$ colony forming units). Survival, days post challenge are shown by the plots.

Figure 4 Protection from CDI by passive immunisation with ovine anti *Clostridium difficile* Toxins A or B. Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A + B at 10

mg/dose (▼), Toxin A antibodies alone at 10 mg/dose (Δ), Toxin B antibodies alone at 10 mg/dose (**O**) or with a non-specific control antibody (□) at the times indicated. Animals received clindamycin at Day -2 and at Day 0 were challenged with *C. difficile* spores (2 x10^2 colony forming units). Survival, days post challenge are shown by the plots.

Figure 5 Protection from CDI induced by the 027 Ribotype, 'hypervirulent' *Clostridium difficile* (strain R20291, Stoke Mandeville) by passive immunisation with ovine anti *Clostridium difficile* Toxins A and B mixture. Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A and B at 10 mg/dose (▼) or with a non-specific control antibody (■) at the times indicated. Animals received clindamycin at Day -3 and at Day 0 were challenged with *C. difficile* spores (1 x10^3 colony forming units). Disease states at days post challenge are shown by the plots.

Figure 6 Protection from CDI induced by the 078 Ribotype, 'hypervirulent' *Clostridium difficile* (Toxinotype 5) by passive immunisation with ovine anti *Clostridium difficile* Toxins A and B mixture. Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A and B at 10 mg/dose (▼) or with a non-specific control antibody (●) at the times indicated. Animals received clindamycin at Day -2 and at Day 0 were challenged with *C. difficile* spores (2 x10^3 colony forming units). Disease states at days post challenge are shown by the plots.

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 mixtures

Example 11 Assessment of the in vivo efficacy of ovine antibodies for preventing CDI

Example 12 Assessment of the in vivo efficacy of ovine antiserum for treating CDI

Example 13 Clinical uses of antibody formulations

Example 14 Protection from CDI by passive immunisation with ovine anti-*Clostridium difficile* Toxins A and B antibody mixture

Example 15 Protection from CDI by passive immunisation with ovine anti-*Clostridium difficile* Toxins A or B antibodies, or with ovine anti-*Clostridium difficile* Toxins A and B antibody mixture

Example 16 Protection from CDI induced by the 027 Ribotype, 'hypervirulent' *Clostridium difficile* (strain R20291, Stoke Mandeville) by passive immunisation with ovine anti-*Clostridium difficile* Toxins A and B mixture

Example 17 Protection from CDI induced by the 078 Ribotype, 'hypervirulent' *Clostridium difficile* isolate by passive immunisation with ovine anti-*Clostridium difficile* Toxins A and B mixture

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

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 4oC 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 may be 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 may be 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

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) 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 may made 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. Other 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, it will be necessary to first extract the DNA and derive the toxin sequence(s) by standard molecular biology methods. Once the sequence has been derived, the recombinant toxin may be expressed from a synthetic gene as above.

Example 4 Purification of *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 may made by 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. Other chromatographic techniques well known to the art of protein purification, such as size exclusion chromatography and/or affinity chromatography, may be used. Alternatively, 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.

If the peptides are produced in an insoluble form then they may be expressed with a histidine-6 purification tag 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 between peptides and final conditions may be fine-tuned on the basis of outcome of protective efficacy evaluations.

Example 6 Preparation of antiserum

A number of 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
number of and interval between doses

As a result of optimising 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 may be 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 is 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

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

These data (below) 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. The assays employed are described immediately above 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 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

Table 4 - Neutralisation Titres of Ovine Anti Toxin B against equivalent concentrations of Toxin B from Toxinotype 0 and Toxinotype III

These data (below) compare the capacity of ovine antibody raised against Toxoid B derived from Toxin B (Toxinotype 0) to neutralise equivalent amounts of Toxin B (Toxinotype 0) and Toxin B (Toxinotype III). Toxin B (Toxinotype 0) and Toxin B (Toxinotype III) were purified from *C. difficile* strains VPI 10463 and R20291, respectively.

Cell assays were performed as described immediately above in Example 8 using equivalent cytotoxic amounts of each Toxin B type. Titrations were carried out on Toxin B fixed at 4 units (4 fold the amount required to induce cell death) in one experiment and 10 units in another experiment.

The data show that the ovine antibody raised against Toxin B, using a Toxoid B derived from Toxinotype 0 strain, neutralised Toxin B (Toxinotype 0) and Toxin B (Toxinotype III) with the same efficacy.

Toxinotype	**Ovine anti Toxin B - Neutralisation Titre**	
	4 units Toxin B	**10 units toxin B**
Toxin B - Toxinotype 0	2560	1280
Toxin B - Toxinotype III	2560	1280
Relative titre (0:III)	1	1

Table 5. Neutralisation Titres of Ovine Anti Toxin B against equivalent concentrations of Toxin B from Toxinotype 0 and Toxinotype V

These data compare the capacity of ovine antibody raised against Toxoid B derived from Toxin B (Toxinotype 0) to neutralise equivalent amounts of Toxin B (Toxinotype 0) and Toxin B (Toxinotype V). Toxin B (Toxinotype 0) and Toxin B (Toxinotype V) were purified from *C. difficile* strains VPI 10463 and a *C. difficile* ribotype 078 isolate, respectively.

Cell assays were performed as in Example 8 using equivalent cytotoxic amounts of each Toxin B type. Titrations were carried out on Toxin B fixed at 4 units (4 fold the amount required to induce cell death) in one experiment and 10 units in another experiment.

The data show that the ovine antibody raised against Toxin B, using a Toxoid B derived from Toxinotype 0 strain, neutralised Toxin B (Toxinotype 0) and also Toxin B (Toxinotype V) with 2-fold reduced efficiency.

Toxinotype	**Ovine anti Toxin B - Neutralisation Titre**	
	4 units Toxin B	**10 units toxin B**
Toxin B - Toxinotype 0	2560	1280
Toxin B - Toxinotype V	1280	640
Relative titre (0:V)	2	2

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

Column Preparation

The required amount of 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 must be 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. However 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 mixtures

Antibodies will be blended to form the final product such that the required neutralising activities against Toxins A and B and their various isoforms (toxinotypes) are present in the final mixture.

Antibodies prepared against the various *C. difficile* toxinotypes may be blended in order to provide similar cross-protective efficacy as assessed in Example 10. For example, to provide similar cross-protection against *C. difficile* Toxin B derived from Toxinotypes 0 and III, antibodies may be mixed in a various proportions (e.g. 1:1, 2:1, 1:2 ratios) in order to provide similar specific neutralising activity against each of the Toxin B toxinotypes.

Example 11 Assessment of the *in vivo* efficacy of ovine antibodies for preventing CDI

To demonstrate the prophylactic (preventative) efficacy of antibodies against CDI in vivo, Syrian hamsters are passively immunised with ovine antibodies which have neutralising activity against one or more of the toxins of *C. difficile*. For assessing the prophylactic efficacy, hamsters will be given antibody either intravenously or by the intraperitoneal route at various times from 96 hours pre-challenge to 240 hours post challenge with *C. difficile*

Passively immunised hamsters are administered with a broad spectrum antibiotic (e.g. clindamycin) and 12-72 h later challenged with *C. difficile* spores by mouth. Animals are then monitored for up to 15 days for symptoms of *C. difficile*-associated disease. Control, non-immunised animals develop signs of the disease (e.g. diarrhoea, swollen abdomen, lethargy, ruffled fur) while those passively immunised with ovine antibody appear normal.

Example 12 Assessment of the *in vivo* efficacy of ovine antiserum for treating CDI

To demonstrate the efficacy of antiserum to treat CDI in vivo, Syrian hamsters are passively immunised with ovine antibodies which have neutralising activity against one or more of the toxins of *C. difficile*. For assessing the efficacy of a treatment formulation, hamsters will be given antibody either intravenously or by the intraperitoneal route at various times from 6 hours post-challenge to 240 hours post challenge with *C. difficile*

Prior to passively immunisation hamsters are administered with a broad spectrum antibiotic (e.g. clindamycin) and 12-72 h later challenged with *C. difficile* spores by mouth. Animals are then monitored for up to 15 days for symptoms of *C. difficile*-associated disease. Control, non-immunised animals develop signs of the disease (e.g. diarrhoea, swollen abdomen, lethargy, ruffled fur) while those treated with ovine antibody appear normal.

Example 13 Clinical use of antibody formulations

Three examples serve to illustrate the therapeutic value of the systemic ovine products in patients with differing degrees of seventy in their CDI.

Mild CDI

A 67 year old male was admitted to a coronary care unit following a severe myocardial infarction. He was making an uneventful recovery when he developed a mild diarrhoea without any other signs or symptoms. Because there had been recent episodes of CDI in the hospital, a faecal sample was sent immediately for testing and found to contain both Toxin A and Toxin B. After isolation to a single room with its own toilet he received 250mg of the ovine $F(ab')_2$ intravenously followed by a second injection two days later. His diarrhoea stopped quickly and he made a full recovery without the need of either metranidazole or vancomycin.

Severe CDI with risk of relapse

A female aged 81 fell in her home and sustained a fractured left hip. She was immediately admitted to hospital and the hip was pinned successfully. Her frail condition prevented early discharge and, a few days later, she developed a productive cough for which she was given a wide spectrum antibiotic. After a further eight days she developed profuse diarrhoea with abdominal pain and tenderness and CDI was diagnosed by the appropriate faecal tests. At the time there was also evidence of systemic manifestations of the infection including a markedly raised white blood cell count, and of significant fluid loss with dehydration. The patient was started immediately on oral vancomycin and, at the same time, received the first of five daily injections of 250mg of the ovine $F(ab')_2$ - based product intravenously. There was a rapid resolution of the signs and symptoms and of the laboratory manifestations of CDI. However, in order to avoid the risk of relapse of her CDI following stopping vancomycin, she continued to be treated for a further two weeks on an oral form of the antibody therapy. She experienced no relapse.

Severe CDI with complications

An 87 year old female developed bronchopneumonia while resident in long-stay care facilities. The local general practitioner started her on a course of antibiotic therapy with immediate benefit. However, eight days after stopping the antibiotic she experienced severe diarrhoea. Her condition started to deteriorate necessitating admission to hospital where Toxin A was detected in her faeces by an ELISA test. By this time she was extremely ill with evidence

of circulatory failure and her diarrhoea had stopped. The latter was found to be due a combination of paralytic ileus and toxic megacolon and an emergency total colectomy was considered essential. Since such surgery is associated with a mortality in excess of 60% she received intravenous replacement therapy together with the contents of two ampoules (500mg) of product. By the time she was taken to the operating theatre four hours later, her general condition had improved significantly and she survived surgery.

Example 14 Protection from CDI by passive immunisation with ovine anti-*Clostridium difficile* Toxins A and B antibody mixture

Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A and B at 10 mg/dose (▼), 2 mg/dose (■) or with a non-specific control antibody (●) at the times indicated. Animals received clindamycin at Day -2 and at Day 0 were challenged with *C. difficile* spores (2 $x10^2$ colony forming units). Survival, days post challenge are shown by the plots.

The data (see Figure 3) clearly show that passive immunisation with a mixture of ovine antibodies to Toxins A and B affords protection from CDI. In this experiment, 90% of animal given the high antibody dose were asymptomatic at 12 days post challenge.

Example 15 Protection from CDI by passive immunisation with ovine anti-*Clostridium difficile* Toxins A or B antibodies, or with ovine anti-*Clostridium difficile* Toxins A and B antibody mixture

Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A + B at 10 mg/dose (▼), Toxin A antibodies alone at 10 mg/dose (Δ), Toxin B antibodies alone at 10 mg/dose (**O**) or with a non-specific control antibody (□) at the times indicated. Animals received clindamycin at Day -2 and at Day 0 were challenged with *C. difficile* spores (2 $x10^2$ colony forming units). Survival, days post challenge are shown by the plots.

The data (see Figure 4) show that with respect to protection against CDI, the efficacy of a mixture of Toxin A + B antibodies is significantly better than either antibody given alone.

Example 16 Protection from CDI induced by the 027 Ribotype, 'hypervirulent' *Clostridium difficile* (strain R20291, Stoke Mandeville) by passive immunisation with ovine anti-*Clostridium difficile* Toxins A and B mixture

Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A and B at 10 mg/dose (▼) or with a non-specific control antibody (■) at the times indicated. Animals received clindamycin at Day -3 and at Day 0 were challenged with *C. difficile* spores (1 $x10^3$ colony forming units). Disease states at days post challenge are shown by the plots.

The data (see Figure 5) clearly show that passive immunisation with a mixture of ovine antibodies to Toxins A and B affords protection from CDI induced by the 027, 'hypervirulent C. difficile (strain R20291, stoke Mandeville). In this experiment, 90% of animal given Toxin A/B antibody were asymptomatic at 18 days post challenge while 80% of the non-immunised controls showed severe symptoms of CDI.

Example 17 Protection from CDI induced by the 078 Ribotype, 'hypervirulent' *Clostridium difficile* isolate by passive immunisation with ovine anti-*Clostridium difficile* Toxins A and B mixture

Syrian hamsters (groups of 10) were passively immunised (i.p.) with either ovine antibodies to Toxin A and B at 10 mg/dose (▼) or with a non-specific control antibody (●) at the times indicated. Animals received clindamycin at Day -2 and at Day 0 were challenged with *C. difficile* spores (2 $x10^3$ colony forming units). Disease states at days post challenge are shown by the plots.

The data (see Figure 6) clearly show that passive immunisation with a mixture of ovine antibodies to Toxins A and B affords protection from CDI induced by the 078, 'hypervirulent C. difficile. In this experiment, 100% of animal given Toxin A/B antibody were asymptomatic at 18 days post challenge while 80% of the non-immunised controls showed severe symptoms of CDI.

SEQ ID NOs

1. *Clostridium difficile* Toxin A - Toxinotype 0

MSLISKEELIKLAYSIRPRENEYKTILTNLDEYNKLTTNNNENKYLQLKKLNESIDVFMNKYKTSSRNR
ALSNLKKDILKEVILIKNSNTSPVEKNLHFVWIGGEVSDIALEYIKQWADINAEYNIKLWYDSEAFLVN
TLKKAIVESSTTEALQLLEEEIQNPQFDNMKFYKKRMEFIYDRQKRFINYYKSQINKPTVPTIDDIIKS
HLVSEYNRDETVLESYRTNSLRKINSNHGIDIRANSLFTEQELLNIYSQELLNRGNLAAASDIVRLLAL
KNFGGVYLDVDMLPGIHSDLFKTISRPSSIGLDRWEMIKLEAIMKYKKYINNYTSENFDKLDQQLKDNF
KLIIESKSEKSEIFSKLENLNVSDLEIKIAFALGSVINQALISKQGSYLTNLVIEQVKNRYQFLNQHLN
PAIESDNNFTDTTKIFHDSLFNSATAENSMFLTKIAPYLQVGFMPEARSTISLSGPGAYASAYYDFINL
QENTIEKTLKASDLIEFKFPENNLSQLTEQEINSLWSFDQASAKYQFEKYVRDYTGGSLSEDNGVDFNK
NTALDKNYLLNNKIPSNNVEEAGSKNYVHYIIQLQGDDISYEATCNLFSKNPKNSIIIQRNMNESAKSY
FLSDDGESILELNKYRIPERLKNKEKVKVTFIGHGKDEFNTSEFARLSVDSLSNEISSFLDTIKLDISP
KNVEVNLLGCNMFSYDFNVEETYPGKLLLSIMDKITSTLPDVNKNSITIGANQYEVRINSEGRKELLAH
SGKWINKEEAIMSDLSSKEYIFFDSIDNKLKAKSKNIPGLASISEDIKTLLLDASVSPDTKFILNNLKL
NIESSIGDYIYYEKLEPVKNIIHNSIDDLIDEFNLLENVSDELYELKKLNNLDEKYLISFEDISKNNST
YSVRFINKSNGESVYVETEKEIFSKYSEHITKEISTIKNSIITDVNGNLLDNIQLDHTSQVNTLNAAFF
IQSLIDYSSNKDVLNDLSTSVKVQLYAQLFSTGLNTIYDSIQLVNLISNAVNDTINVLPTITEGIPIVS
TILDGINLGAAIKELLDEHDPLLKKELEAKVGVLAINMSLSIAATVASIVGIGAEVTIFLLPIAGISAG
IPSLVNNELILHDKATSVVNYFNHLSESKKYGPLKTEDDKILVPIDDLVISEIDFNNNSIKLGTCNILA
MEGGSGHTVTGNIDHFFSSPSISSHIPSLSIYSAIGIETENLDFSKKIMMLPNAPSRVFWWETGAVPGL
RSLENDGTRLLDSIRDLYPGKFYWRFYAFFDYAITTLKPVYEDTNIKIKLDKDTRNFIMPTITTNEIRN
KLSYSFDGAGGTYSLLLSSYPISTNINLSKDDLWIFNIDNEVREISIENGTIKKGKLIKDVLSKIDINK
NKLIIGNQTIDFSGDIDNKDRYIFLTCELDDKISLIIEINLVAKSYSLLLSGDKNYLISNLSNTIEKIN
TLGLDSKNIAYNYTDESNNKYFGAISKTSQKSIIHYKKDSKNILEFYNDSTLEFNSKDFIAEDINVFMK
DDINTITGKYYVDNNTDKSIDFSISLVSKNQVKVNGLYLNESVYSSYLDFVKNSDGHHNTSNFMNLFLD
NISFWKLFGFENINFVIDKYFTLVGKTNLGYVEFICDNNKNIDIYFGEWKTSSSKSTIFSGNGRNVVVE
PIYNPDTGEDISTSLDFSYEPLYGIDRYINKVLIAPDLYTSLININTNYYSNEYYPEIIVLNPNTFHKK
VNINLDSSSFEYKWSTEGSDFILVRYLEESNKKILQKIRIKGILSNTQSFNKMSIDFKDIKKLSLGYIM
SNFKSFNSENELDRDHLGFKIIDNKTYYYDEDSKLVKGLININNSLFYFDPIEFNLVTGWQTINGKKYY
FDINTGAALTSYKIINGKHFYFNNDGVMQLGVFKGPDGFEYFAPANTQNNNIEGQAIVYQSKFLTLNGK
KYYFDNNSKAVTGWRIINNEKYYFNPNNAIAAVGLQVIDNNKYYFNPDTAIISKGWQTVNGSRYYFDTD
TAIAFNGYKTIDGKHFYFDSDCVVKIGVFSTSNGFEYFAPANTYNNNIEGQAIVYQSKFLTLNGKKYYF
DNNSKAVTGLQTIDSKKYYFNTNTAEAATGWQTIDGKKYYFNTNTAEAATGWQTIDGKKYYFNTNTAIA
STGYTIINGKHFYFNTDGIMQIGVFKGPNGFEYFAPANTDANNIEGQAILYQNEFLTLNGKKYYFGSDS
KAVTGWRIINNKKYYFNPNNAIAAIHLCTINNDKYYFSYDGILQNGYITIERNNFYFDANNESKMVTGV
FKGPNGFEYFAPANTHNNNIEGQAIVYQNKFLTLNGKKYYFDNDSKAVTGWQTIDGKKYYFNLNTAEAA
TGWQTIDGKKYYFNLNTAEAATGWQTIDGKKYYFNTNTFIASTGYTSINGKHFYFNTDGIMQIGVFKGP
NGFEYFAPANTDANNIEGQAILYQNKFLTLNGKKYYFGSDSKAVTGLRTIDGKKYYFNTNTAVAVTGWQ
TINGKKYYFNTNTSIASTGYTIISGKHFYFNTDGIMQIGVFKGPDGFEYFAPANTDANNIEGQAIRYQN

RFLYLHDNIYYFGNNSKAATGWVTIDGNRYYFEPNTAMGANGYKTIDNKNFYFRNGLPQIGVFKGSNGF
EYFAPANTDANNIEGQAIRYQNRFLHLLGKIYYFGNNSKAVTGWQTINGKVYYFMPDTAMAAAGGLFEI
DGVIYFFGVDGVKAPGIYG

2. Protein Sequence of *C. difficile* Toxin B - Toxinotype 0

MSLVNRKQLEKMANVRFRTQEDEYVAILDALEEYHNMSENTVVEKYLKLKDINSLTDIYIDTYKKSGRN
KALKKFKEYLVTEVLELKNNNLTPVEKNLHFVWIGGQINDTAINYINQWKDVNSDYNVNVFYDSNAFLI
NTLKKTVVESAINDTLESFRENLNDPRFDYNKFFRKRMEIIYDKQKNFINYYKAQREENPELIIDDIVK
TYLSNEYSKEIDELNTYIEESLNKITQNSGNDVRNFEEFKNGESFNLYEQELVERWNLAAASDILRISA
LKEIGGMYLDVDMLPGIQPDLFESIEKPSSVTVDFWEMTKLEAIMKYKEYIPEYTSEHFDMLDEEVQSS
FESVLASKSDKSEIFSSLGDMEASPLEVKIAFNSKGIINQGLISVKDSYCSNLIVKQIENRYKILNNSL
NPAISEDNDFNTTTNTFIDSIMAEANADNGRFMMELGKYLRVGFFPDVKTTINLSGPEAYAAAYQDLLM
FKEGSMNIHLIEADLRNFEISKTNISQSTEQEMASLWSFDDARAKAQFEEYKRNYFEGSLGEDDNLDFS
QNIVVDKEYLLEKISSLARSSERGYIHYIVQLQGDKISYEAACNLFAKTPYDSVLFQKNIEDSEIAYYY
NPGDGEIQEIDKYKIPSIISDRPKIKLTFIGHGKDEFNTDIFAGFDVDSLSTEIEAAIDLAKEDISPKS
IEINLLGCNMFSYSINVEETYPGKLLLKVKDKISELMPSISQDSIIVSANQYEVRINSEGRRELLDHSG
EWINKEESIIKDISSKEYISFNPKENKITVKSKNLPELSTLLQEIRNNSNSSDIELEEKVMLTECEINV
ISNIDTQIVEERIEEAKNLTSDSINYIKDEFKLIESISDALCDLKQQNELEDSHFISFEDISETDEGFS
IRFINKETGESIFVETEKTIFSEYANHITEEISKIKGTIFDTVNGKLVKKVNLDTTHEVNTLNAAFFIQ
SLIEYNSSKESLSNLSVAMKVQVYAQLFSTGLNTITDAAKVVELVSTALDETIDLLPTLSEGLPIIATI
IDGVSLGAAIKELSETSDPLLRQEIEAKIGIMAVNLTTATTAIITSSLGIASGFSILLVPLAGISAGIP
SLVNNELVLRDKATKVVDYFKHVSLVETEGVFTLLDDKIMMPQDDLVISEIDFNNNSIVLGKCEIWRME
GGSGHTVTDDIDHFFSAPSITYREPHLSIYDVLEVQKEELDLSKDLMVLPNAPNRVFAWETGWTPGLRS
LENDGTKLLDRIRDNYEGEFYWRYFAFIADALITTLKPRYEDTNIRINLDSNTRSFIVPIITTEYIREK
LSYSFYGSGGTYALSLSQYNMGINIELSESDVWIIDVDNVVRDVTIESDKIKKGDLIEGILSTLSIEEN
KIILNSHEINFSGEVNGSNGFVSLTFSILEGINAIIEVDLLSKSYKLLISGELKILMLNSNHIQQKIDY
IGFNSELQKNIPYSFVDSEGKENGFINGSTKEGLFVSELPDVVLISKVYMDDSKPSFGYYSNNLKDVKV
ITKDNVNILTGYYLKDDIKISLSLTLQDEKTIKLNSVHLDESGVAEILKFMNRKGNTNTSDSLMSFLES
MNIKSIFVNFLQSNIKFILDANFIISGTTSIGQFEFICDENDNIQPYFIKFNTLETNYTLYVGNRQNMI
VEPNYDLDDSGDISSTVINFSQKYLYGIDSCVNKVVISPNIYTDEINITPVYETNNTYPEVIVLDANYI
NEKINVNINDLSIRYVWSNDGNDFILMSTSEENKVSQVKIRFVNVFKDKTLANKLSFNFSDKQDVPVSE
IILSFTPSYYEDGLIGYDLGLVSLYNEKFYINNFGMMVSGLIYINDSLYYFKPPVNNLITGFVTVGDDK
YYFNPINGGAASIGETIIDDKNYYFNQSGVLQTGVFSTEDGFKYFAPANTLDENLEGEAIDFTGKLIID
ENIYYFDDNYRGAVEWKELDGEMHYFSPETGKAFKGLNQIGDYKYYFNSDGVMQKGFVSINDNKHYFDD
SGVMKVGYTEIDGKHFYFAENGEMQIGVFNTEDGFKYFAHHNEDLGNEEGEEISYSGILNFNNKIYYFD
DSFTAVVGWKDLEDGSKYYFDEDTAEAYIGLSLINDGQYYFNDDGIMQVGFVTINDKVFYFSDSGIIES
GVQNIDDNYFYIDDNGIVQIGVFDTSDGYKYFAPANTVNDNIYGQAVEYSGLVRVGEDVYYFGETYTIE
TGWIYDMENESDKYYFNPETKKACKGINLIDDIKYYFDEKGIMRTGLISFENNNYYFNENGEMQFGYIN
IEDKMFYFGEDGVMQIGVFNTPDGFKYFAHQNTLDENFEGESINYTGWLDLDEKRYYFTDEYIAATGSV
IIDGEEYYFDPDTAQLVISE

3. Protein Sequence of *C. difficile* Toxin A - Toxinotype III

MSLISKEELIKLAYSIRPRENEYKTILTNLDEYNKLTTNNNENKYLQLKKLNESIDVFMNKYKNSSRNR
ALSNLKKDILKEVILIKNSNTSPVEKNLHFVWIGGEVSDIALEYIKQWADINAEYNIKLWYDSEAFLVN
TLKKAIVESSTTEALQLLEEEIQNPQFDNMKFYKKRMEFIYDRQKRFINYYKSQINKPTVPTIDDIIKS
HLVSEYNRDETLLESYRTNSLRKINSNHGIDIRANSLFTEQELLNIYSQELLNRGNLAAASDIVRLLAL
KNFGGVYLDVDMLPGIHSDLFKTIPRPSSIGLDRWEMIKLEAIMKYKKYINNYTSENFDKLDQQLKDNF
KLIIESKSEKSEIFSKLENLNVSDLEIKIAFALGSVINQALISKQGSYLTNLVIEQVKNRYQFLNQHLN
PAIESDNNFTDTTKIFHDSLFNSATAENSMFLTKIAPYLQVGFMPEARSTISLSGPGAYASAYYDFINL
QENTIEKTLKASDLIEFKFPENNLSQLTEQEINSLWSFDQASAKYQFEKYVRDYTGGSLSEDNGVDFNK
NTALDKNYLLNNKIPSNNVEEAGSKNYVHYIIQLQGDDISYEATCNLFSKNPKNSIIIQRNMNESAKSY
FLSDDGESILELNKYRIPERLKNKEKVKVTFIGHGKDEFNTSEFARLSVDSLSNEISSFLDTIKLDISP
KNVEVNLLGCNMFSYDFNVEETYPGKLLLSIMDKITSTLPDVNKDSITIGANQYEVRINSEGRKELLAH
SGKWINKEEAIMSDLSSKEYIFFDSIDNKLKAKSKNIPGLASISEDIKTLLLDASVSPDTKFILNNLKL
NIESSIGDYIYYEKLEPVKNIIHNSIDDLIDEFNLLENVSDELYELKKLNNLDEKYLISFEDISKNNST
YSVRFINKSNGESVYVETEKEIFSKYSEHITKEISTIKNSIITDVNGNLLDNIQLDHTSQVNTLNAAFF
IQSLIDYSSNKDVLNDLSTSVKVQLYAQLFSTGLNTIYDSIQLVNLISNAVNDTINVLPTITEGIPIVS
TILDGINLGAAIKELLDEHDPLLKKELEAKVGVLAINMSLSIAATVASIVGIGAEVTIFLLPIAGISAG
IPSLVNNELILHDKATSVVNYFNHLSESKEYGPLKTEDDKILVPIDDLVISEIDFNNNSIKLGTCNILA
MEGGSGHTVTGNIDHFFSSPYISSHIPSLSVYSAIGIKTENLDFSKKIMMLPNAPSRVFWWETGAVPGL
RSLENNGTKLLDSIRDLYPGKFYWRFYAFFDYAITTLKPVYEDTNTKIKLDKDTRNFIMPTITTDEIRN
KLSYSFDGAGGTYSLLLSSYPISMNINLSKDDLWIFNIDNEVREISIENGTIKKGNLIEDVLSKIDINK
NKLIIGNQTIDFSGDIDNKDRYIFLTCELDDKISLIIEINLVAKSYSLLLSGDKNYLISNLSNTIEKIN
TLGLDSKNIAYNYTDESNNKYFGAISKTSQKSIIHYKKDSKNILEFYNGSTLEFNSKDFIAEDINVFMK
DDINTITGKYYVDNNTDKSIDFSISLVSKNQVKVNGLYLNESVYSSYLDFVKNSDGHHNTSNFMNLFLN
NISFWKLFGFENINFVIDKYFTLVGKTNLGYVEFICDNNKNIDIYFGEWKTSSSKSTIFSGNGRNVVVE
PIYNPDTGEDISTSLDFSYEPLYGIDRYINKVLIAPDLYTSLININTNYYSNEYYPEIIVLNPNTFHKK
VNINLDSSSFEYKWSTEGSDFILVRYLEESNKKILQKIRIKGILSNTQSFNKMSIDFKDIKKLSLGYIM
SNFKSFNSENELDRDHLGFKIIDNKTYYYDEDSKLVKGLININNSLFYFDPIESNLVTGWQTINGKKYY
FDINTGAASTSYKIINGKHFYFNNNGVMQLGVFKGPDGFEYFAPANTQNNNIEGQAIVYQSKFLTLNGK
KYYFDNDSKAVTGWRIINNEKYYFNPNNAIAAVGLQVIDNNKYYFNPDTAIISKGWQTVNGSRYYFDTD
TAIAFNGYKTIDGKHFYFDSDCVVKIGVFSGSNGFEYFAPANTYNNNIEGQAIVYQSKFLTLNGKKYYF
DNNSKAVTGWQTIDSKKYYFNTNTAEAATGWQTIDGKKYYFNTNTAEAATGWQTIDGKKYYFNTNTSIA
STGYTIINGKYFYFNTDGIMQIGVFKVPNGFEYFAPANTHNNNIEGQAILYQNKFLTLNGKKYYFGSDS
KAITGWQTIDGKKYYFNPNNAIAATHLCTINNDKYYFSYDGILQNGYITIERNNFYFDANNESKMVTGV
FKGPNGFEYFAPANTHNNNIEGQAIVYQNKFLTLNGKKYYFDNDSKAVTGWQTIDSKKYYFNLNTAVAV
TGWQTIDGEKYYFNLNTAEAATGWQTIDGKRYYFNTNTYIASTGYTIINGKHFYFNTDGIMQIGVFKGP
DGFEYFAPANTHNNNIEGQAILYQNKFLTLNGKKYYFGSDSKAVTGLRTIDGKKYYFNTNTAVAVTGWQ
TINGKKYYFNTNTYIASTGYTIISGKHFYFNTDGIMQIGVFKGPDGFEYFAPANTDANNIEGQAIRYQN
RFLYLHDNIYYFGNDSKAATGWATIDGNRYYFEPNTAMGANGYKTIDNKNFYFRNGLPQIGVFKGPNGF
EYFAPANTDANNIDGQAIRYQNRFLHLLGKIYYFGNNSKAVTGWQTINSKVYYFMPDTAMAAAGGLFEI
DGVIYFFGVDGVKAPGIYG

4. Protein Sequence of *C. difficile* Toxin B - Toxinotype III

MSLVNRKQLEKMANVRFRVQEDEYVAILDALEEYHNMSENTVVEKYLKLKDINSLTDIYIDTYKKSGRN
KALKKFKEYLVTEVLELKNNNLTPVEKNLHFVWIGGQINDTAINYINQWKDVNSDYNVNVFYDSNAFLI
NTLKKTIVESATNDTLESFRENLNDPRFDYNKFYRKRMEIIYDKQKNFINYYKTQREENPDLIIDDIVK
IYLSNEYSKDIDELNSYIEESLNKVTENSGNDVRNFEEFKGGESFKLYEQELVERWNLAAASDILRISA
LKEVGGVYLDVDMLPGIQPDLFESIEKPSSVTVDFWEMVKLEAIMKYKEYIPGYTSEHFDMLDEEVQSS
FESVLASKSDKSEIFSSLGDMEASPLEVKIAFNSKGIINQGLISVKDSYCSNLIVKQIENRYKILNNSL
NPAISEDNDFNTTTNAFIDSIMAEANADNGRFMMELGKYLRVGFFPDVKTTINLSGPEAYAAAYQDLLM
FKEGSMNIHLIEADLRNFEISKTNISQSTEQEMASLWSFDDARAKAQFEEYKKNYFEGSLGEDDNLDFS
QNTVVDKEYLLEKISSLARSSERGYIHYIVQLQGDKISYEAACNLFAKTPYDSVLFQKNIEDSEIAYYY
NPGDGEIQEIDKYKIPSIISDRPKIKLTFIGHGKDEFNTDIFAGLDVDSLSTEIETAIDLAKEDISPKS
IEINLLGCNMFSYSVNVEETYPGKLLLRVKDKVSELMPSISQDSIIVSANQYEVRINSEGRRELLDHSG
EWINKEESIIKDISSKEYISFNPKENKIIVKSKNLPELSTLLQEIRNNSNSSDIELEEKVMLAECEINV
ISNIDTQVVEGRIEEAKSLTSDSINYIKNEFKLIESISDALYDLKQQNELEESHFISFEDILETDEGFS
IRFIDKETGESIFVETEKAIFSEYANHITEEISKIKGTIFDTVNGKLVKKVNLDATHEVNTLNAAFFIQ
SLIEYNSSKESLSNLSVAMKVQVYAQLFSTGLNTITDAAKVVELVSTALDETIDLLPTLSEGLPVIATI
IDGVSLGAAIKELSETSDPLLRQEIEAKIGIMAVNLTAATTAIITSSLGIASGFSILLVPLAGISAGIP
SLVNNELILRDKATKVVDYFSHISLAESEGAFTSLDDKIMMPQDDLVISEIDFNNNSITLGKCEIWRME
GGSGHTVTDDIDHFFSAPSITYREPHLSIYDVLEVQKEELDLSKDLMVLPNAPNRVFAWETGWTPGLRS
LENDGTKLLDRIRDNYEGEFYWRYFAFIADALITTLKPRYEDTNIRINLDSNTRSFIVPVITTEYIREK
LSYSFYGSGGTYALSLSQYNMNINIELNENDTWVIDVDNVVRDVTIESDKIKKGDLIENILSKLSIEDN
KIILDNHEINFSGTLNGGNGFVSLTFSILEGINAVIEVDLLSKSYKVLISGELKTLMANSNSVQQKIDY
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VEPNYDLDDSGDISSTVINFSQKYLYGIDSCVNKVIISPNIYTDEINITPIYEANNTYPEVIVLDTNYI
SEKININDLSIRYVWSNDGSDFILMSTDEENKVSQVKIRFTNVFKGNTISDKISFNFSDKQDVSINK
VISTFTPSYYVEGLLNYDLGLISLYNEKFYINNFGMMVSGLVYINDSLYYFKPPIKNLITGFTTIGDDK
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DSFTAVVGWKDLEDGSKYYFDEDTAEAYIGISIINDGKYYFNDSGIMQIGFVTINNEVFYFSDSGIVES
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TGWIYDMENESDKYYFDPETKKAYKGINVIDDIKYYFDENGIMRTGLITFEDNHYYFNEDGIMQYGYLN
IEDKTFYFSEDGIMQIGVFNTPDGFKYFAHQNTLDENFEGESINYTGWLDLDEKRYYFTDEYIAATGSV
IIDGEEYYFDPDTAQLVISE

5. Protein Sequence of *C. difficile* Binary toxin fragment A

MKKFRKHKRISNCISILLILYLTLGGLLPNNIYAQDLQSYSEKVCNTTYKAPIESFLKDKEKAKEWERK
EAERIEQKLERSEKEALESYKKDSVEISKYSQTRNYFYDYQIEANSREKEYKELRNAISKNKIDKPMYV
YYFESPEKFAFNKVIRTENQNEISLEKFNEFKETIQNKLFKQDGFKDISLYEPGKGDEKPTPLLMHLKL

PRNTGMLPYTNTNNVSTLIEQGYSIKIDKIVRIVIDGKHYIKAEASVVNSLDFKDDVSKGDSWGKANYN
DWSNKLTPNELADVNDYMRGGYTAINNYLISNGPVNNPNPELDSKITNIENALKREPIPTNLTVYRRSG
PQEFGLTLTSPEYDFNKLENIDAFKSKWEGQALSYPNFISTSIGSVNMSAFAKRKIVLRITIPKGSPGA
YLSAIPGYAGEYEVLLNHGSKFKINKIDSYKDGTITKLIVDATLIP

6. Protein Sequence of *C. difficile* Binary toxin fragment B

MKIQMRNKKVLSFLTLTAIVSQALVYPVYAQTSTSNHSNKKKEIVNEDILPNNGLMGYYFSDEHFKDLK
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VNMKKGKEYKVRIELQDKNLGSIDNLSSPNLYWELDGMKKIIPEENLFLRDYSNIEKDDPFIPNNNFFD
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QNGFTANVTTNYSHTTDNSTAVQDSNGESWNTGLSINKGESAYINANVRYYNTGTAPMYKVTPTTNLVL
DGDTLSTIKAQENQIGNNLSPGDTYPKKGLSPLALNTMDQFSSRLIPINYDQLKKLDAGKQIKLETTQV
SGNFGTKNSSGQIVTEGNSWSDYISQIDSISASIILDTENESYERRVTAKNLQDPEDKTPELTIGEAIE
KAFGATKKDGLLYFNDIPIDESCVELIFDDNTANKIKDSLKTLSDKKIYNVKLERGMNILIKTPTYFTN
FDDYNNYPSTWSNVNTTNQDGLQGSANKLNGETKIKIPMSELKPYKRYVFSGYSKDPLTSNSIIVKIKA
KEEKTDYLVPEQGYTKFSYEFETTEKDSSNIEITLIGSGTTYLDNLSITELNSTPEILDEPEVKIPTDQ
EIMDAHKIYFADLNFNPSTGNTYINGMYFAPTQTNKEALDYIQKYRVEATLQYSGFKDIGTKDKEMRNY
LGDPNQPKTNYVNLRSYFTGGENIMTYKKLRIYAITPDDRELLVLSVD

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.

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

3. The antibody composition according to any preceding claim, wherein the antibodies bind to at least one *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 according to any Claim 3, wherein the antibodies bind to *C. difficile* Toxin A and to *C. difficile* Toxin B.

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

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

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

8. The antibody composition according to any of Claims 1-3, Claim 4 or Claim 6, wherein the *C. difficile* toxin is a Toxin B is selected from the following group: Toxinotype 0, Toxinotype III and Toxinotype V.

9. A pharmaceutical composition comprising the antibody composition according to Claims 1 to 8, together with at least one component

selected from a pharmaceutically acceptable carrier, excipient, and/ or salt for parenteral administration.

10. A method for prevention or treatment of *C. difficile* infection, said method comprising administering the antibody composition according to Claims 1 to 8 or a pharmaceutical composition according to Claim 9.

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

12. A method of producing ovine antibodies for use in the antibody composition according to Claims 1 to 7 or for use in a pharmaceutical composition according to Claim 8, 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.

13. A method according to Claim 11 or Claim 12, wherein the immunogen is a *C. difficile* toxoid.

Figure 1

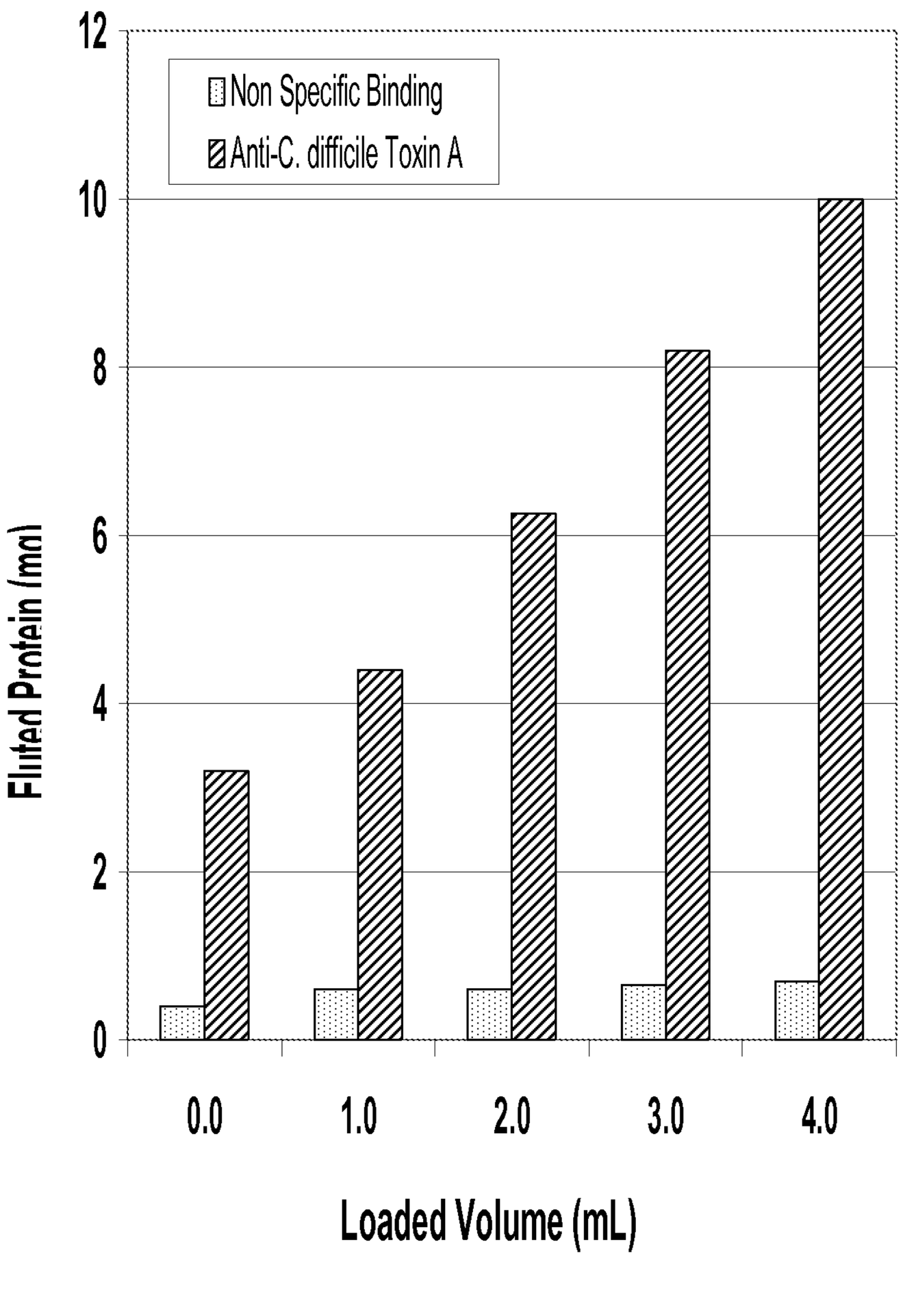

Non Specific Binding
Anti-C. difficile Toxin A
Eluted Protein (mg)
12
10
8
6
4
2
0
0.0
1.0
2.0
3.0
4.0
Loaded Volume (mL)

Figure 2

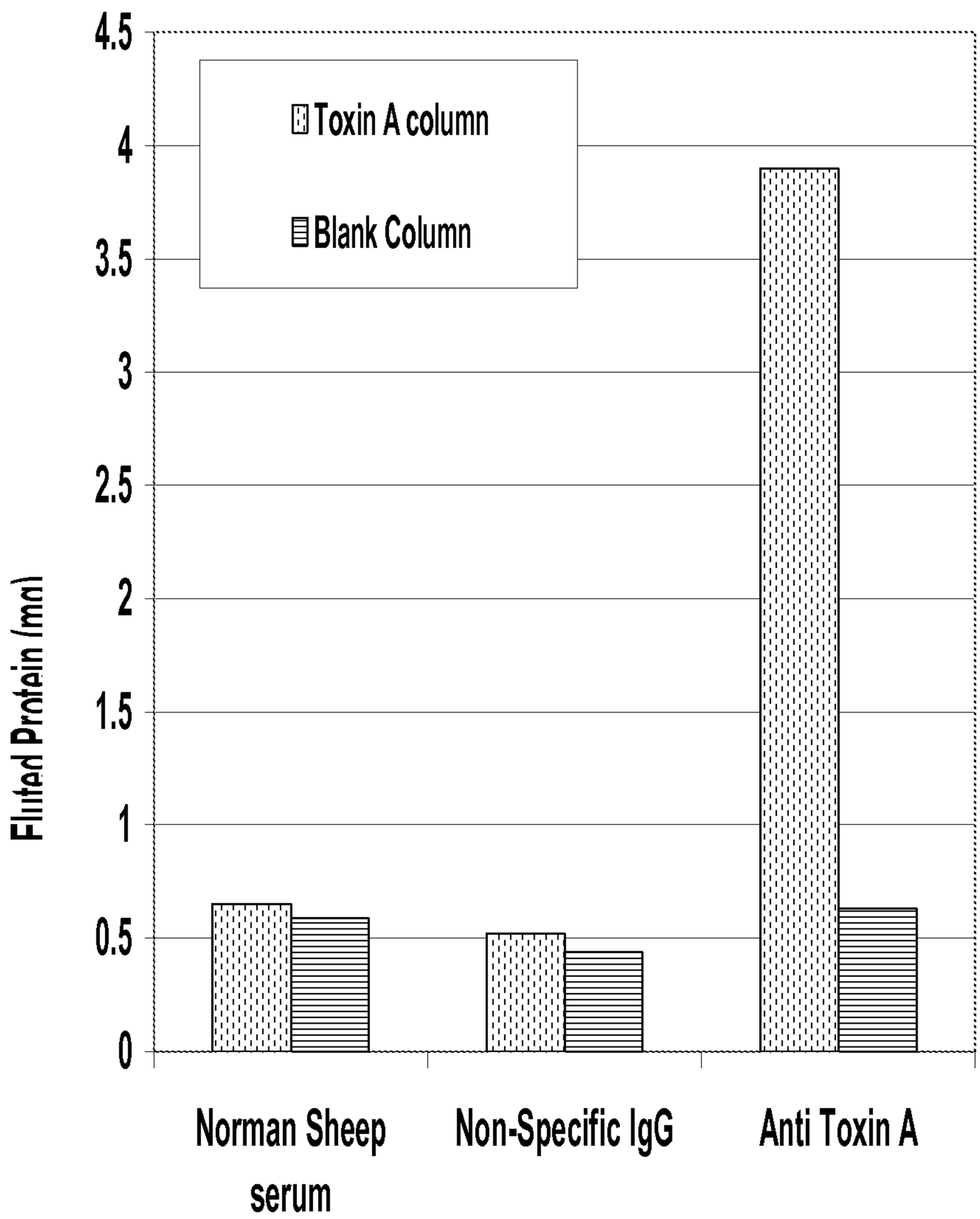

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Toxin A column
Blank Column
Eluted Protein (mg)
Norman Sheep
Non-Specific IgG
Anti Toxin A
serum

Figure 3

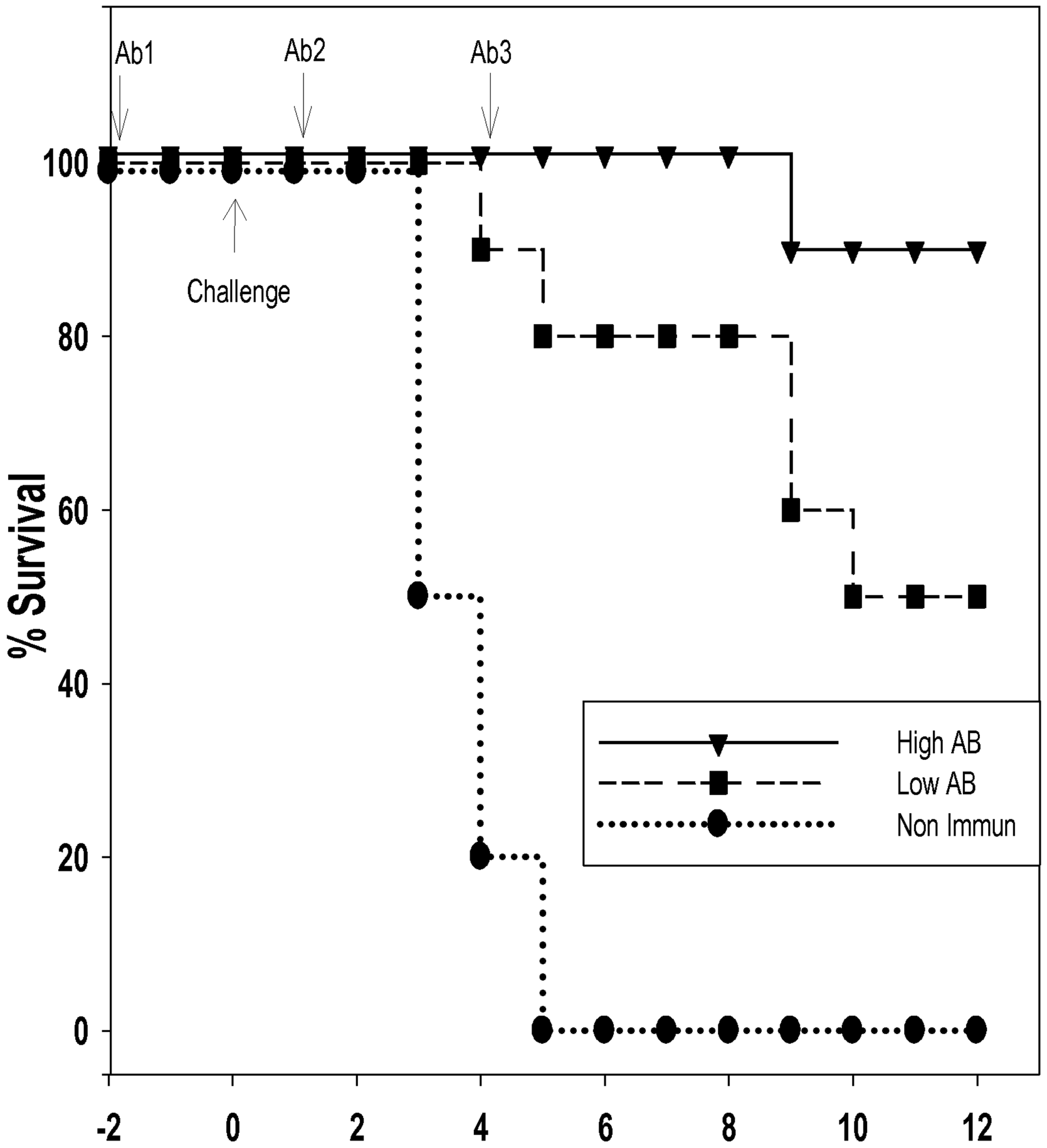

Ab1
Ab2
Ab3
Challenge
100
80
60
40
20
0
% Survival
High AB
Low AB
Non Immun
-2
0
2
4
6
8
10
12
Days Post Challenge

Figure 4

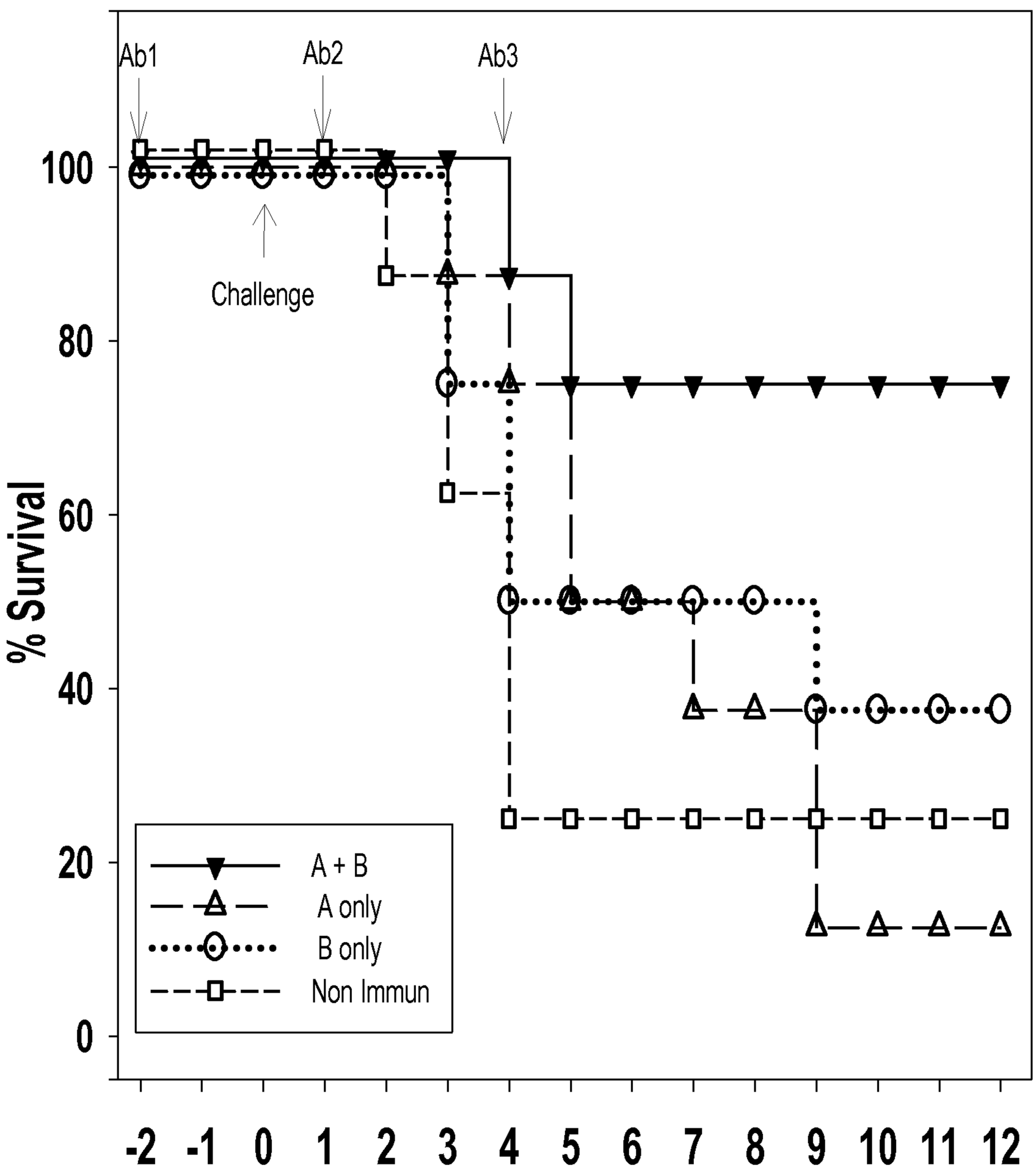

Ab1
Ab2
Ab3
Challenge
100
80
60
40
20
0
% Survival
A + B
A only
B only
Non Immun
-2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12
Days Post Challenge

Figure 5

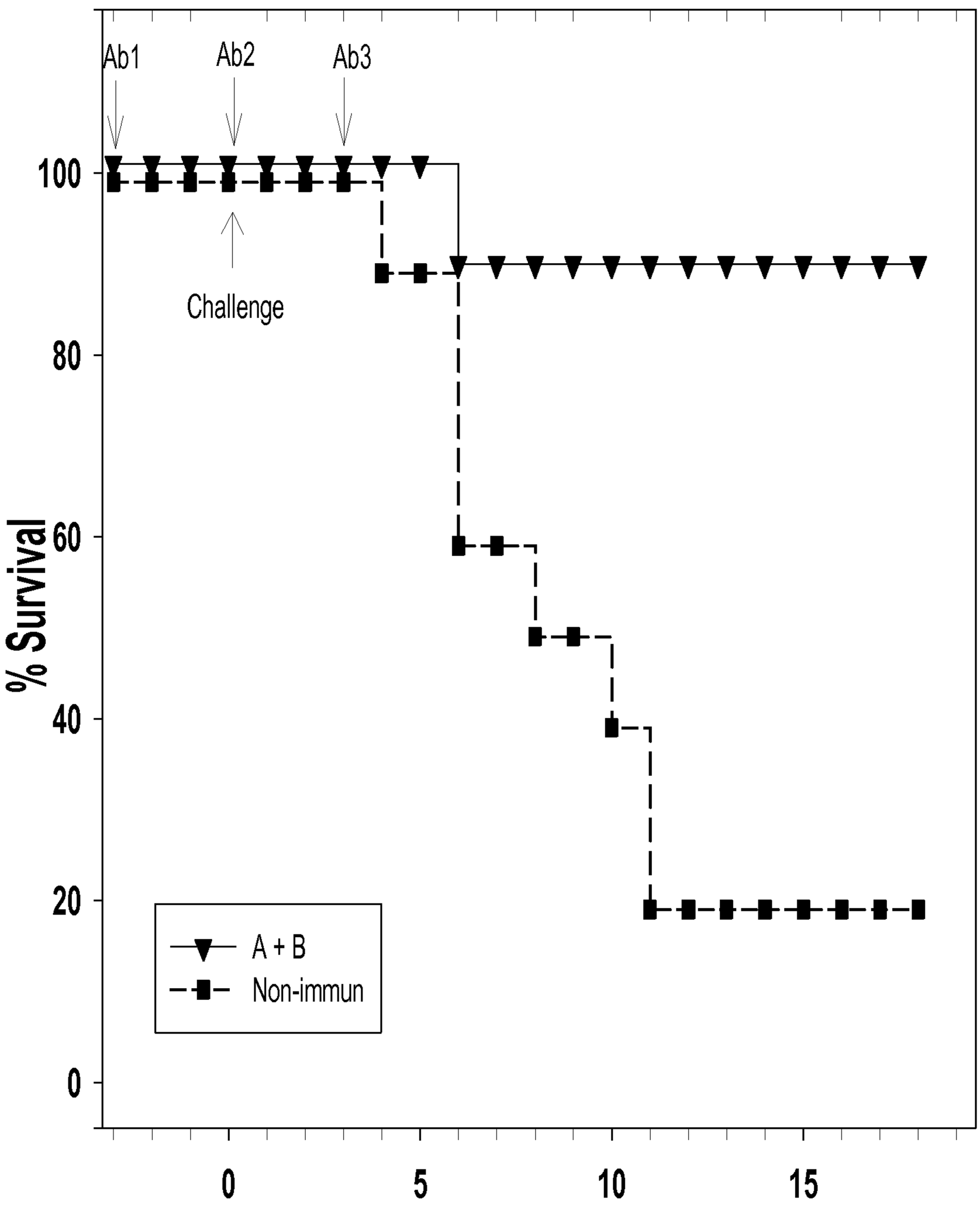

Ab1
Ab2
Ab3
Challenge
100
80
60
40
20
0
% Survival
A + B
Non-immun
0
5
10
15
Days Post Challenge

Figure 6

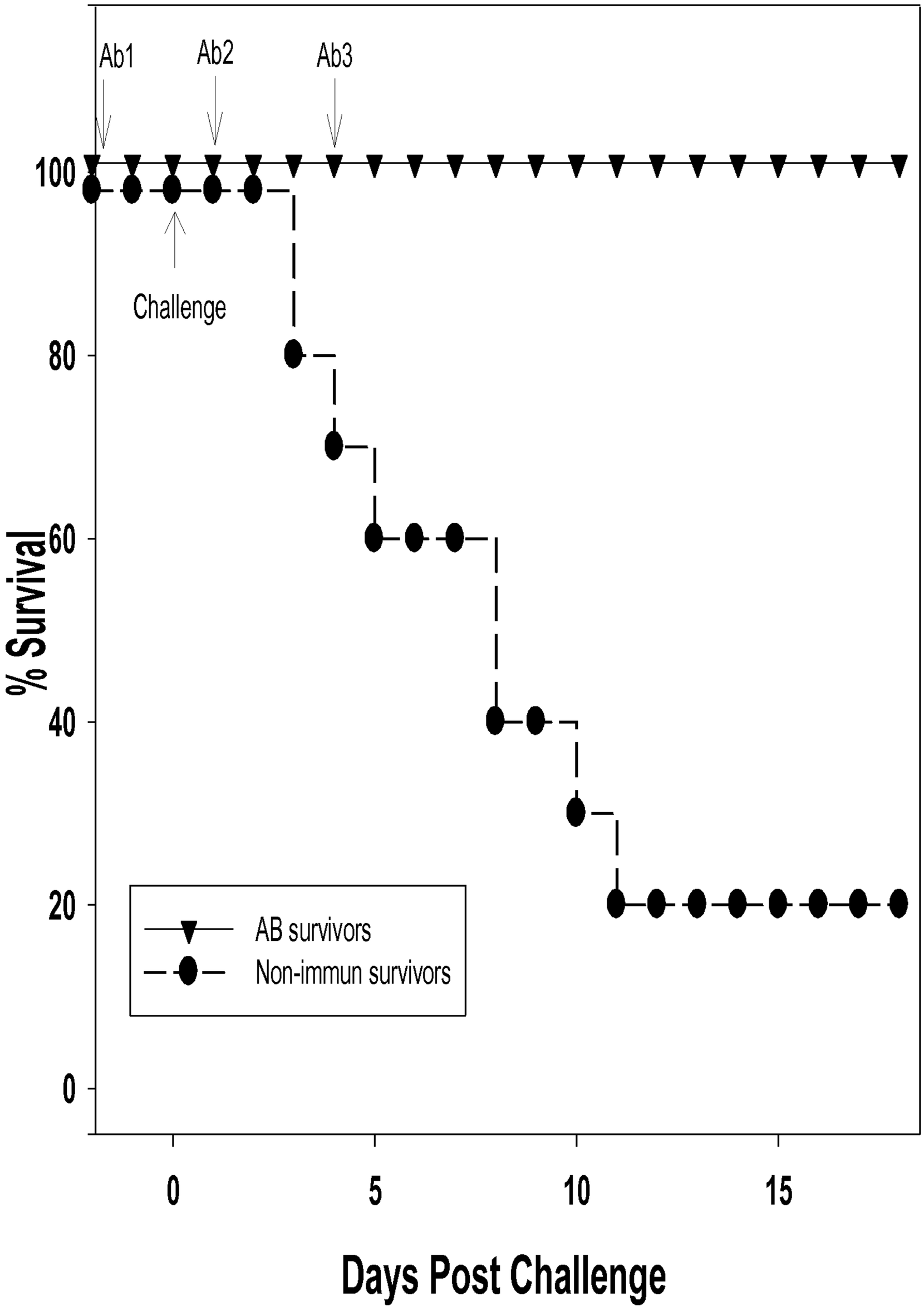
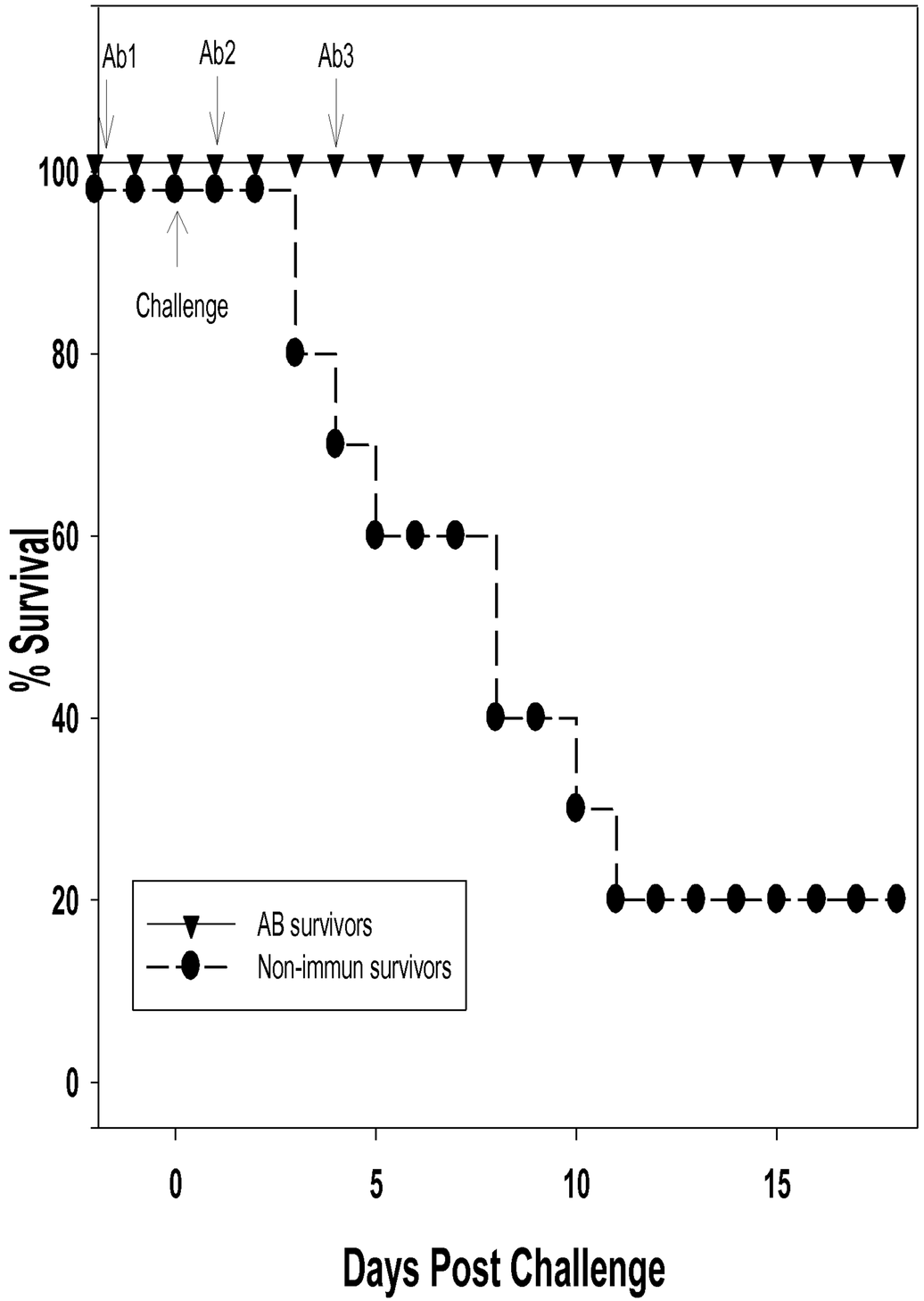

Ab1
Ab2
Ab3
Challenge
100
80
60
40
20
0
% Survival
AB survivors
Non-immun survivors
0
5
10
15
Days Post Challenge

Figure 6


Ab1
Ab2
Ab3
100
80
60
40
20
0
% Survival
Challenge
AB survivors
Non-immun survivors
0
5
10
15
Days Post Challenge