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(54) Title: POLYPEPTIDE COMPRISING AN IMMUNOGLOBULIN CHAIN VARIABLE DOMAIN WHICH BINDS TO CLOSTRIDIUM DIFFICILE TOXIN B

(57) Abstract: The application concerns polypeptides comprising an immunoglobulin chain variable domain which binds to Clostridium difficile toxin B and toxin A. These are derived from llama antibodies isolated using a phage display library. Modifications were introduced and their effect on the potency and protease stability was assayed. In some embodiments, dimeric and tetrameric constructs, quadrahead constructs, were produced.



POLYPEPTIDE COMPRISING AN IMMUNOGLOBULIN CHAIN VARIABLE DOMAIN WHICH BINDS
TO CLOSTRIDIUM DIFFICILE TOXIN B

FIELD OF THE INVENTION

5 The present invention relates to polypeptides comprising an immunoglobulin chain variable domain (or 'ICVD') which binds to *Clostridium difficile* toxin B ('TcdB' or 'toxin B') as well as to constructs and pharmaceutical compositions comprising these polypeptides. The present invention also relates to nucleic acids encoding such polypeptides, to methods for preparing such polypeptides, to cDNA and vectors comprising nucleic acids encoding such polypeptides,
10 to host cells expressing or capable of expressing such polypeptides and to uses of such polypeptides, pharmaceutical compositions or constructs.

BACKGROUND OF THE INVENTION

15 *Clostridium difficile*, a spore forming anaerobic bacillus is the causative agent of *C. difficile* infection. The hospital environment and patients undergoing antibiotic treatment provide a discrete ecosystem where *C. difficile* persists and selected virulent clones thrive. Consequently, *C. difficile* is the most frequent cause of nosocomial diarrhoea worldwide. Given the continued use of broad-spectrum antibiotics and the rising numbers of
20 immunocompromised and elderly patients, the problems associated with *C. difficile* infection are unlikely to recede.

The pathology of *C. difficile* is mediated by two toxins, toxin A and toxin B and it has been demonstrated that *C. difficile* ribotypes which lack these toxins do not cause pathogenic
25 infection. Each toxin alone is capable of causing the symptoms of disease. It is believed that the toxins mediate their effect locally by entering the epithelial cells lining the lumen of the colon resulting in cell death, with consequent fluid loss and diarrhoea. There is no consistent pathology associated with toxin entering the systemic circulation and therefore neutralisation of the toxins in the lumen of the colon may provide an effective therapy for this debilitating
30 condition.

Alarming, in the last 10 years a new group of highly virulent *C. difficile* ribotypes has emerged to cause outbreaks of increased disease severity. The most prevalent of the hypervirulent ribotypes is ribotype 027 (strain R20291, Stabler et al 2009 *Genome Biology* 10:R102) and
35 patients infected with this ribotype exhibit more severe diarrhoea, higher mortality and more recurrences. In Canada, 027 ribotypes were undetected in 2000 but reached 75.2% of all ribotypes isolated by 2003. The 027 ribotype also caused the outbreak of *C. difficile* at the Stoke Mandeville hospital in the UK. In less than three years a total of 498 patients acquired *C. difficile* at the hospital of whom 127 died.

40 The burden of *C. difficile* disease represented by ribotype 027, other newly emerging hypervirulent ribotypes such as PCR-ribotype 078 (strain 'M120', He et al 2010. *PNAS*. 107 (16) Table S1) and ribotypes such as 087 (strain VPI 10463, Zaib et al 2009 *BMC Microbiology*

9:6), 017 (strain M68, He et al 2010 *PNAS*. 107 (16)), 106 (strain Liv22) and 001 (strain Liv24) continue to be a major concern in hospitals throughout the developed world.

Current therapy for *C. difficile* involves a course of either vancomycin or metronidazole, which in the majority of patients resolves the symptoms and diarrhoea. Unfortunately on cessation of these antibiotics, 20-30% of patients relapse and a vicious cycle of further antibiotic courses and relapses can follow. Recurrence rates are worse with patients infected with the hypervirulent 027 ribotype.

Whilst there are new treatments in development for *C. difficile*, effectiveness against multiple ribotypes remains a challenge. In 2011 a new antibiotic, Fidaxomicin, the first new treatment for *C. difficile* in more than 20 years, was approved by the FDA and EMEA. Although Fidaxomicin demonstrated a reduced rate of recurrence in non-027 infected patients, it did not show any superiority versus vancomycin for patients infected with the 027 hypervirulent ribotype.

Merck have completed a Phase 2 trial of a combination of anti toxin A and anti toxin B antibodies administered systemically. These antibodies were originally developed by Medarex. Comparative experiments provided herein demonstrate that constructs of the present invention have a higher potency than Medarex-developed antibody mAb 124 against toxins of multiple ribotypes including 027.

There is, therefore, an overwhelming unmet need for therapies that will reduce relapse rates in patients infected with *C. difficile* and especially those infected with the hypervirulent 027 ribotype. Safe and effective therapeutics must be developed which are capable of neutralising TcdB from a broad range of *C. difficile* ribotypes, including TcdB from ribotype 027. This is particularly challenging due to the significant sequence variation of toxins produced by different ribotypes of *C. difficile* (see Table 1B below).

WO 2006/121422 discloses antibodies that specifically bind to toxins of *C. difficile*, antigen binding portions thereof, and methods of making and using said antibodies and antigen binding portions. WO 2011/130650 discloses regents, compositions and therapies with which to treat *C. difficile* infection and related disease conditions and pathologies, including in particular antibodies or antigen-binding fragments thereof that bind specifically to TcdA and/or TcdB of *C. difficile* and neutralise the activities of these toxins. WO 2012/055030 discloses *C. difficile* toxin-specific antibodies, compositions and uses thereof, which toxin-specific antibodies may be specific for either TcdA or TcdB.

Polypeptides of the present invention may, in at least some embodiments, have one or more of the following advantages compared to anti-TcdB substances of the prior art:

- (i) increased affinity for TcdB;
- (ii) increased specificity for TcdB;

- (iii) increased neutralising capability against TcdB;
 - (iv) increased cross-reactivity with TcdB from multiple different ribotypes of *C. difficile*, particularly ribotypes 027, 087, 078, 106, 001 and 017;
 - (v) reduced immunogenicity when administered to a human;
 - 5 (vi) increased stability in the presence of proteases, for example (a) in the presence of proteases present in the small and/or large intestine and/or *C. difficile*-specific proteases and/or inflammatory proteases, for example enteropeptidase, trypsin, chymotrypsin and/or (b) in the presence of proteases from gut commensal microflora and/or pathogenic bacteria, actively secreted and/or released by lysis
 - 10 of microbial cells;
 - (vii) increased stability to protease degradation during production (for example resistance to yeast proteases);
 - (viii) increased suitability for oral administration;
 - (ix) increased suitability for local delivery to the intestinal tract following oral
 - 15 administration;
 - (x) increased suitability for expression, in a heterologous host such as bacteria such as *Escherichia coli* and/or a yeast such as a yeast belonging to the genera *Aspergillus*, *Saccharomyces*, *Kluyveromyces*, *Hansenula* or *Pichia*, such as *Saccharomyces cerevisiae* or *Pichia pastoris*;
 - 20 (xi) suitability for, and improved properties for, use in a pharmaceutical;
 - (xii) suitability for, and improved properties for, use in a functional food;
 - (xiii) increased suitability for formatting in a multispecific format;
 - (xiv) binding to novel, advantageous epitopes.
- 25 Advantages (i) to (xiv) above may potentially be realised by the polypeptides of the present invention in a monovalent format or in a multivalent format such as a bihead format (for example homobihead or heterobihead formats) or a quadrahead format.

SUMMARY OF THE INVENTION

30 The present inventors have produced surprisingly advantageous polypeptides comprising immunoglobulin chain variable domains which bind to TcdB. These polypeptides, in preferred embodiments, benefit from surprisingly high potency against TcdB from multiple ribotypes of *C. difficile* and more particularly remain stable on exposure to proteases such as trypsin,

35 chymotrypsin and/or proteases present in the small and large intestine. In one embodiment, these polypeptides have undergone further enhancement by engineering. It is expected that these further enhanced polypeptides benefit from the above advantages, retain their TcdB-neutralising activity during passage through the intestinal tract and further resist degradation and/or inactivation by proteases present in the intestinal tract. It may be expected that these

40 polypeptides have particular utility in the prevention or treatment of *C. difficile* infection, particularly when administered orally.

The present invention provides a polypeptide comprising an immunoglobulin chain variable domain which binds to *Clostridium difficile* toxin B, wherein the immunoglobulin chain variable domain comprises three complementarity determining regions (CDR1-CDR3) and four framework regions (FR1-FR4), wherein:

(a) CDR1 comprises a sequence sharing 40% or greater sequence identity with SEQ ID NO: 1, CDR2 comprises a sequence sharing 55% or greater sequence identity with SEQ ID NO: 2 and CDR3 comprises a sequence sharing 50% or greater sequence identity with SEQ ID NO: 3; or

(b) CDR1 comprises a sequence sharing 40% or greater sequence identity with SEQ ID NO: 4, CDR2 comprises a sequence sharing 55% or greater sequence identity with SEQ ID NO: 5 and CDR3 comprises a sequence sharing 60% or greater sequence identity with SEQ ID NO: 6.

In order to facilitate understanding of the invention and with no limiting effect, option (a) relates to the Q10F1arm ICVD sequence and option (b) relates to the Q31B1arm and/or Q35H8 ICVD sequences.

DESCRIPTION OF THE FIGURES

- Figure 1 Example TcdB dose-response curve on Vero cells
- Figure 2 Dose response curves of TcdB ribotypes 027 and 087 by B10F1
- Figure 3 Dose response curves of TcdB ribotypes 027 and 087 by Q31B1
- 25 Figure 4 Dose response curve of TcdB ribotype 027 by Q35H8
- Figure 5 Dose response curves of TcdB ribotypes 027 and 087 by ID1B, ID24B, ID25B and ID27B
- Figure 6 Dose response curves of TcdB ribotypes 027 and 087 by ID2B, ID20B, ID21B and ID22B
- 30 Figure 7 Dose response curves of TcdB ribotypes 027, 087, 106, 001 and 078 by ID41B
- Figure 8 Dose response curves of TcdB ribotype 017 by ID41B and ID43B
- Figure 9 Dose response curves of TcdB ribotype 017 by ID45B, ID46B and ID49B
- Figure 10 Results of incubation of ID11B and ID43B with trypsin and chymotrypsin beads
- Figure 11 Results of incubation of ID11B and ID43B in faecal supernatant pools
- 35 Figure 12 Dose response curves of TcdB ribotype 106, 001, 078, 017, 027 and 087 by ID11B and Mab124
- Figure 13 Dose response curves of TcdB ribotype 106, 001, 078, 017, 027 and 087 by ID12B and Mab124
- Figure 14 Dose response curves of TcdB ribotype 106, 001, 078, 017, 027 and 087 by ID43B and Mab124
- 40 Figure 15 Dose response curves of multiple TcdA and TcdB ribotypes by ID1C and ID3C
- Figure 16 Dose response curves of ribotype 027 TcdA and TcdB by ID5C
- Figure 17 Dose response curves of multiple TcdA ribotypes by ID8C compared to ID33A

- Figure 18 Dose response curves of multiple TcdB ribotypes by ID8C compared to ID43A
 Figure 19 Dose response curves of ribotype 027 TcdA and TcdB by ID6C
 Figure 20 Dose response curves of multiple TcdA ribotypes by ID7C compared to ID17A
 Figure 21 Dose response curves of multiple TcdB ribotypes by ID7C compared to ID41A
 5 Figure 22 Dose response curves of ribotype 027 and 087 TcdA and TcdB by ID11C

DESCRIPTION OF THE SEQUENCES

- SEQ ID NO: 1 – Polypeptide sequence of B10F1arm CDR1
 10 SEQ ID NO: 2 – Polypeptide sequence of B10F1arm CDR2
 SEQ ID NO: 3 – Polypeptide sequence of B10F1arm CDR3
 SEQ ID NO: 4 – Polypeptide sequence of Q31B1arm CDR1
 SEQ ID NO: 5 – Polypeptide sequence of Q31B1arm CDR2
 SEQ ID NO: 6 – Polypeptide sequence of Q31B1arm CDR3
 15 SEQ ID NO: 7 – Polypeptide sequence of Q35H8 CDR1
 SEQ ID NO: 8 – Polypeptide sequence of Q35H8 CDR2
 SEQ ID NO: 9 – Polypeptide sequence of Q35H8 CDR3
 SEQ ID NO: 10 – Polypeptide sequence of B10F1arm
 SEQ ID NO: 11 – Polypeptide sequence of Q31B1arm
 20 SEQ ID NO: 12 – Polypeptide sequence of Q35H8
 SEQ ID NO: 13 – Polypeptide sequence of ID1B
 SEQ ID NO: 14 – Polypeptide sequence of ID2B
 SEQ ID NO: 15 – Polypeptide sequence of ID3B
 SEQ ID NO: 16 – Polypeptide sequence of ID11B
 25 SEQ ID NO: 17 – Polypeptide sequence of ID12B
 SEQ ID NO: 18 – Polypeptide sequence of ID20B
 SEQ ID NO: 19 – Polypeptide sequence of ID21B
 SEQ ID NO: 20 – Polypeptide sequence of ID22B
 SEQ ID NO: 21 – Polypeptide sequence of ID24B
 30 SEQ ID NO: 22 – Polypeptide sequence of ID25B
 SEQ ID NO: 23 – Polypeptide sequence of ID27B
 SEQ ID NO: 24 – Polypeptide sequence of ID41B
 SEQ ID NO: 25 – Polypeptide sequence of ID43B
 SEQ ID NO: 26 – Polypeptide sequence of ID45B
 35 SEQ ID NO: 27 – Polypeptide sequence of ID46B
 SEQ ID NO: 28 – Polypeptide sequence of ID49B
 SEQ ID NO: 29 – Polypeptide sequence of B10F1
 SEQ ID NO: 30 – Polypeptide sequence of Q31B1
 SEQ ID NO: 31 – Polynucleotide sequence of 3' primer mentioned in Preparative Methods
 40 section
 SEQ ID NO: 32 – Polynucleotide sequence of M13.rev used in Example 2
 SEQ ID NO: 33 – Polynucleotide sequence of M13.fw used in Example 2
 SEQ ID NO: 34 – Polynucleotide sequence encoding ID11B

- SEQ ID NO: 35 – Polynucleotide sequence encoding ID12B
 SEQ ID NO: 36 – Polynucleotide sequence encoding ID41B
 SEQ ID NO: 37 – Polynucleotide sequence encoding ID43B
 SEQ ID NO: 38 – Polynucleotide sequence encoding B10F1arm (in ID43B)
 5 SEQ ID NO: 39 – Polynucleotide sequence encoding Q31B1arm (in ID43B)
 SEQ ID NO: 40 – Polynucleotide sequence encoding Q35H8arm (in ID12B)
 SEQ ID NO: 41 – Polypeptide sequence of ID1C
 SEQ ID NO: 42 – Polypeptide sequence of ID3C
 SEQ ID NO: 43 – Polypeptide sequence of ID5C
 10 SEQ ID NO: 44 – Polypeptide sequence of ID6C
 SEQ ID NO: 45 – Polypeptide sequence of ID7C
 SEQ ID NO: 46 – Polypeptide sequence of ID8C
 SEQ ID NO: 47 – Polypeptide sequence of ID11C
 SEQ ID NO: 48 – Polypeptide sequence of Q34A3 (anti-TcdA ICVD)
 15 SEQ ID NO: 49 – Polypeptide sequence of B4F10 (anti-TcdA ICVD)
 SEQ ID NO: 50 – Polypeptide sequence of ID33A (anti-TcdA bihead)
 SEQ ID NO: 51 – Polypeptide sequence of ID17A (anti-TcdA bihead)
 SEQ ID NO: 52 – Polypeptide sequence of TcdB from *C. difficile* ribotype 087
 SEQ ID NO: 53 – Polypeptide sequence of TcdB from *C. difficile* ribotype 078
 20 SEQ ID NO: 54 – Polypeptide sequence of TcdB from *C. difficile* ribotype 017
 SEQ ID NO: 55 – Polypeptide sequence of TcdB from *C. difficile* ribotype 027

DETAILED DESCRIPTION OF THE INVENTION

25 Polypeptides including antibodies and antibody fragments including ICVDs such as the VH and VHH

A conventional antibody or immunoglobulin (Ig) is a protein comprising four polypeptide chains: two heavy (H) chains and two light (L) chains. Each chain is divided into a constant region and
 30 a variable domain. The heavy chain variable domains are abbreviated herein as VHC, and the light (L) chain variable domains are abbreviated herein as VLC. These domains, domains related thereto and domains derived therefrom, are referred to herein as immunoglobulin chain variable domains. The VHC and VLC domains can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDRs"), interspersed with
 35 regions that are more conserved, termed "framework regions" ("FRs"). The framework and complementarity determining regions have been precisely defined (Kabat et al 1991 Sequences of Proteins of Immunological Interest, *Fifth Edition U.S. Department of Health and Human Services*, NIH Publication Number 91-3242, herein incorporated by reference in its entirety). In a conventional antibody, each VHC and VLC is composed of three CDRs and four
 40 FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The conventional antibody tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains is formed with the heavy and the light immunoglobulin chains inter-connected by e.g. disulfide bonds, and the heavy chains

similarity connected. The heavy chain constant region includes three domains, CH1, CH2 and CH3. The light chain constant region is comprised of one domain, CL. The variable domain of the heavy chains and the variable domain of the light chains are binding domains that interact 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 (C1q) of the classical complement system. The term antibody includes immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be kappa or lambda types. The overall structure of immunoglobulin-gamma (IgG) antibodies assembled from two identical heavy (H)-chain and two identical light (L)-chain polypeptides is well established and highly conserved in mammals (Padlan 1994 *Mol Immunol* 31:169-217).

An exception to conventional antibody structure is found in sera of Camelidae. In addition to conventional antibodies, these sera possess special IgG antibodies. These IgG antibodies, known as heavy-chain antibodies (HCAbs), are devoid of the L chain polypeptide and lack the first constant domain (CH1). At its N-terminal region, the H chain of the homodimeric protein contains an immunoglobulin chain variable domain, referred to as the VHH, which serves to associate with its cognate antigen (Muyldermans 2013 *Annu Rev Biochem* 82:775-797, Hamers-Casterman et al 1993 *Nature* 363(6428):446-448, Muyldermans et al 1994 *Protein Eng* 7(9):1129-1135, herein incorporated by reference in their entirety).

An antigen-binding fragment (or "antibody fragment" or "immunoglobulin fragment") as used herein refers to a portion of an antibody that specifically binds to TcdB (e.g. a molecule in which one or more immunoglobulin chains is not full length, but which specifically binds to TcdB). Examples of binding fragments encompassed within the term antigen-binding fragment include:

- (i) a Fab fragment (a monovalent fragment consisting of the VLC, VHC, CL and CH1 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 Fd fragment (consisting of the VHC and CH1 domains);
- (iv) a Fv fragment (consisting of the VLC and VHC domains of a single arm of an antibody);
- (v) an scFv fragment (consisting of VLC and VHC domains 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);
- (vi) a VH (an immunoglobulin chain variable domain consisting of a VHC domain (Ward et al *Nature* 1989 341:544-546);
- (vii) a VL (an immunoglobulin chain variable domain consisting of a VLC domain);
- (viii) a V-NAR (an immunoglobulin chain variable domain consisting of a VHC domain from chondrichthyes IgNAR (Roux et al 1998 *Proc Natl Acad Sci USA* 95:11804-11809 and Griffiths et al 2013 *Antibodies* 2:66-81, herein incorporated by reference in their entirety)
- (ix) a VHH.

The total number of amino acid residues in an immunoglobulin chain variable domain such as a VHH or VH may be in the region of 110-130.

Immunoglobulin chain variable domains of the invention may for example be obtained by preparing a nucleic acid encoding an immunoglobulin chain variable domain using techniques for nucleic acid synthesis, followed by expression of the nucleic acid thus obtained. According to a specific embodiment, an immunoglobulin chain variable domain of the invention does not have an amino acid sequence which is exactly the same as (i.e. shares 100% sequence identity with) the amino acid sequence of a naturally occurring polypeptide such as a VH or VHH domain of a naturally occurring antibody.

Substituting at least one amino acid residue in the framework region of a non human immunoglobulin variable domain with the corresponding residue from a human variable domain is humanisation. Humanisation of a variable domain may reduce immunogenicity in humans.

Suitably the polypeptide of the present invention consists of an immunoglobulin chain variable domain. Suitably, the polypeptide of the present invention is an antibody or an antibody fragment. Suitably the antibody fragment is a VHH, a VH, a VL, a V-NAR, a Fab fragment, a VL or a F(ab')₂ fragment (such as a VHH or VH, most suitably a VHH).

Specificity, affinity, avidity and cross-reactivity

Specificity refers to the number of different types of antigens or antigenic determinants to which a particular antigen-binding polypeptide can bind. The specificity of an antigen-binding polypeptide is the ability of the antigen-binding polypeptide to recognise a particular antigen as a unique molecular entity and distinguish it from another.

Affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding polypeptide (K_d), is a measure of the binding strength between an antigenic determinant and an antigen-binding site on the antigen-binding polypeptide: the lesser the value of the K_d , the stronger the binding strength between an antigenic determinant and the antigen-binding polypeptide (alternatively, the affinity can also be expressed as the affinity constant (K_a), which is $1/K_d$). Affinity can be determined by known methods, depending on the specific antigen of interest.

Avidity is the measure of the strength of binding between an antigen-binding polypeptide and the pertinent antigen. Avidity is related to both the affinity between an antigenic determinant and its antigen binding site on the antigen-binding polypeptide and the number of pertinent binding sites present on the antigen-binding polypeptide.

Suitably, antigen-binding polypeptides of the invention will bind with a dissociation constant (K_d) of 10^{-6} to 10^{-12} M, more suitably 10^{-7} to 10^{-12} M, more suitably 10^{-8} to 10^{-12} M and more suitably 10^{-9} to 10^{-12} M.

Any K_d value less than 10^{-6} is considered to indicate binding. Specific binding of an antigen-binding polypeptide to an antigen or antigenic determinant can be determined in any suitable known manner, including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known in the art.

An anti-TcdB polypeptide, a polypeptide which interacts with TcdB, or a polypeptide against TcdB, are all effectively polypeptides which bind to TcdB. A polypeptide of the invention may bind to a linear or conformational epitope on TcdB. The term "binds to TcdB" means binding to any one or more of the N-terminal, hydrophobic or C-terminal domains of TcdB.

Suitably, the polypeptide of the invention is capable of neutralising TcdB from multiple ribotypes of *C. difficile*. More suitably, the polypeptide of the invention is capable of neutralising TcdA from ribotypes 087, 027, 078, 017, 106 and 001. More suitably, the polypeptide of the invention will neutralise TcdB from ribotype 027.

Suitably the polypeptide of the invention is directed against one or more epitopes on TcdB such that said polypeptide of the invention, upon binding to TcdB, is capable of inhibiting or reducing the cytotoxic effect that is mediated by said TcdB. Suitably, the polypeptide of the invention binds to the cell binding domain of *Clostridium difficile* toxin B.

The polypeptides of the present invention bind to one or more epitope(s) on TcdB. In one aspect of the invention there is provided a polypeptide which binds to the same epitope on TcdB as B10F1, Q31B1, Q35H8, ID1B, ID2B, ID3B, ID11B, ID12B, ID20B, ID21B, ID22B, ID24B, ID25B, ID27B, ID41B, ID43B, Q31B1arm, B10F1arm, ID45B, ID46B or ID49B.

Suitably, the polypeptide of the invention is isolated. An "isolated" polypeptide is one that is removed from its original environment. For example, a naturally-occurring polypeptide of the invention is isolated if it is separated from some or all of the coexisting materials in the natural system.

Potency, inhibition and neutralisation

Potency is a measure of the activity of a therapeutic agent expressed in terms of the amount required to produce an effect of given intensity. A highly potent agent evokes a greater response at low concentrations compared to an agent of lower potency that evokes a smaller response at low concentrations. Potency is a function of affinity and efficacy. Efficacy refers to the ability of therapeutic agent to produce a biological response upon binding to a target ligand and the quantitative magnitude of this response. The term half maximal effective concentration (EC50) refers to the concentration of a therapeutic agent which causes a response halfway between the baseline and maximum after a specified exposure time. The therapeutic agent may cause inhibition or stimulation. It is commonly used, and is used herein, as a measure of potency.

A neutralising polypeptide for the purposes of the invention is a polypeptide which defends a cell from the effects of TcdB by, for example, inhibiting the biological effect of TcdB. The effectiveness of an anti-TcdB therapeutic agent can be ascertained using a neutralisation assay. A particularly suitable neutralisation assay is the measurement of Vero cell viability using Alamar Blue (Fields and Lancaster *American Biotechnology Laboratory* 1993 11(4):48-50). Using a range of known concentrations of anti-TcdB polypeptide, this assay can be performed to ascertain the ability of an anti-TcdB polypeptide to neutralise the effects of TcdB cytotoxicity by producing a dose-response curve and/or by ascertaining the half maximal effective concentration (EC50) of the anti-TcdB polypeptide. This standard Vero cell assay is used herein and detailed further in the Examples section below.

Suitably the polypeptide or construct of the invention neutralises TcdB cytotoxicity (such as TcdB ribotype 087, 027, 078, 017, 106 and 001) in the standard Vero cell assay with an EC50 of 50000 pM or less, such as 40000 pM or less, such as 30000 pM or less, such as 20000 pM or less, such as 10000 pM or less, such as 5000 pM or less, such as 4000 pM or less, such as 3000 pM or less, such as 2000 pM or less, such as 1000 pM or less, such as 500 pM or less, such as 250 pM or less, such as 100 pM or less, such as 80 pM or less, such as 60 pM or less, such as 40 pM or less, such as 30 pM or less, such as 20 pM or less, such as 10 pM or less.

In one aspect of the invention there is provided a VH or VHH which specifically binds to and has neutralising activity against *Clostridium difficile* toxin B. More suitably there is provided a VH or VHH which specifically binds to and has neutralising activity against toxin B of more than one strain of *C. difficile*. More specifically there is provided a VH or VHH which specifically binds to and has neutralising activity against toxin B of two or more of *C. difficile* ribotypes 027, 087, 078, 106, 001 and 017.

Polypeptide and polynucleotide sequences

For the purposes of comparing two closely-related polypeptide sequences, the "% sequence identity" between a first polypeptide sequence and a second polypeptide sequence may be calculated using NCBI BLAST v2.0, using standard settings for polypeptide sequences (BLASTP). For the purposes of comparing two closely-related polynucleotide sequences, the "% sequence identity" between a first nucleotide sequence and a second nucleotide sequence may be calculated using NCBI BLAST v2.0, using standard settings for nucleotide sequences (BLASTN).

Polypeptide or polynucleotide sequences are said to be the same as or identical to other polypeptide or polynucleotide sequences, if they share 100% sequence identity over their entire length. Residues in sequences are numbered from left to right, i.e. from N- to C-terminus for polypeptides; from 5' to 3' terminus for polynucleotides.

A "difference" between sequences refers to an insertion, deletion or substitution of a single amino acid residue in a position of the second sequence, compared to the first sequence. Two polypeptide sequences can contain one, two or more such amino acid differences. Insertions, deletions or substitutions in a second sequence which is otherwise identical (100% sequence identity) to a first sequence result in reduced % sequence identity. For example, if the identical sequences are 9 amino acid residues long, one substitution in the second sequence results in a sequence identity of 88.9%. If the identical sequences are 17 amino acid residues long, two substitutions in the second sequence results in a sequence identity of 88.2%. If the identical sequences are 7 amino acid residues long, three substitutions in the second sequence results in a sequence identity of 57.1%. If first and second polypeptide sequences are 9 amino acid residues long and share 6 identical residues, the first and second polypeptide sequences share greater than 66% identity (the first and second polypeptide sequences share 66.7% identity). If first and second polypeptide sequences are 17 amino acid residues long and share 16 identical residues, the first and second polypeptide sequences share greater than 94% identity (the first and second polypeptide sequences share 94.1% identity). If first and second polypeptide sequences are 7 amino acid residues long and share 3 identical residues, the first and second polypeptide sequences share greater than 42% identity (the first and second polypeptide sequences share 42.9% identity).

Alternatively, for the purposes of comparing a first, reference polypeptide sequence to a second, comparison polypeptide sequence, the number of additions, substitutions and/or deletions made to the first sequence to produce the second sequence may be ascertained. An addition is the addition of one amino acid residue into the sequence of the first polypeptide (including addition at either terminus of the first polypeptide). A substitution is the substitution of one amino acid residue in the sequence of the first polypeptide with one different amino acid residue. A deletion is the deletion of one amino acid residue from the sequence of the first polypeptide (including deletion at either terminus of the first polypeptide).

For the purposes of comparing a first, reference polynucleotide sequence to a second, comparison polynucleotide sequence, the number of additions, substitutions and/or deletions made to the first sequence to produce the second sequence may be ascertained. An addition is the addition of one nucleotide residue into the sequence of the first polynucleotide (including addition at either terminus of the first polynucleotide). A substitution is the substitution of one nucleotide residue in the sequence of the first polynucleotide with one different nucleotide residue. A deletion is the deletion of one nucleotide residue from the sequence of the first polynucleotide (including deletion at either terminus of the first polynucleotide).

A "conservative" amino acid substitution is an amino acid substitution in which an amino acid residue is replaced with another amino acid residue of similar chemical structure and which is expected to have little influence on the function, activity or other biological properties of the polypeptide. Such conservative substitutions suitably are substitutions in which one amino acid within the following groups is substituted by another amino acid residue from within the same group:

Group	Amino acid residue
Non-polar aliphatic	Glycine
	Alanine
	Valine
	Leucine
	Isoleucine
Aromatic	Phenylalanine
	Tyrosine
	Tryptophan
Polar uncharged	Serine
	Threonine
	Asparagine
	Glutamine
Negatively charged	Aspartate
	Glutamate
Positively charged	Lysine
	Arginine

As used herein, numbering of polypeptide sequences and definitions of CDRs and FRs are as defined according to the Kabat system (Kabat et al 1991 Sequences of Proteins of Immunological Interest, Fifth Edition U.S. Department of Health and Human Services, NIH Publication Number 91-3242, in conjunction with the methods for analysis of antibody sequence and structure described in Martin 2010 'Protein sequence and structure of antibody variable domains', *Antibody Engineering* volume 2, both herein incorporated by reference in their entirety). A "corresponding" amino acid residue between a first and second polypeptide sequence is an amino acid residue in a first sequence which shares the same position

according to the Kabat system with an amino acid residue in a second sequence, whilst the amino acid residue in the second sequence may differ in identity from the first. Suitably corresponding residues will share the same number (and letter) if the framework and CDRs are the same length according to Kabat definition. Alignment can be achieved manually or by using, for example, a known computer algorithm for sequence alignment such as NCBI BLAST v2.0 (BLASTP or BLASTN) using standard settings.

Suitably, the polynucleotides used in the present invention are isolated. An "isolated" polynucleotide is one that is removed from its original environment. For example, a naturally-occurring polynucleotide is isolated if it is separated from some or all of the coexisting materials in the natural system. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of its natural environment or if it is comprised within cDNA.

In one aspect of the invention there is provided a polynucleotide encoding the polypeptide or construct of the invention. Suitably the polynucleotide comprises or consists of a sequence sharing 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater, such as 99% or greater sequence identity with any one of SEQ ID NOs: 34-40. More suitably the polynucleotide comprises or consists of any one of SEQ ID NOs: 34-40. In a further aspect there is provided a cDNA comprising said polynucleotide.

In one aspect of the invention there is provided a polynucleotide comprising or consisting of a sequence sharing 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater, such as 99% or greater sequence identity with any one of the portions of any one of SEQ ID NOs: 34-40 which encodes CDR1, CDR2 or CDR3 of the encoded immunoglobulin chain variable domain.

Suitably, the polypeptide sequence of the present invention contains at least one alteration with respect to a native sequence. Suitably, the polynucleotide sequences of the present invention contain at least one alteration with respect to a native sequence. Suitably the alteration to the polypeptide sequence or polynucleotide sequence is made to increase stability of the polypeptide or encoded polypeptide to proteases present in the intestinal tract.

Table 1A: Kabat characterisation system applied to ICVD and ICVD construct sequences

CDRs 1, 2 and 3 are the first, second and third underlined portions of each ICVD. FRs 1, 2, 3 and 4 are the first, second, third and fourth portions joining the CDRs of each ICVD. The linker is also shown in the case of homobiheads or heterobiheads. Bold residues are substitutions of wild type residues. Substitution descriptions in brackets are referred-to by N-to-C-terminal numbering (as opposed to Kabat numbering).

B10F1 (unmodified) (SEQ ID NO: 29)

QVQLQESGGGLVQAGGSLRLSCAASGRTFS SYVMG WFRQAPGKEREFVA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQNLNLPEDTAVVYCAA SLTYGGRSARYDY WGQGTQVTVSS

10 Q31B1 (unmodified) (SEQ ID NO: 30)

EVQLVESGGGLVQAGDSLRLSCAASGRTL SYTMG WFRQAPEKEREFVA GSSRDGRTNYVANSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA HTTSGVPVRERSYAY WGQGTQVTVSS

Q35H8 (unmodified) (SEQ ID NO: 12)

EVQLVESGGGWVQPGDSLRLSCVASGRPLS SFTMG WFRQAPEKEREFVG GKSRDGRITTYSNSVKG RFTIDRDDAQNTVYLQMNSLNPDDTAVVYCAA HTTSGVPVRVKSAY WGQGTQVTVSS

15

ID1B (B10F1 with Q1D and R27A) (SEQ ID NO: 13)

DVQLQESGGGLVQAGGSLRLSCAASGATFS SYVMG WFRQAPGKEREFVA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQNLNLPEDTAVVYCAA SLTYGGRSARYDY WGQGTQVTVSS

20 ID2B (Q31B1 with E1D, V5Q and R27A) (SEQ ID NO: 14)

DVQLQESGGGLVQAGDSLRLSCAASGATLS SYTMG WFRQAPEKEREFVA GSSRDGRTNYVANSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA HTTSGVPVRERSYAY WGQGTQVTVSS

ID3B (Q35H8 with E1D, V5Q, P14A and R27A) (SEQ ID NO: 15)

25

DVQLQESGGGWVQAGDSLRLSCVASGAPLS SFTMG WFRQAPEKEREFVG GKSRDGRITTYSNSVKG RFTIDRDDAQNTVYLQMNSLNPDDTAVVYCAA HTTSGVPVRVKSAY WGQGTQVTVSS

15

ID11B (Q31B1 x B10F1 hetero bihead with [G₄S]₄ linker) (SEQ ID NO: 16)

DVQLQESGGGLVQAGDSLRLSCAASGRTL SYTMG WFRQAPEKEREFA GSSRDGRTNYANSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA HTTSGVPVRERSYAY WGQGTQVTSS
GGGGGGGGGGGGGGGGGG

5 DVQLQESGGGLVQAGDSLRLSCAASGRTFS SYIMG WFRQAPGKEREFA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA SLTYGRSARYDY WGQGTQVTSS

ID12B (Q35H8 x B10F1 hetero bihead with [G₄S]₄ linker) (SEQ ID NO: 17)

DVQLQESGGGWVQAGDSLRLSCVASGRPLS SFTMG WFRQAPEKEREFLG GKSRDGRTTYSNSVKG RFTIDRDDAQNTVYLQMNSLNPDDTAVVYCAA HTTSGVPVRVKSAY WGQGTQVTSS
GGGGGGGGGGGGGGGGGG

10 DVQLQESGGGLVQAGDSLRLSCAASGRTFS SYIMG WFRQAPGKEREFA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA SLTYGRSARYDY WGQGTQVTSS

ID20B (ID2B with M34I, R53H, R56H) (SEQ ID NO: 18)

15 DVQLQESGGGLVQAGDSLRLSCAASGATLS SYTIG WFRQAPEKEREFA GSSHDGHTNYANSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA HTTSGVPVRERSYAY WGQGTQVTSS

ID21B (ID2B with M34I, R107H) (SEQ ID NO: 19)

DVQLQESGGGLVQAGDSLRLSCAASGATLS SYTIG WFRQAPEKEREFA GSSRDGRTNYANSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA HTTSGVPVHERSYAY WGQGTQVTSS

20

ID22B (ID2B with M34I, R109H) (SEQ ID NO: 20)

DVQLQESGGGLVQAGDSLRLSCAASGATLS SYTIG WFRQAPEKEREFA GSSRDGRTNYANSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA HTTSGVPVREHSYAY WGQGTQVTSS

25 ID24B (ID1B with M34I, R58H) (SEQ ID NO: 21)

DVQLQESGGGLVQAGDSLRLSCAASGATFS SYIIG WFRQAPGKEREFA AINGSGGNHISADSVKG RFTISRDNAKNTVYLQMNSLKPEDTAVVYCAA SLTYGRSARYDY WGQGTQVTSS

16

ID25B (ID1B with M34I, R108H) (SEQ ID NO: 22)

DVQLQESGGGLVQAGGSLRLSCAASGATFS SYVIG WFRQAPGKEREFVA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA SLTYYGSRSAHYDY WGQGTQVTSS

5 ID27B (ID1B with M34I, R105H) (SEQ ID NO: 23)

DVQLQESGGGLVQAGGSLRLSCAASGATFS SYVIG WFRQAPGKEREFVA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA SLTYYGHSAHYDY WGQGTQVTSS

ID41B ((ID2B with R107H) x (ID1B with R105H), with [G₄S]₄ linker) (SEQ ID NO: 24)

10

DVQLQESGGGLVQAGGSLRLSCAASGATLS SYTMG WFRQAPEKEREFVA GSSRDGRITNYANSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA HTTSGVPVHERSYAY WGQGTQVTSS
GGGSGGGGGGGGGGGG

DVQLQESGGGLVQAGGSLRLSCAASGATFS SYVMG WFRQAPGKEREFVA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA SLTYYGHSAHYDY WGQGTQVTSS

15 ID43B ID21B ((ID2B with R108H) x (ID1B with R105H), with [G₄S]₄ linker) (SEQ ID NO: 25)

DVQLQESGGGLVQAGGSLRLSCAASGATLS SYTMG WFRQAPEKEREFVA GSSRDGRITNYANSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA HTTSGVPVHERSYAY WGQGTQVTSS
GGGSGGGGGGGGGGGG

DVQLQESGGGLVQAGGSLRLSCAASGATFS SYVMG WFRQAPGKEREFVA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA SLTYYGHSAHYDY WGQGTQVTSS

20

Q31B1arm (modified Q31B1 arm of 43B) (SEQ ID NO: 11)

DVQLQESGGGLVQAGGSLRLSCAASGATLS SYTMG WFRQAPEKEREFVA GSSRDGRITNYANSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA HTTSGVPVHERSYAY WGQGTQVTSS

25 B10F1arm (modified B10F1 arm of 43B) (SEQ ID NO: 10)

DVQLQESGGGLVQAGGSLRLSCAASGATFS SYVMG WFRQAPGKEREFVA AINGSGGNRISADSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA SLTYYGHSAHYDY WGQGTQVTSS

ID45B (ID2B with D1E and Q5V, wild type R107) (SEQ ID NO: 26)

EVQLVESGGGLVQAGGSLRLSCAASGATLS SYTMG WFRQAPEKEREFVA GSSRDGRITNYANSVKG RFTISRDNAKNTVYLQNSLKPEDTAVVYCAA HTTSGVPVHERSYAY WGQGTQVTSS

30

17

ID46B (ID45B with R107H) (SEQ ID NO: 27)

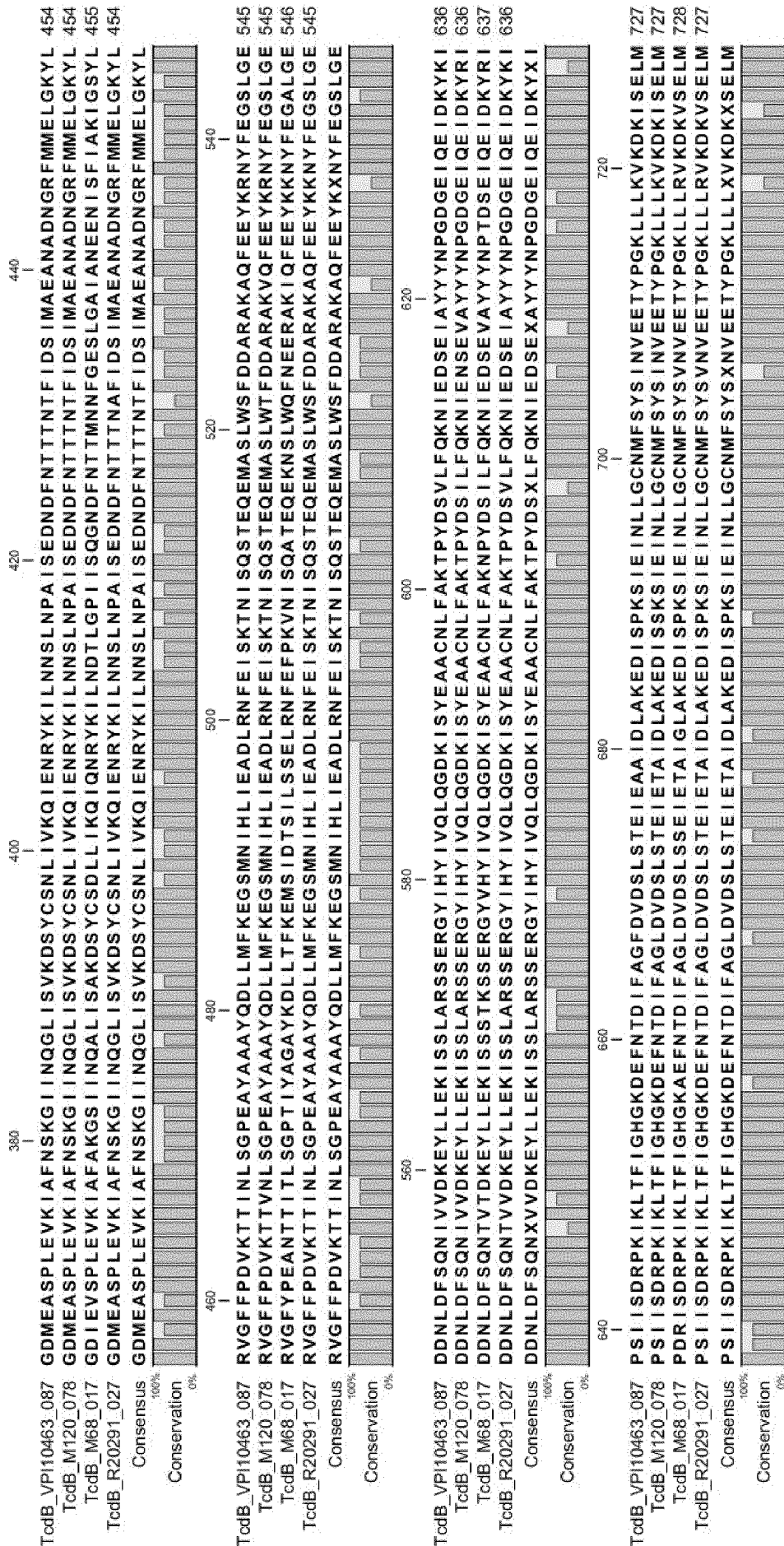
EVQLVESGGGLVQAGDSLRLSCAASGATLS SYTMG WFRQAPEKEREFEVA GSSRDGRTNYYANSVKG RFTISRDNAKNTVYVYLMNSLKPEDTAVWYCAA HTTSGVPVHERSYAY WGQGTQVTVSS

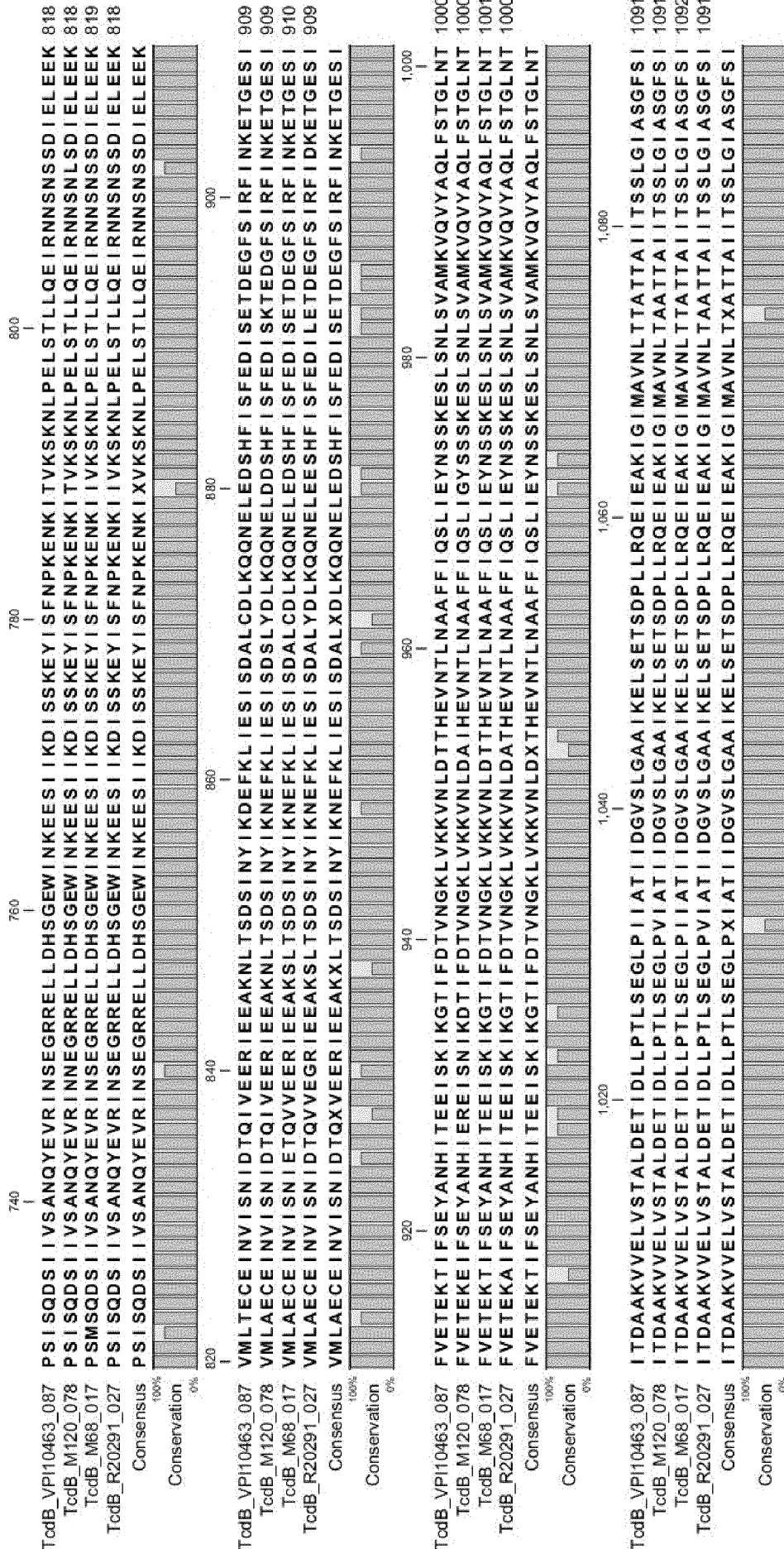
5 ID49B (ID45B with R107F) (SEQ ID NO: 28)

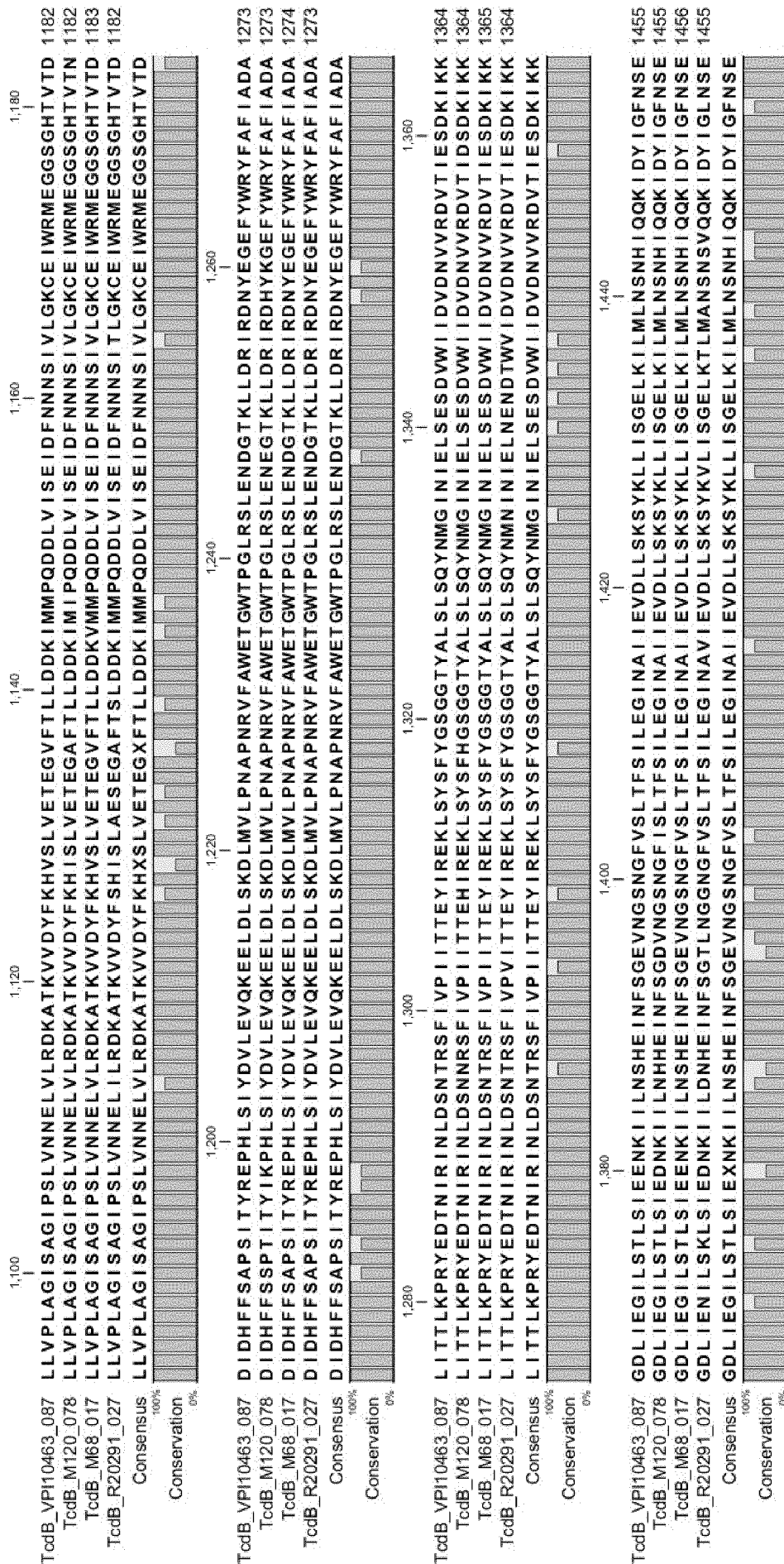
EVQLVESGGGLVQAGDSLRLSCAASGATLS SYTMG WFRQAPEKEREFEVA GSSRDGRTNYYANSVKG RFTISRDNAKNTVYVYLMNSLKPEDTAVWYCAA HTTSGVPVFERSYAY WGQGTQVTVSS

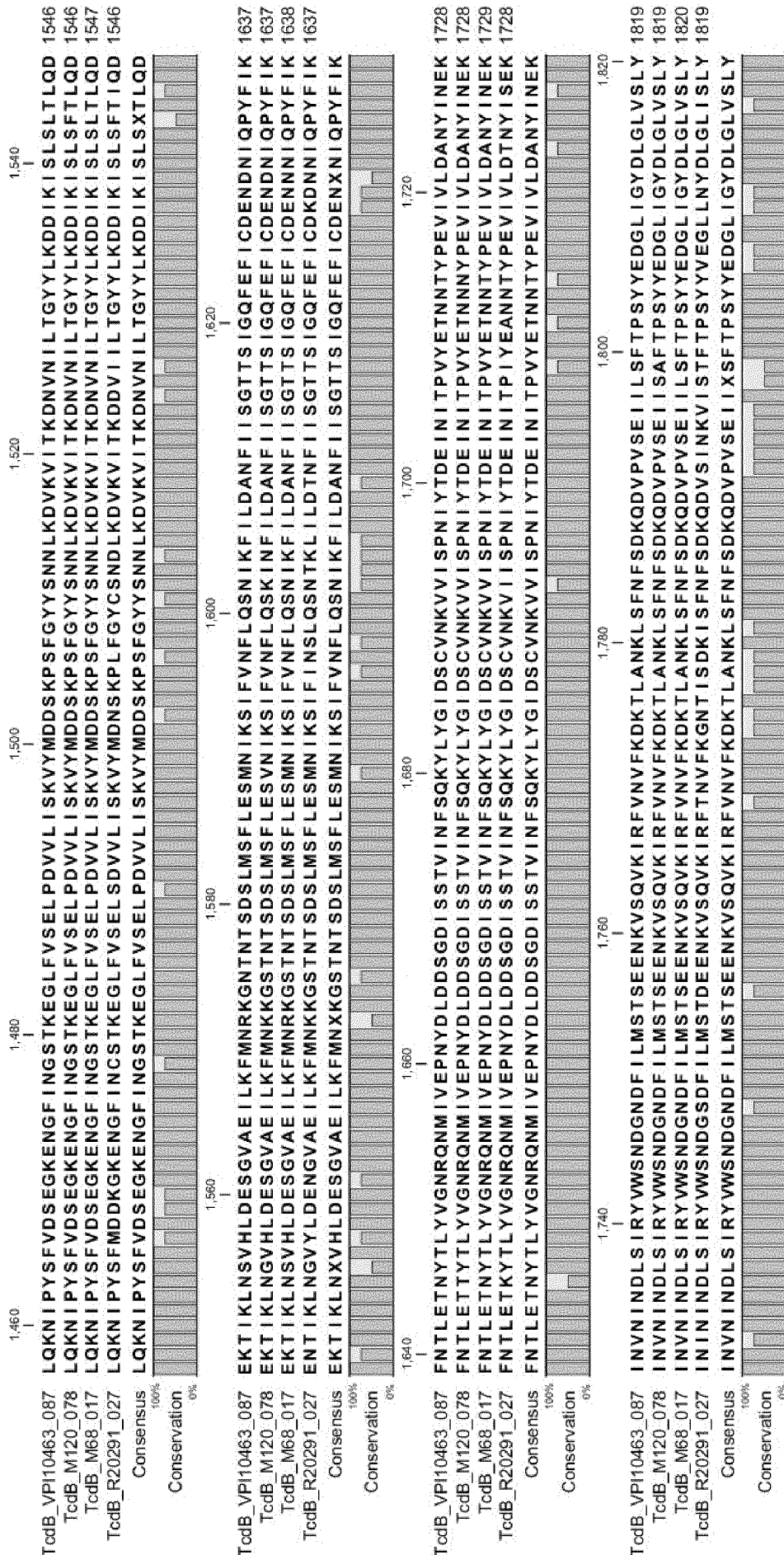
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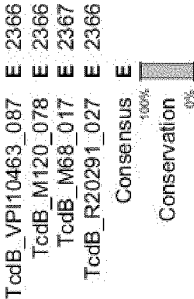
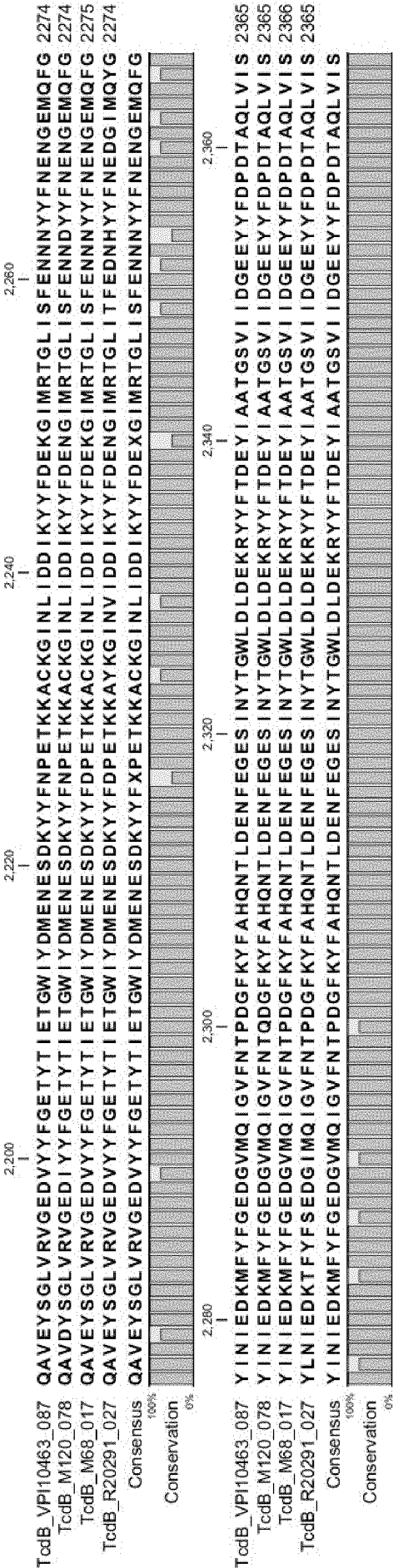
[illegible]











Suitably the polypeptide of the invention comprises an immunoglobulin chain variable domain which binds to *Clostridium difficile* toxin B, wherein the immunoglobulin chain variable domain comprises three complementarity determining regions (CDR1-CDR3) and four framework regions (FR1-FR4), wherein:

5 (a) CDR1 comprises a sequence sharing 60% or greater, such as 80% or greater sequence identity with SEQ ID NO: 1, CDR2 comprises a sequence sharing 60% or greater, such as 70% or greater, such as 75% or greater, such as 80% or greater, such as 85% or greater, such as 90% or greater sequence identity with SEQ ID NO: 2 and CDR3 comprises a sequence
10 sharing 60% or greater, such as 65% or greater, such as 75% or greater, such as 80% or greater, such as 90% or greater sequence identity with SEQ ID NO: 3; suitably any residues of CDR1, CDR2 or CDR3 differing from their corresponding residues in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3, respectively, are conservative substitutions with respect to their corresponding residues; suitably CDR1, CDR2 and/or CDR3 are devoid of K or R; suitably
15 CDR1, CDR2 and/or CDR3 have been mutated to replace one or more R or K residues with an H residue or

(b) CDR1 comprises a sequence sharing 60% or greater, such as 80% or greater sequence identity with SEQ ID NO: 4, CDR2 comprises a sequence sharing 60% or greater, such as
20 70% or greater, such as 75% or greater, such as 80% or greater, such as 85% or greater, such as 90% or greater sequence identity with SEQ ID NO: 5 and CDR3 comprises a sequence sharing 65% or greater, such as 70% or greater, such as 75% or greater, such as 85% or greater, such as 90% or greater sequence identity with SEQ ID NO: 6; suitably any residues of CDR1, CDR2 or CDR3 differing from their corresponding residues in SEQ ID NO: 4, SEQ ID
25 NO: 5 or SEQ ID NO: 6, respectively, are conservative substitutions with respect to their corresponding residue; suitably CDR1, CDR2 and/or CDR3 are devoid of K or R; suitably CDR1, CDR2 and/or CDR3 have been mutated to replace one or more R or K residues with an H residue.

30 Suitably the polypeptide of the invention comprises an immunoglobulin chain variable domain which binds to *Clostridium difficile* toxin B, wherein the immunoglobulin chain variable domain comprises three complementarity determining regions (CDR1-CDR3) and four framework regions (FR1-FR4), wherein CDR1 comprises or consists of SEQ ID NO: 1, CDR2 comprises or consists of SEQ ID NO: 2 and CDR3 comprises or consists of SEQ ID NO: 3; or CDR1
35 comprises or consists of SEQ ID NO: 4, CDR2 comprises or consists of SEQ ID NO: 5 and CDR3 comprises or consists of SEQ ID NO: 6; or CDR1 comprises or consists of SEQ ID NO: 7, CDR2 comprises or consists of SEQ ID NO: 8 and CDR3 comprises or consists of SEQ ID NO: 9.

40 Suitably the polypeptide of the invention comprises an immunoglobulin chain variable domain which binds to *Clostridium difficile* toxin B, wherein the immunoglobulin chain variable domain comprises three complementarity determining regions (CDR1-CDR3) and four framework regions (FR1-FR4), wherein: CDR1 comprises or more suitably consists of a sequence having

no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 1, CDR2 comprises or more suitably consists of a sequence having no more than 7, more suitably no more than 6, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 2 and CDR3 comprises or more suitably consists of a sequence having no more than 6, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 3; or

CDR1 comprises or more suitably consists of a sequence having no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 4, CDR2 comprises or more suitably consists of a sequence having no more than 7, more suitably no more than 6, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 5 and CDR3 comprises or more suitably consists of a sequence having no more than 6, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 6; or

CDR1 comprises or more suitably consists of a sequence having no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 7, CDR2 comprises or more suitably consists of a sequence having no more than 7, more suitably no more than 6, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 8 and CDR3 comprises or more suitably consists of a sequence having no more than 6, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to SEQ ID NO: 9.

Suitably the polypeptide of the invention comprises an immunoglobulin chain variable domain which binds to *Clostridium difficile* toxin B, wherein the immunoglobulin chain variable domain comprises three complementarity determining regions (CDR1-CDR3) and four framework regions (FR1-FR4), wherein CDR3 is devoid of K or R, more suitably CDR1, CDR2 and CDR3 are devoid of K or R. Suitably, CDR1, CDR2 and/or CDR3 have been mutated to replace one or more R or K residues with an H residue.

Suitably, FR1, FR2, FR3 and FR4 each comprise a sequence sharing 40% or greater, such as 60% or greater, such as 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater sequence identity with FR1, FR2, FR3 and FR4 of SEQ ID NO 10, respectively; or

FR1, FR2, FR3 and FR4 each comprise a sequence sharing 40% or greater, such as 60% or greater, such as 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater sequence identity with FR1, FR2, FR3 and FR4 of SEQ ID NO 11, respectively;

or

FR1, FR2, FR3 and FR4 each comprise a sequence sharing 40% or greater, such as 60% or greater, such as 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater sequence identity with FR1, FR2, FR3 and FR4 of SEQ ID NO 12, respectively.

Suitably FR1 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 15, more suitably no more than 10, more suitably no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR1 of SEQ ID NO 10; FR2 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR2 of SEQ ID NO 10; FR3 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 15, more suitably no more than 10, more suitably no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR3 of SEQ ID NO 10; and FR4 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR4 of SEQ ID NO 10.

Alternatively FR1 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 15, more suitably no more than 10, more suitably no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR1 of SEQ ID NO 11; FR2 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR2 of SEQ ID NO 11; FR3 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 15, more suitably no more than 10, more suitably no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR3 of SEQ ID NO 11; and FR4 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no

more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR4 of SEQ ID NO 11.

5 Alternatively FR1 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 16, more suitably no more than 10, more suitably no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR1 of SEQ ID NO 12; FR2 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR2 of SEQ ID NO 12; FR3 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 15, more suitably no more than 10, more suitably no more than 7, more suitably no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR3 of SEQ ID NO 12; and FR4 of the polypeptide of the invention comprises or more suitably consist of a sequence having no more than 5, more suitably no more than 4, more suitably no more than 3, more suitably no more than 2, more suitably no more than 1 addition(s), deletion(s) and/or substitutions(s) compared to FR4 of SEQ ID NO 12.

Suitably residue 1 of FR1 is D, E or Q; and/or residue 5 of FR1 is V in the inventive polypeptide or each polypeptide of a multimeric construct.

25 Suitably the polypeptide of the invention comprises or more suitably consists of: SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

30 Suitably the *Clostridium difficile* toxin B bound by the polypeptide of the invention comprises or more suitably consists of a sequence sharing 40% or greater, such as 60% or greater, such as 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater, such as 100% sequence identity with any one of SEQ ID NOs: 52-55.

35 **Linkers and multimers**

A construct according to the invention comprises multiple polypeptides and therefore may suitably be multivalent. Such a construct may comprise at least two identical polypeptides according to the invention. A construct consisting of two identical polypeptides according to the invention is a "homobihead". In one aspect of the invention there is provided a construct comprising two or more identical polypeptides of the invention.

Alternatively, a construct may comprise a polypeptide of the invention and at least one further polypeptide which is different, but still a polypeptide according to the invention (a "heterobihead"). Suitably, the different polypeptide in such a construct is selected from: SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30. Suitably such a construct is selected from SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 24 or SEQ ID NO: 25.

Alternatively, such a construct may comprise (a) at least one polypeptide according to the invention and (b) at least one polypeptide such as an antibody or antigen-binding fragment thereof, which is not a polypeptide of the invention (also a "heterobihead"). The at least one polypeptide of (b) may bind TcdB (for example via a different epitope to that of (a)), or alternatively may bind to a target other than TcdB. Suitably the different polypeptide (b) binds to *Clostridium difficile* toxin A.

Constructs can be multivalent and/or multispecific. A multivalent construct (such as a bivalent construct) comprises two or more binding polypeptides therefore presents two or more sites at which attachment to one or more antigens can occur. An example of a multivalent construct could be a homobihead or a heterobihead. A multispecific construct (such as a bispecific construct) comprises two or more different binding polypeptides which present two or more sites at which either (a) attachment to two or more different antigens can occur or (b) attachment to two or more different epitopes on the same antigen can occur. An example of a multispecific construct could be a heterobihead. A multispecific construct is multivalent.

A construct of the invention may comprise, or more suitably consist of, one or more polypeptides according to the invention and suitably additionally comprise, or more suitably consist of, one, two, three, four, five, six, seven, eight, nine or more further polypeptides wherein each of the further polypeptides binds to a target, such as a target selected from the list consisting of *Clostridium difficile* toxin A and *Clostridium difficile* toxin B.

A construct consisting of a total of four polypeptides which each bind a target is known as a 'quadrahead'. The format of such a construct according to the invention may be, from N- to C-terminal, suitably A-A-A-A, A-A-A-B, A-A-B-B, A-B-B-B, B-A-A-A, B-B-A-A, A-B-A-B, B-A-B-A, A-B-B-A or B-A-A-B, more suitably A-A-B-B, B-B-A-A, A-B-A-B, B-A-B-A, A-B-B-A or B-A-A-B, wherein A is a polypeptide which binds to *Clostridium difficile* toxin A and B is a polypeptide which binds to *Clostridium difficile* toxin B, wherein the polypeptides which bind to *Clostridium difficile* toxin A are identical or different and the polypeptides which bind to *Clostridium difficile* toxin B are identical or different.

Suitably the construct is of the format A-A'-B-B', A-B-B'-A' or B-A-A'-B', wherein B is a polypeptide according to the invention, B' is a different polypeptide according to the invention, A is a polypeptide which binds to *Clostridium difficile* toxin A and A' is a different polypeptide

which binds to *Clostridium difficile* toxin A. Suitably the construct is selected from: SEQ ID NOs: 41-47.

Suitably, the polypeptides comprised within the construct are antibody fragments. More suitably, the polypeptides comprised within the construct are selected from the list consisting of: a VHH, a VH, a VL, a V-NAR, a Fab fragment and a F(ab')₂ fragment. More suitably, the polypeptides comprised within the construct are VHs or VHHs.

The polypeptides of the invention can be linked to each other directly (i.e. without use of a linker) or via a linker. The linker is suitably a polypeptide and will be selected so as to allow binding of the polypeptides to their epitopes. If used for therapeutic purposes, the linker is suitably non-immunogenic in the subject to which the polypeptides are administered. Suitably the linkers are of the format (G₄S)_x. Most suitably x is 6.

Vectors and Hosts

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian and yeast vectors). Other vectors (e.g. non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions, and also bacteriophage and phagemid systems. The invention also relates to nucleotide sequences that encode polypeptide sequences or multivalent and/or multispecific constructs. The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. Such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell.

In one aspect of the invention there is provided a vector comprising the polynucleotide encoding the polypeptide or construct of the invention or cDNA comprising said polynucleotide. In a further aspect of the invention there is provided a host cell transformed with said vector, which is capable of expressing the polypeptide or construct of the invention. Suitably the host

cell is a bacteria such as *E. coli* or a yeast such a yeast belonging to the genera *Aspergillus*, *Saccharomyces*, *Kluyveromyces*, *Hansenula* or *Pichia*, such as *Saccharomyces cerevisiae* or *Pichia pastoris*.

5 Stability

Suitably, the polypeptide or construct of the present invention substantially retains neutralisation ability and/or potency when delivered orally and after exposure to the intestinal tract (for example, after exposure to proteases present in the small and/or large intestine, *C. difficile*-specific proteases and inflammatory proteases). Such proteases include enteropeptidase, trypsin and chymotrypsin. Proteases present in, or produced in, the small and/or large intestine include proteases sourced from intestinal tract commensal microflora and/or pathogenic bacteria, for example wherein the proteases are cell membrane-attached proteases, secreted proteases and proteases released on cell lysis). Most suitably the proteases are trypsin and chymotrypsin. Suitably the polypeptide or construct of the invention is substantially resistant to one or more proteases.

Suitably the intestinal tract is the intestinal tract of a human. The small intestine suitably consists of the duodenum, jejunum and ileum. The large intestine suitably consists of the cecum, colon, rectum and anal canal.

The polypeptide or construct of the present invention substantially retains neutralisation ability when suitably 10%, more suitably 20%, more suitably 30%, more suitably 40%, more suitably 50%, more suitably 60%, more suitably 70%, more suitably 80%, more suitably 90%, more suitably 95%, more suitably 100% of the original neutralisation ability of the polypeptide of the invention or construct is retained after exposure to proteases present in the small and/or large intestine such as trypsin or chymotrypsin.

Suitably the polypeptide or construct of the invention substantially retains neutralisation ability after exposure to proteases present in the small and/or large intestine such as trypsin or chymotrypsin for, for example, up to at least 15, more suitably up to at least 30, more suitably up to at least 45, more suitably up to at least 60 minutes at 37 degrees C.

Suitably 10% or more, more suitably 20% or more, more suitably 30% or more, more suitably 40% or more, more suitably 50% or more, more suitably 60% or more, more suitably 70% or more, more suitably 80% or more of the neutralisation ability of the polypeptide or construct of the invention is retained after up to 1 or more suitably up to 4 hours of exposure to conditions of the intestinal tract, more suitably the small or large intestine, more suitably human faecal supernatant.

40

Therapeutic use and delivery

A therapeutically effective amount of a polypeptide, pharmaceutical composition or construct of the invention, is an amount which is effective, upon single or multiple dose administration to a subject, in neutralising TcdB to a significant extent in a subject. A therapeutically effective amount may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the polypeptide, pharmaceutical composition or construct to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the polypeptide of the invention, pharmaceutical composition or construct are outweighed by the therapeutically beneficial effects. The polypeptide or construct of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. The polypeptide or construct of the invention can be in the form of a pharmaceutically acceptable salt.

A pharmaceutical composition of the invention may suitably be formulated for oral, intramuscular, subcutaneous, intravenous, intrarectal or enema delivery. The pharmaceutical compositions of the invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. Solid dosage forms are preferred. The polypeptide of the invention, pharmaceutical composition or construct may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like.

Typically, the pharmaceutical composition comprises a polypeptide or construct of the invention and a pharmaceutically acceptable diluent or carrier. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the polypeptide or construct of the invention. Pharmaceutical compositions may include antiadherents, binders, coatings, disintegrants, flavours, colours, lubricants, sorbents, preservatives, sweeteners, freeze dry excipients (including lyoprotectants) or compression aids. Suitably, the polypeptide or construct of the invention is lyophilised before being incorporated into a pharmaceutical composition.

A polypeptide of the invention may also be provided with an enteric coating. An enteric coating is a polymer barrier applied on oral medication which protects the polypeptide from the low pH of the stomach. Materials used for enteric coatings include fatty acids, waxes, shellac, plastics, and plant fibers. Suitable enteric coating components include methyl acrylate-methacrylic acid copolymers, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate (hypromellose acetate succinate), polyvinyl acetate phthalate (PVAP), methyl methacrylate-methacrylic acid copolymers, sodium

alginate and stearic acid. Suitable enteric coatings include pH-dependent release polymers. These are polymers which are insoluble at the highly acidic pH found in the stomach, but which dissolve rapidly at a less acidic pH. Thus, suitably, the enteric coating will not dissolve in the acidic juices of the stomach (pH ~3), but will do so in the higher pH environment present in the small intestine (pH above 6) or in the colon (pH above 7.0). The pH-dependent release polymer is selected such that the polypeptide or construct of the invention will be released at about the time that the dosage reaches the small intestine.

A polypeptide, construct or pharmaceutical composition of the invention can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or non-aqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilisers, isotonic agents, suspending agents, emulsifying agents, stabilisers and preservatives. Acceptable carriers, excipients and/or stabilisers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid, glutathione, cysteine, methionine and citric acid; preservatives (such as ethanol, benzyl alcohol, phenol, m-cresol, p-chlor-m-cresol, methyl or propyl parabens, benzalkonium chloride, or combinations thereof); amino acids such as arginine, glycine, ornithine, lysine, histidine, glutamic acid, aspartic acid, isoleucine, leucine, alanine, phenylalanine, tyrosine, tryptophan, methionine, serine, proline and combinations thereof; monosaccharides, disaccharides and other carbohydrates; low molecular weight (less than about 10 residues) polypeptides; proteins, such as gelatin or serum albumin; chelating agents such as EDTA; sugars such as trehalose, sucrose, lactose, glucose, mannose, maltose, galactose, fructose, sorbose, raffinose, glucosamine, N-methylglucosamine, galactosamine, and neuraminic acid; and/or non-ionic surfactants such as polysorbates, POE ethers, poloxamers, Triton-X, or polyethylene glycol.

For all modes of delivery, the polypeptide, pharmaceutical composition or construct of the invention may be formulated in a buffer, in order to stabilise the pH of the composition, at a concentration between 5-50, or more suitably 15-40 or more suitably 25-30 g/litre. Examples of suitable buffer components include physiological salts such as sodium citrate and/or citric acid. Suitably buffers contain 100-200, more suitably 125-175 mM physiological salts such as sodium chloride. Suitably the buffer is selected to have a pKa close to the pH of the composition or the physiological pH of the patient.

Exemplary polypeptide or construct concentrations in a pharmaceutical composition may range from about 1 mg/mL to about 200 mg/ml or from about 50 mg/mL to about 200 mg/mL, or from about 150 mg/mL to about 200 mg/mL.

An aqueous formulation of the polypeptide, construct or pharmaceutical composition of the invention may be prepared in a pH-buffered solution, e.g., at pH ranging from about 4.0 to about 7.0, or from about 5.0 to about 6.0, or alternatively about 5.5. Examples of suitable buffers include phosphate-, histidine-, citrate-, succinate-, acetate-buffers and other organic

acid buffers. The buffer concentration can be from about 1 mM to about 100 mM, or from about 5 mM to about 50 mM, depending, for example, on the buffer and the desired tonicity of the formulation.

5 The tonicity of the pharmaceutical composition may be altered by including a tonicity modifier. Such tonicity modifiers can be charged or uncharged chemical species. Typical uncharged tonicity modifiers include sugars or sugar alcohols or other polyols, preferably trehalose, sucrose, mannitol, glycerol, 1,2-propanediol, raffinose, sorbitol or lactitol (especially trehalose, mannitol, glycerol or 1,2-propanediol). Typical charged tonicity modifiers include salts such as
10 a combination of sodium, potassium or calcium ions, with chloride, sulfate, carbonate, sulfite, nitrate, lactate, succinate, acetate or maleate ions (especially sodium chloride or sodium sulphate); or amino acids such as arginine or histidine. Suitably, the aqueous formulation is isotonic, although hypertonic or hypotonic solutions may be suitable. The term "isotonic" denotes a solution having the same tonicity as some other solution with which it is compared,
15 such as physiological salt solution or serum. Tonicity agents may be used in an amount of about 5 mM to about 350 mM, e.g., in an amount of 1 mM to 500 mM. Suitably, at least one isotonic agent is included in the composition.

A surfactant may also be added to the pharmaceutical composition to reduce aggregation of
20 the formulated polypeptide or construct and/or minimize the formation of particulates in the formulation and/or reduce adsorption. Exemplary surfactants include polyoxyethylensorbitan fatty acid esters (Tween), polyoxyethylene alkyl ethers (Brij), alkylphenylpolyoxyethylene ethers (Triton-X), polyoxyethylene-polyoxypropylene copolymer (Poloxamer, Pluronic), and sodium dodecyl sulfate (SDS). Examples of suitable polyoxyethylenesorbitan-fatty acid esters
25 are polysorbate 20, and polysorbate 80. Exemplary concentrations of surfactant may range from about 0.001% to about 10% w/v.

A lyoprotectant may also be added in order to protect the polypeptide or construct of the invention against destabilizing conditions during the lyophilization process. For example,
30 known lyoprotectants include sugars (including glucose, sucrose, mannose and trehalose); polyols (including mannitol, sorbitol and glycerol); and amino acids (including alanine, glycine and glutamic acid). Lyoprotectants can be included in an amount of about 10 mM to 500 mM.

The dosage ranges for administration of the polypeptide of the invention, pharmaceutical
35 composition or construct of the invention are those to produce the desired therapeutic effect. The dosage range required depends on the precise nature of the polypeptide of the invention, pharmaceutical composition or construct, 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
40 dosage levels can be adjusted using standard empirical routines for optimisation.

Suitable daily dosages of polypeptide of the invention, pharmaceutical composition or construct of the invention are in the range of 50ng-50mg per kg, such as 50ug-40mg per kg,

such as 5-30mg 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 more frequently, for example 2, 3 or 4 times per day or less frequently for example every other day or once per week.

5 In one aspect of the invention there is provided the use of the polypeptide, pharmaceutical composition or construct of the invention in the manufacture of a medicament for the treatment of *C. difficile* infection. In a further aspect of the invention there is provided a method of treating *C. difficile* infection comprising administering to a person in need thereof a
10 therapeutically effective amount of the polypeptide, pharmaceutical composition or construct of the invention.

The word 'treatment' is intended to embrace prophylaxis as well as therapeutic treatment. Treatment of infection also embraces treatment of exacerbations thereof and also embraces
15 treatment of patients in remission from infection symptoms to prevent relapse of symptoms.

Combination therapy

A pharmaceutical composition of the invention may also comprise one or more active agents
20 (e.g. active agents suitable for treating *C. difficile* infection). It is within the scope of the invention to use the pharmaceutical composition of the invention in therapeutic methods for the treatment of *C. difficile* infection as an adjunct to, or in conjunction with, other established therapies normally used in the treatment of *C. difficile* infection, such as antibiotics.

25 Possible combinations include combinations with, for example, one or more active agents selected from the list comprising *C. difficile* toxoid vaccine, ampicillin, amoxicillin, vancomycin, metronidazole, fidaxomicin, linezolid, nitazoxanide, rifaximin, ramoplanin, difimicin, clindamycin, cephalosporins (such as second and third generation cephalosporins),
30 fluoroquinolones (such as gatifloxacin or moxifloxacin), macrolides (such as erythromycin, clarithromycin, azithromycin), penicillins, aminoglycosides, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline, imipenem, meropenem, antibacterial agents, bactericides, or bacteriostats. Possible combinations also include combinations with one or more active agents which are probiotics, for example *Saccharomyces boulardii* or *Lactobacillus rhamnosus* GG.
35

Hence another aspect of the invention provides a pharmaceutical composition of the invention in combination with one or more further active agents, for example one or more active agents described above.

40 In a further aspect of the invention, the polypeptide, pharmaceutical composition or construct is administered sequentially, simultaneously or separately with at least one active agent selected from the list above. In a further aspect of the invention, the polypeptide, pharmaceutical composition or construct is administered sequentially, simultaneously or separately with fecal

microbiota transplantation (i.e. fecal bacteriotherapy, fecal transfusion, fecal transplant, stool transplant, fecal enema, human probiotic infusion).

Similarly, another aspect of the invention provides a combination product comprising:

- 5 (A) a polypeptide, pharmaceutical composition or construct of the present invention; and
(B) one or more other active agents,
wherein each of components (A) and (B) is formulated in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier. In this aspect of the invention, the combination product may be either a single (combination) formulation or a kit-of-parts. Thus, this aspect of the
10 invention encompasses a combination formulation including a polypeptide, pharmaceutical composition or construct of the present invention and another therapeutic agent, in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier.

The invention also encompasses a kit of parts comprising components:

- 15 (i) a polypeptide, pharmaceutical composition or construct of the present invention in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier; and
(ii) a formulation including one or more other active agents, in admixture with a pharmaceutically-acceptable adjuvant, diluent or carrier, which components (i) and (ii) are each provided in a form that is suitable for administration in conjunction with the other.

20 Component (i) of the kit of parts is thus component (A) above in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier. Similarly, component (ii) is component (B) above in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier. The one or more other active agents (i.e. component (B) above) may be, for example,
25 any of the agents mentioned above in connection with the treatment of *C. difficile* infection. If component (B) is more than one further active agent, these further active agents can be formulated with each other or formulated with component (A) or they may be formulated separately. In one embodiment component (B) is one other therapeutic agent. In another embodiment component (B) is two other therapeutic agents. The combination product (either
30 a combined preparation or kit-of-parts) of this aspect of the invention may be used in the treatment or prevention of *C. difficile* infection.

Suitably the polypeptide, pharmaceutical composition or construct of the invention is for use as a medicament and more suitably for use in the treatment, prevention, diagnosis and/or
35 detection of *C. difficile* infection, most suitably for use in the treatment of *C. difficile* infection.

Preparative Methods

40 Polypeptides of the invention can be obtained and manipulated using the techniques disclosed for example in Green and Sambrook 2012 *Molecular Cloning: A Laboratory Manual* 4th Edition Cold Spring Harbour Laboratory Press.

Monoclonal antibodies can be produced using hybridoma technology, by fusing a specific antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture and for an absence of antibody chain synthesis (Köhler and Milstein 1975 *Nature* 256:495-497 and Nelson et al 2000 *Molecular Pathology* 53(3):111-117, herein incorporated by reference in their entirety).

A monoclonal antibody directed against a determined antigen can, for example, be obtained by:

- a) immortalizing lymphocytes obtained from the peripheral blood of an animal previously immunized with a determined antigen, with an immortal cell and preferably with myeloma cells, in order to form a hybridoma,
- b) culturing the immortalized cells (hybridoma) formed and recovering the cells producing the antibodies having the desired specificity.

Alternatively, the use of a hybridoma cell is not required. Accordingly, monoclonal antibodies can be obtained by a process comprising the steps of:

- a) cloning into vectors, especially into phages and more particularly filamentous bacteriophages, DNA or cDNA sequences obtained from lymphocytes especially peripheral blood lymphocytes of an animal (suitably previously immunized with determined antigens),
- b) transforming prokaryotic cells with the above vectors in conditions allowing the production of the antibodies,
- c) selecting the antibodies by subjecting them to antigen-affinity selection,
- d) recovering the antibodies having the desired specificity.

Methods for immunizing camelids, cloning the VHH repertoire of B cells circulating in blood (Chomezynski and Sacchi 1987 *Anal Biochem* 162:156-159), and isolation of antigen-specific VHHs from immune (Arbabi-Ghahroudi et al 1997 *FEBS Lett* 414:521-526) and nonimmune (Tanha et al 2002 *J Immunol Methods* 263:97-109) libraries using phage, yeast, or ribosome display are known (WO92/01047, Nguyen et al 2001 *Adv Immunol* 79:261-296 and Harmsen et al 2007 *Appl Microbiol Biotechnol* 77(1):13-22). These references are herein incorporated by reference in their entirety.

Antigen-binding fragments of antibodies such as the scFv and Fv fragments can be isolated and expressed in *E. coli* (Miethe et al 2013 *J Biotech* 163(2):105-111, Skerra et al 1988 *Science* 240(4855):1038-1041 and Ward et al *Nature* 1989 341:544-546, herein incorporated by reference in their entirety).

Mutations can be made to the DNA or cDNA that encode polypeptides which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli* and *S. cerevisiae*, are known.

Modification of polypeptides can be achieved for example by substitutions, additions or deletions to a nucleic acid encoding the polypeptide. The substitutions, additions or deletions to a nucleic acid encoding the polypeptide can be introduced by many methods, including for example error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis (Ling et al 1997 *Anal Biochem* 254(2):157-178, herein incorporated by reference in its entirety), gene reassembly, Gene Site Saturation Mutagenesis (GSSM), synthetic ligation reassembly (SLR) or a combination of these methods. The modifications, additions or deletions to a nucleic acid can also be introduced by a method comprising recombination, recursive sequence recombination, phosphothioate-modified DNA mutagenesis, uracil-containing template mutagenesis, gapped duplex mutagenesis, point mismatch repair mutagenesis, repair-deficient host strain mutagenesis, chemical mutagenesis, radiogenic mutagenesis, deletion mutagenesis, restriction-selection mutagenesis, restriction-purification mutagenesis, ensemble mutagenesis, chimeric nucleic acid multimer creation, or a combination thereof.

In particular, artificial gene synthesis may be used (Nambiar et al 1984 *Science* 223:1299-1301, Sakamar and Khorana 1988 *Nucl. Acids Res* 14:6361-6372, Wells et al 1985 *Gene* 34:315-323 and Grundstrom et al 1985 *Nucl. Acids Res* 13:3305-3316, herein incorporated by reference in their entirety). A gene encoding a polypeptide of the invention can be synthetically produced by, for example, solid-phase DNA synthesis. Entire genes may be synthesized de novo, without the need for precursor template DNA. To obtain the desired oligonucleotide, the building blocks are sequentially coupled to the growing oligonucleotide chain in the order required by the sequence of the product. Upon the completion of the chain assembly, the product is released from the solid phase to solution, deprotected, and collected. Products can be isolated by high-performance liquid chromatography (HPLC) to obtain the desired oligonucleotides in high purity (Verma and Eckstein 1998 *Annu Rev Biochem* 67:99-134)

Expression of immunoglobulin chain variable domains such as VHs and VHHs can be achieved using a suitable expression vector such as a prokaryotic cell such as bacteria, for example *E. coli* (for example according to the protocols disclosed in WO94/04678, which is incorporated herein by reference and detailed further below). Expression of immunoglobulin chain variable domains such as VHs and VHHs can also be achieved using eukaryotic cells, for example insect cells, CHO cells, Vero cells or suitably yeast cells such as yeasts belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula* or *Pichia*. Suitably *S. cerevisiae* is used (for example according to the protocols disclosed in WO94/025591, which is incorporated herein by reference and detailed further below).

Specifically, VHHs can be prepared according to the methods disclosed in WO94/04678 using *E. coli* cells by a process comprising the steps of:

a) cloning in a Bluescript vector (Agilent Technologies) a DNA or cDNA sequence coding for the VHH (for example obtained from lymphocytes of camelids or produced synthetically) optionally including a His-tag,

b) recovering the cloned fragment after amplification using a 5' primer specific for the VHH containing an XhoI site and a 3' primer containing the SpeI site having the sequence
 5 TC TTA ACT AGT GAG GAG ACG GTG ACC TG (SEQ ID NO: 31),

c) cloning the recovered fragment in phase in the Immuno PBS vector (Huse et al 1989 *Science* 246 (4935):1275-1281, herein incorporated by reference in its entirety) after digestion of the vector with XhoI and SpeI restriction enzymes,

10 d) transforming host cells, especially *E. coli* by transfection with the recombinant Immuno PBS vector of step c,

e) recovering the expression product of the VHH coding sequence, for instance by affinity purification such as by chromatography on a column using Protein A, cation exchange, or a nickel-affinity resin if the VHH includes a His-tag.

15 Alternatively, immunoglobulin chain variable domains such as VHs and VHHs are obtainable by a process comprising the steps of:

a) obtaining a DNA or cDNA sequence coding for a VHH, having a determined specific antigen binding site,

20 b) amplifying the obtained DNA or cDNA, using a 5' primer containing an initiation codon and a HindIII site, and a 3' primer containing a termination codon having a XhoI site,

c) recombining the amplified DNA or cDNA into the HindIII (position 2650) and XhoI (position 4067) sites of a plasmid pMM984 (Merchinsky et al 1983 *J. Virol.* 47:227-232, herein incorporated by reference in its entirety),

25 d) transfecting permissive cells especially NB-E cells (Faisst et al 1995 *J Virol* 69:4538-4543, herein incorporated by reference in its entirety) with the recombinant plasmid,

e) recovering the obtained products.

30 Further, immunoglobulin chain variable domains such as VHHs or VHs can be produced using *E. coli* or *S. cerevisiae* according to the methods disclosed in Frenken et al 2000 *J Biotech* 78:11-21 and WO99/23221 (herein incorporated by reference in their entirety) as follows:

35 After taking a blood sample from an immunised llama and enriching the lymphocyte population via Ficoll (a neutral, highly branched, high-mass, hydrophilic polysaccharide which dissolves readily in aqueous solutions - Pharmacia) discontinuous gradient centrifugation, isolating total RNA by acid guanidium thiocyanate extraction (Chomezynski and Sacchi 1987 *Anal Biochem* 162:156-159), and first strand cDNA synthesis (e.g. using a cDNA kit such as RPN 1266 (Amersham)), DNA fragments encoding VHH and VH fragments and part of the short or long hinge region are amplified by PCR using the specific primers detailed on pages 22 and 23 of
 40 WO99/23221. Upon digestion of the PCR fragments with PstI and HindIII or BstEII, the DNA fragments with a length between about 300 and 450 bp are purified via agarose gel electrophoresis and ligated in the *E. coli* phagemid vector pUR4536 or the episomal *S. cerevisiae* expression vector pUR4548, respectively. pUR4536 is derived from pHEN

(Hoogenboom et al 1991 *Nucl Acid Res* 19:4133-4137, herein incorporated by reference in its entirety) and contains the *lacI^q* gene and unique restriction sites to allow the cloning of the llama VHH and VH genes. pUR4548 is derived from pSY1 (Harmsen et al 1993 *Gene* 125:115-123, herein incorporated by reference in its entirety). From this plasmid, the BstEII site in the *leu2* gene is removed via PCR and the cloning sites between the SUC2 signal sequence and the terminator are replaced in order to facilitate the cloning of the VH/VHH gene fragments. The VH/VHHs have the c-myc tag at the C-terminus for detection. Individual *E. coli* JM109 colonies are transferred to 96 well microtiter plates containing 150 ml 2TY medium supplemented with 1% glucose and 100 mg L⁻¹ ampicillin. After overnight growth (37 degrees C), the plates are duplicated in 2TY medium containing 100 mg L⁻¹ ampicillin and 0.1 mM IPTG. After another overnight incubation and optionally freezing and thawing, cells are centrifuged and pelleted and the supernatant can be used in an ELISA. Individual *S. cerevisiae* colonies are transferred to test tubes containing selective minimal medium (comprising 0.7% yeast nitrogen base, 2% glucose, supplemented with the essential amino acids and bases) and are grown for 48 h at 30 degrees C. Subsequently, the cultures are diluted ten times in YPGal medium (comprising 1% yeast extract, 2% bacto peptone and 5% galactose). After 24 and 48 h of growth, the cells are pelleted and the culture supernatant can be analysed in an ELISA. Absorbance at 600 nm (OD600) is optionally measured.

Further, immunoglobulin chain variable domains such as VH/VHHs can be produced using *S. cerevisiae* using the procedure as follows:

Isolate a naturally-occurring DNA sequence encoding the VH/VHH or obtain a synthetically produced DNA sequence encoding the VH/VHH, including a 5'-UTR, signal sequence, stop codons and flanked with SacI and HindIII sites (such a synthetic sequence can be produced as outlined above or for example may be ordered from a commercial supplier such as Geneart (Life Technologies)).

Use the restriction sites for transfer of the VH/VHH gene to the multi-copy integration (MCI) vector pUR8569 or pUR8542, as follows. Cut the DNA sequence encoding the VHH optionally contained within a shuttle vector, cassette or other synthetic gene construct and the MCI vector with SacI and HindIII using: 25 ul VHH DNA (Geneart plasmid or MCI vector), 1 ul SacI, 1 ul HindIII, 3 ul of a suitable buffer for double digestion such as NEB buffer 1 (New England Biolabs) overnight at 37 degrees C. Run 25 ul of digested DNA encoding the VHH and 25 ul of digested MCI vector on a 1.5% agarose gel with 1xTAE buffer and then perform gel extraction for example using QIAquick Gel Extraction Kit (Qiagen)). Set-up a ligation of digested MCI vector and digested DNA encoding the VH/VHH as follows: 100 ng vector, 30 ng VHH gene, 1.5 ul 10x ligase buffer, 1 ul T4 DNA ligase, and ddH₂O. Then perform ligation overnight at 16 degrees C.

Next transform the *E. coli* cells. For chemical competent XL-1 blue cells, thaw 200 ul heat competent XL-1 blue cells and add 5 ul ligation mix on ice for about 30 minutes followed by heat shock for 90 seconds at 42 degrees C. Then add 800 ul Luria-Bertani low salt medium

supplemented with 2% glucose and recover cells for 2 hours at 37 degrees C. Plate cells on Luria-Bertani agar and ampicillin (100 ug/ml) plates and keep overnight at 37 degrees C. For electro competent TG1 *E. coli* cells, use an electroporation cuvette. In the electroporation cuvette: thaw 50 ul electro competent TG1 cells and 1 ul ligation mix on ice for about 15 minutes. Place the cuvette in the holder and pulse. Add 500 ul of 2TY medium and recover cells for 30 minutes at 37 degrees C. Plate 100 ul of cells on Luria-Bertani, agar, containing ampicillin (100 ug/ml) and 2% glucose plates. Keep plates at 37 degrees C overnight.

After cloning of the VH/VHH gene into *E. coli* as detailed above, *S. cerevisiae* can be transformed with the linearized MCI vector. Before transformation is carried out, some steps are performed: (i) the DNA should be changed from circular to linear by digestion or else the DNA cannot be integrated into the yeast genome and (ii) the digested DNA should be cleaned of impurities by ethanol precipitation. Also, during the transformation process, the yeast cells are made semi-permeable so the DNA can pass the membrane.

Preparation for yeast transformation: perform a HpaI digestion of the midi-prep prepared from the selected *E. coli* colony expressing the VH/VHH gene as follows. Prepare a 100 ul solution containing 20ng of midi-prep, 5ul HpaI, 10ul of appropriate buffer such as NEB4 buffer (BioLabs), and ddH₂O.

Cut the DNA with the HpaI at room temperature overnight. Next perform an ethanol precipitation (and put to one side a 5ul sample from HpaI digestion). Add 300 ul ethanol 100% to 95ul HpaI digested midiprep, vortex, and spin at full speed for 5 minutes. Carefully decant when a pellet is present, add 100ul of ethanol 70%, then spin again for 5 minutes at full speed. Decant the sample again, and keep at 50-60 degrees C until the pellet is dry. Re-suspend the pellet in 50 ul ddH₂O. Run 5ul on a gel beside the 5ul HpaI digested sample.

Yeast transformation: prepare YNBglu plates. Use 10 g agar + 425ml water (sterilised), 25ml filtered 20× YNB (3.35g YNB (yeast nitrogen base) in 25ml sterilized H₂O) and 50ml sterile 20% glucose and pour into petri dishes. Pick one yeast colony from the masterplate and grow in 3 ml YPD (Yeast Extract Peptone Dextrose) overnight at 30 degrees C. Next day prepare about 600ml YPD and use to fill 3 flasks with 275ml, 225ml and 100ml YPD. Add 27.5 ul yeast YPD culture to the first flask and mix gently. Take 75 ml from the first flask and put this in the second flask, mix gently. Take 100 ml from the second flask and put in the third one, mix gently. Grow until reaching an OD₆₆₀ of between 1 and 2. Divide the flask reaching this OD over 4 Falcon tubes, ± 45ml in each. Spin for 2 minutes at 4200rpm. Discard the supernatant. Dissolve the pellets in two Falcon tubes with 45ml H₂O (reducing the number of tubes from 4 to 2). Spin for 2 minutes at 4200rpm. Dissolve the pellets in 45ml H₂O (from 2 tubes to 1). Spin for 2 minutes at 4200rpm. Gently dissolve the pellets in 5ml lithium acetate (LiAc) (100mM), and spin for a few seconds. Carefully discard some LiAc, but retain over half of the LiAc in the tube. Vortex the cells, boil carrier DNA for 5 minutes and quickly chill in ice-water. Add to a 15ml tube containing: 240ul PEG, 50ul cells, 36uLiAc (1M), 25ul carrier DNA, 45ul ethanol precipitated VH/VHH. Mix gently after each step (treat the blank sample the same, only

without ethanol precipitated VH/VHH). Incubate for 30 minutes at 30 degrees C, gently invert the tube 3-4 times, then heat shock for 20-25 minutes at 42 degrees C. Spin up to 6000rpm for a brief time. Gently remove the supernatant and add 250ul ddH₂O and mix. Streak all of it on an YNBglu plate until plates are dry and grow for 4-5 days at 30 degrees C. Finally, prepare YNBglu plates by dividing plates in 6 equal parts, number the parts 1 to 6, inoculate the biggest colony and streak out number 1. Repeat for other colonies from big to small from 1 to 6. Grow at 30 degrees C for 3-4 days large until colonies are produced. The VH/VHH clones are grown using glucose as a carbon source, and induction of VH/ VHH expression is done by turning on the Galactose-7-promoter by adding 0.5% galactose. Perform a 3mL small scale culture to test the colonies and choose which one shows the best expression of the VH or VHH. This colony is then used in purification.

Purification: the VH/VHH is purified by cation exchange chromatography with a strong anion resin (such as Capto S). On day 1, inoculate the selected yeast colony expressing the VH/VHH in 5ml YPD medium (YP medium + 2% glucose) and grow the cells in 25mL sealed sterile tubes at 30 degrees C overnight (shaking at 180 rpm). On day 2, dilute the 5 ml overnight culture in 50mL freshly prepared YP medium + 2% glucose + 0.5% galactose, grow the cells in 250ml aerated baffled flasks at 30 degrees C for two nights (shaking at 180 rpm). On day 4, spin the cells down in a centrifuge at 4200rpm for 20 min. Cation exchange purification step using a strong anion resin: adjust the pH of the supernatant containing the ligand to 3.5. Wash 0.75 ml resin (+/-0.5mL slurry) per of 50mL supernatant with 50mL of ddH₂O followed by three washes with binding buffer. Add the washed resin to the supernatant and incubate the suspension at 4 degrees C on a shaker for 1.5 hours. Pellet the resin-bound VH/VHH by centrifugation at 500g for 2 minutes and wash it with wash buffer. Decant supernatant and re-suspend the resin with 10mL of binding buffer. Put a filter in a PD-10 column, pour the resin in the column and let the resin settle for a while, then add a filter above the resin. Wait until all binding buffer has run through. Elute the VH/VHH with 6 x 0.5 ml elution buffer. Collect the elution fractions in eppendorf tubes. Measure the protein concentration of the 6 eluted fractions with a Nanodrop. Pool the fractions that contain the VHH and transfer the solution into a 3,500 Da cutoff dialysis membrane. Dialyze the purified protein solution against 3 L of PBS overnight at 4 degrees C. On day 5, dialyze the purified protein solution against 2 L of fresh PBS for an additional 2 hours at 4 degrees C. Finally, calculate the final concentration by BCA.

Although discussed in the context of the VH/VHH, the techniques described above could also be used for scFv, Fab, Fv and other antibody fragments if required.

Multiple antigen-binding fragments (suitably VH/VHHs) can be fused by chemical cross-linking by reacting amino acid residues with an organic derivatising agent such as described by Blattler et al *Biochemistry* 24:1517-1524 (herein incorporated by reference in its entirety). Alternatively, the antigen-binding fragments may be fused genetically at the DNA level i.e. a polynucleotide construct formed which encodes the complete polypeptide construct comprising one or more antigen-binding fragments. One way of joining multiple antigen-binding fragments

via the genetic route is by linking the antigen-binding fragment coding sequences either directly or via a peptide linker. For example, the carboxy-terminal end of the first antigen-binding fragment may be linked to the amino-terminal end of the next antigen-binding fragment. This linking mode can be extended in order to link antigen-binding fragments for the construction of tri-, tetra-, etc. functional constructs. A method for producing multivalent (such as bivalent) VHH polypeptide constructs is disclosed in WO 96/34103 (herein incorporated by reference in its entirety).

Suitably, the polypeptide of the invention can be produced in a fungus such as a yeast (for example, *S. cerevisiae*) comprising growth of the fungus on a medium comprising a carbon source wherein 50-100 wt% of said carbon source is ethanol, according to the methods disclosed in WO02/48382. Large scale production of VHH fragments in *S. cerevisiae* is described in Thomassen et al 2002 *Enzyme and Micro Tech* 30:273-278 (herein incorporated by reference in its entirety).

In one aspect of the invention there is provided a process for the preparation of the polypeptide or construct of the invention comprising the following steps:

- i) cloning into a vector, such as a plasmid, the polynucleotide of the invention,
- ii) transforming a cell, such as a bacterial cell or a yeast cell capable of producing the polypeptide or construct of the invention, with said vector in conditions allowing the production of the polypeptide or construct,
- iii) recovering the polypeptide or construct, such as by affinity chromatography.

The present invention will now be further described by means of the following non-limiting examples.

EXAMPLES

Example 1: Llama immunisation

Llama immunisations were carried out using two different immunisation protocols to optimise the chance of obtaining potent cross-strain neutralising antibodies against TcdB.

Under the first protocol, two llamas were primed with 100ug of TcdB toxoids prepared by formalin inactivation of purified TcdB from a *C. difficile* 027 strain, as well as with 10^7 formalin inactivated spores from *C. difficile* strain 017 (M68) using Specol adjuvant. Llama 2 was boosted at 7, 14, 21, 28, 35 days with the same antigens, except that from day 14, gamma irradiated spores rather than formalin inactivated spores were used. In addition, the adjuvant was changed to IMS1312 for the last two boosts. Llama 1 had a similar immunisation protocol except that two further boosts were given on days 42 and 49. For Llama 1, formalin inactivated spores were used on days 0, 7, 14 and 21, and Specol was the adjuvant. However, thereafter the adjuvant was changed to IMS1312 and gamma irradiated spores were used. Intramuscular injections were used for priming and boosting both llamas, except for the last boost which was

administered subcutaneously. Blood samples were taken on days 28 (both llamas), 39 (llama 2, terminal sample), 42 (llama 1) and 53 (llama 1, terminal sample). On days 53 and 39 llamas 1 and 2 respectively were culled and lymph nodes removed. Lymphocytes were prepared from lymph nodes and blood samples to maximise the diversity of the immune response being sampled.

Under the second protocol, a llama was primed with TcdB toxoid and then boosted with 100µg/injection of *C.difficile* 630 recombinant TcdB cell binding domain (CBD) on days 14, 28 and 35. Antigens were dissolved in 1ml PBS + 1ml of Stimune and were injected intramuscularly. Blood samples were taken on days 0, 28 and 43. The llama was rested for 2.5 months then re-immunised three further times at 14 day intervals with TcdB toxoid prepared from *C. difficile* 027. Two days after the final boost, blood was removed from the llama for lymphocyte preparation.

Example 2: Phage display, ICVD selection and production

RNA extracted from the llama lymphocytes was transcribed into cDNA using a reverse transcriptase kit. The cDNA was cleaned on PCR cleaning columns. IgH (both conventional and heavy chain) fragments were amplified using primers annealing at the leader sequence region and at the CH2 region. Two DNA fragments (~700 bp and 900 bp) were amplified representing VHHs and VHs, respectively. The 700 bp fragment was cut from the gel and purified. A sample was used as a template for nested PCR. The amplified fragment was cleaned on a column and eluted. The eluted DNA was digested with BstEII and SfiI, and the 400 bp fragment was isolated from the gel. The fragments were ligated into the phagemid pUR8100 and transformed into *E. coli* TG1. Bacteria from overnight grown cultures of the libraries were collected and stored. The optical density at 600 nm (OD600) of these stocks was measured. The insert frequency was determined by picking multiple different clones from each of the library transformations and running colony PCR.

Phages were rescued from the bacteria containing libraries from the llamas by inoculating in medium containing glucose and ampicillin. When cultures were at log-phase, helper phage was added to infect the cultures and produce phages. Next day, produced phages were precipitated from the culture supernatant using a PEG/NaCl solution. The number of phages was determined by titration of the solution and infecting log-phase *E. coli* TG1 with the different phage dilutions. TcdB from ribotype 027 and strain VPI10463 were coated into wells of maxisorb plates, overnight. The following amounts were used: 500 ng, 167ng, 55 ng and 0 ng (non-coated well). Next day wells of the maxisorb plates were blocked with 4% Marvel in PBS, then phage from the libraries were added to the wells. After extensive washing with PBS-Tween and PBS, bound phages were eluted using alkaline pH shock and neutralized with 1 M Tris-HCl pH7.5. About half the eluted phages were rescued by infecting log-phase *E. coli* TG1 and selecting in medium containing ampicillin and glucose.

The sequences of the selected ICVDs are provided in Table 1A above. Selected ICVDs were B10F1, Q31B1 and Q35H8.

- 5 Selected variable domains were subcloned from the phagemid vector into the expression plasmid pMEK222 (pMEK222 is a gene3 deleted version of the phagemid pUR8100, and where the cloned variable domain is followed by FLAG-6His tags, two stop codons and the M13 terminator sequence (see WO2013/064701)). The variable domain genes were digested with SfiI and Eco91I (or BstEII) and ligated into pMEK222 cut with the same restriction enzymes. *E. coli* strain BL21 DE3 was transformed by the ligations and plated on LB-agar
- 10 plates supplemented with ampicillin and 2% glucose. Transformants were screened using colony PCR. Amplifications using the primers M13.rev (SEQ ID NO: 32) and M13.fw (SEQ ID NO: 33) led to the generation of plasmids containing inserts of 700 bp and of ~350 bp (empty plasmids) observed by PCR.
- 15 Variable domains were produced from pMEK222 by inoculation of a fresh overnight grown culture at 1/100 dilution in 800 ml 2X YT, 0.1% glucose and 100 ug/ml ampicillin and grown for 2h at 37 degrees C. Subsequently, 1 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG) was added and the culture was grown for an additional 5 h at 37 degrees C. Bacteria were harvested by centrifugation and resuspended into 30 mL PBS. Bacteria were frozen by
- 20 incubation at -20 degrees C overnight. Bacteria were thawed at room temperature and fractionated by centrifugation. To the soluble fraction, which contains the variable domain, Co2+ agarose beads, for example, Talon resin (Thermo Scientific) were added to bind His-tagged variable domain. After washing the beads, bound variable domains were eluted with PBS supplemented with 150 mM imidazole. Finally, fractions containing the variable domains
- 25 were dialyzed against PBS to remove the imidazole.

Example 3: Modification of ICVDs

- A series of modified anti-TcdB ICVDs were produced by yeast expression of DNA constructs (see Preparative Methods section, above). Heterobiheads were linked using a [Gly₄Ser]₄ amino acid linker. The modified anti-TcdB ICVDs were the following:
- 30

- ID1B (B10F1 with Q1D and R27A)
 ID2B (Q31B1 with E1D, V5Q and R27A)
 35 ID11B (Q31B1 x B10F1 hetero bihead with [G₄S]₄ linker)
 ID12B (Q35H8 x B10F1 hetero bihead with [G₄S]₄ linker)

The sequences of these modified ICVDs are provided in Table 1A.

Example 4: Determining the potency of unmodified anti-TcdB ICVDs, modified anti-TcdB ICVDs and anti-TcdB constructs against multiple ribotypes of TcdB using the standard Vero cell cytotoxicity assay.

5 4.1 - Protocol for preparing the cytotoxicity assay

Culture and maintenance of Vero cells prior to use

Routine subculture of Vero cells can be achieved as follows:

- 10 1. Once a flask of cells has grown to full confluence, aspirate all cell culture medium and apply 2 ml 1x trypsin (dissolved in 0.02% EDTA, Sigma E8008). Once the trypsin has been applied work quickly to prevent loss of cells during washing.
2. Wash the first trypsin application over the surface of the cells and then fully aspirate to remove all traces of cell culture medium (any traces of serum from the medium will
- 15 inhibit trypsin activity).
3. Apply 2 ml of trypsin and wash over the surface of the cells.
4. Remove approximately 1.5-1.7 ml of trypsin from the flask.
5. Tilt the flask so that the remaining 300-500 μ L cover the Vero cells on the surface of the plate.
- 20 6. Incubate the cells at 37 °C 5% CO₂ for 10-12 minutes.
7. To stop trypsin activity add 10 ml Vero cell medium.
8. Resuspend the cells by gently jetting the suspension against the bottom of the flask with a pipette until the medium becomes cloudy (indicating dissipation of cell clumps). 3-4 times should be sufficient. Avoid excessive pipetting as this may harm the cells.
- 25 9. Add 0.2 to 0.5 ml of the cell suspension to 25-30ml fresh Vero cell medium in a 75 cm² cell culture flask (Corning). Incubate the flask at 37 °C 5% CO₂ to allow growth of the cells to full confluence. This should occur in 3-5 days, depending on the inoculum volume and cell count. To obtain finer control over the process, cells may be enumerated using a haemocytometer, as outlined below, and added at a fixed
- 30 inoculum to the medium. Once in a confluent state the cell monolayer should remain healthy for another 1-2 days without medium replacement. To prolong the life of the confluent monolayer for use it is often helpful to refresh 1/3-1/2 of the culture medium (do not replace all the medium as it will have been conditioned with cytokines from the growing Veros). The cells should be split before rounding and detachment starts to
- 35 occur.

Preparing plates for the Vero cell cytotoxicity assay (Day -1)

- 40 Ideally, plates should be prepared the day before use in the cytotoxicity assay. However, plates may also be prepared on the day of use if necessary. If the latter is the case, prepare plates in the morning (for use in the afternoon) and ensure that at least 3 hours are allowed for cell attachment to the microplate prior to use. A fully confluent flask of Vero cells should be used to make the cell suspension for plating.

1. Add 150 µl sterile H₂O to the inter-well spaces and 300 µl to the top and bottom row of a 96-well flat bottomed microplate. This ensures that the cultured cells are hydrated during growth in the microplate.
2. Trypsinise and resuspend (in 10 ml Vero cell culture medium) a confluent flask of Vero cells, as described above.
3. Enumerate the cells using a haemocytometer and light microscope (take four independent counts and use the mean, for example using the four grid corners of a single haemocytometer slide). If there is any concern about cell viability following trypsinisation add Trypan blue dye to the cells before enumeration (1:1 v/v) and multiply the viable cell count x2.
4. Dilute the cells to 5×10^4 cells/ml in the required volume (allow 8 ml per assay plate) of Vero cell culture medium.
5. Using a multichannel pipette, dispense 100 µl of the cell suspension into each well. This is equivalent to 5000 cells/well. If multiple plates are being prepared keep swirling and/or pipetting the cell suspension between consecutive platings to ensure that the cells are evenly distributed.
6. Centrifuge the microplate at 1,000 rpm for 2 minutes at room temperature to fix the cells evenly in place across the bottom of the plate. Spin 2 plates maximum in each arm of the centrifuge to avoid the arms tipping inward and spilling the inter-well water.
7. Visually confirm that cell distribution and number are as expected using a light microscope.
8. Incubate plates at 37 °C 5% CO₂.

Setting up the assay (day 0)

- Note: All solutions described in this section are prepared in Vero cell culture medium. You should calculate the required final volume of each toxin and ICVD to cover the number of plates/combinations before starting the assay. Mix all solutions well (by vortexing and/or multiple inversions) between dilution steps.
1. Prepare the required volume of toxin at double (2x) the final assay concentration. The assay concentration required should be determined beforehand (see preliminary work, below).
 2. Prepare the test ICVDs at double (2x) the top concentration to be tested in the assay. Aim for a top concentration of ICVD that will demonstrate a clear dose-response toxin neutralisation relationship in the assay (see example graph, below).
 3. Prepare 10 serial dilutions (including the undiluted top concentration) of the 2x ICVD stock in a dilution trough. Typically, a 1/3 dilution produces a useful data range.
 4. Use a 96-well round -bottom microplate to prepare mixed solutions before addition to the plates containing Vero cells.
 5. In triplicate, prepare solutions of medium only, toxin only (1x dilution) and Triton-X100 (0.01%) controls and add each to empty plate wells.

6. Attach 10 µl pipette tips to the central 6 rows of an 8-channel aspirator. Carefully remove all medium (around 100 µl per well) from the Vero cell microplate prepared on Day 0.
7. Using a multichannel pipette, add 100 µl from one row of the preparation plate to the cells on the assay plate. Repeat this twice to fill the two adjacent rows on the assay plate (3 replicate rows in total):
8. Once plate feeding is complete incubate at 37 °C for 3 days.

Processing the assay (Day 3)

1. Observe the plates under a light microscope. Check for confluent growth in the medium only control wells and a good toxin response in the Toxin-only control well.
2. Using a multichannel pipette, in the dark, add 10 µl Alamar blue reagent (light sensitive) to each well.
3. Shake the plate for 30 seconds to ensure mixing of the Alamar blue into the culture medium.
4. Incubate the plate for 1 hr 30 minutes at 37 °C 5 % CO₂
5. Following incubation, in the dark, add 50 µl 3% SDS.
6. Read the plate using a plate reader (such as Fluostar Omega), excitation filter 544, emission filter 590, bottom optic. Set the blank (against which the data will be corrected) to the three plate wells treated with Triton X100.
7. Calculate the mean of three replicates for each treatment on the plate. Calculate % toxin neutralisation values using the formula: % Neutralisation = (ICVD treatment - toxin control)*100/(medium control - toxin control).

Preliminary work: determining the optimal amount of toxin to use in the main neutralisation assay

For ease of interpretation in the main assay, the appropriate concentration of toxin to use should be determined beforehand by conducting a toxin dose-response experiment on Vero cells. Prepare 10 serial dilutions of toxin in a 12 well dilution trough. Use the remaining two wells for 0.01% Triton and a medium only control. Prepare a minimum of 330 µL of each solution in the dilution trough (this allows three replicates at 100 µl each). If there is no indication of how potent the toxin preparation is in advance, choose a broad dilution range for the preliminary experiment. This can be repeated over a finer concentration range, if necessary. Apply these solutions to Vero cells in a flat-bottomed microplate, incubate and process the plate as described above.

To assay an ICVD, or full antibody, for neutralising activity against a given concentration of toxin, the minimum concentration of each toxin preparation capable of inducing the maximum reduction in cell viability is selected. An exemplary toxin dose-response curve on Vero cells is provided in Figure 1. The horizontal bar indicates toxin concentrations suitable for use in the main neutralisation assay.

4.2 - Potency determination

Standard Vero cell assays were carried out using the protocol detailed under 4.1 above. Neutralisation curves were produced (Figures) and IC50 values were generated in Graphpad prism using the % neutralisation data and 'log(inhibitor) vs. response -- Variable slope (four parameters)' to fit curves and generate IC50s. In some cases, IC50 was calculated by interpolation of an entered 50% value using the fitted curve. The calculated IC50 values from the different assays were as follows:

Table 2

	% TcdB Neutralisation IC50 (pM)					
	R20291 (027)	10463 (087)	M120 (078)	Liv22 (106)	Liv24 (001)	M68 (017)
B10F1	3,208	195,456	-	-	-	-
Q31B1	520	271,575	-	-	-	-
Q35H8	< 200	< 200	-	-	-	-
ID1B	12,829	187,763	-	-	-	-
ID2B	2,996	12,010	-	-	-	-
ID11B	0.9	1.6	1.1	0.9	0.9	0.4
ID12B	0.6	1.17	0.9	1.03	1.54	0.5

A hyphen denotes an assay which was not performed. It can be seen that the three unmodified ICVDs achieved good potency against both TcdB ribotypes 027 and 087, but B10F1 and Q31B1 were more potent against ribotype 027 than ribotype 087 (Table 2 and Figures 2-4). The substitutions made to B10F1 and Q31B1 in ID1B and ID2B, respectively, resulted in apparently minor reductions in potency against ribotype 027 and an increase in potency against ribotype 087. Heterobiheads ID11B and ID12B demonstrated extremely high potency against ribotypes 027, 087, 078, 106, 001 and 017.

Example 5: Further modification of constructs and impact on potency and protease stability

Genes encoding ICVDs and ICVD constructs were designed based on B10F1 and Q31B1 in which R to H substitutions and an R to F substitution were introduced into the CDRs. These constructs were as follows:

- ID20B (Q31B1 with R27A and M34I, R to H at positions 4 and 7 of CDR2)
- ID21B (Q31B1 with R27A and M34I, R to H at position 9 of CDR3)
- ID22B (Q31B1 with R27A and M34I, R to H at position 11 of CDR3)
- ID24B (B10F1 with R27A and M34I, R to H at position 9 of CDR2)
- ID25B (B10F1 with R27A and M34I, R to H at position 10 of CDR3)

- ID27B (B10F1 with R27A and M34I, R to H at position 7 of CDR3)
- ID41B (ID21B (Q31B1 with R27A, CDR3 R107H) x ID27B (B10F1 with R27A, CDR3 R105H), no M34I substitutions with [G₄S]₄ linker)
- ID43B (ID21B (Q31B1 with R27A, CDR3 R107H) x ID27B (B10F1 with R27A, CDR3 R105H) plus R108H, no M34I substitutions with [G₄S]₄ linker)
- ID45B (modified Q31B1 arm of ID43B with wild type R107)
- ID46B (modified Q31B1 arm of ID43B with R107H)
- ID49B (modified Q31B1 arm of ID43B with R107F)

5.1 – Impact on potency

Neutralising activities of these ICVDs and ICVD constructs were measured using standard Vero cell assays. These data show that the substitutions made in ID24B, ID25B and ID27B with respect to ID1B and the substitutions made in ID20B, ID21B and ID22B with respect to ID2B resulted in slightly reduced potency against ribotypes 027 and 087 (Table 3 and Figures 5 and 6).

ID41B and ID43B, although including single histidine substitutions in CDR3 of their Q31B1 component and a single histidine substitution in CDR3 (ID41B) or a double histidine substitution in CDR3 (ID43B) of their B10F1 components, were extremely potent against ribotypes 027, 087, 078, 106, 001 and 017. The impact of the second histidine substitution in CDR3 of ID43B was limited (Table 3 and Figures 7 and 8).

The potencies of ID46B and ID49B, although including CDR3 arginine to histidine or phenylalanine substitutions respectively, were only slightly reduced compared to ID45B (Table 3 and Figure 9).

Table 3

	% TcdB Neutralisation IC ₅₀ (pM)					
	R20291 (027)	10463 (087)	M120 (078)	Liv22 (106)	Liv24 (001)	M68 (017)
ID20B	57,219	133,544	-	-	-	-
ID21B	12,642	19,476	-	-	-	-
ID22B	7,106	36,985	-	-	-	-
ID24B	79,491	298,794	-	-	-	-
ID25B	75,975	373,841	-	-	-	-
ID27B	178,134	438,066	-	-	-	-
ID41B	5.4	8.6	8.4	8.8	7.6	4.7
ID43B	7.6	17	7.9	13	14	6.4
ID45B	749	-	-	-	-	-
ID46B	4443	-	-	-	-	-
ID49B	3287	-	-	-	-	-

5.2 – Impact on protease stability – exposure to trypsin and chymotrypsin

ID11B and ID43B were incubated with trypsin or chymotrypsin beads for intervals of 0 (untreated control), 15, 30, 45 and 60 minutes. These data demonstrate that ID11B was digested by trypsin after between 15 and 30 minutes. ID43B however remained substantially undigested in trypsin and chymotrypsin (Figure 10). Engineered bi-head construct ID43B therefore has substantial stability against trypsin and chymotrypsin.

5.3 – Impact on protease stability – exposure to faecal supernatant

ID11B and ID43B were incubated in faecal supernatants. The faecal supernatant pools were each produced from 5 faecal samples from either *C.difficile* positive patients (CD+) or *C.difficile* negative patients (CD-). The constructs were digested in the supernatants for 1 hour or as a separate experiment for 4 hours and then measured using an ELISA. ID43B showed much greater % survival than ID11B after incubation for 1 hour (Figure 11). Engineered bi-head construct ID43B therefore has substantial stability in faecal supernatant. The % survival was calculated by dividing the average variable domain concentration for a single time point by the average construct concentration in the 0-time point wells.

Example 6: Comparison of neutralising capabilities of ID11B, ID12B and ID43B relative to Medarex monoclonal antibody Mab124

A standard Vero cell assay was conducted to assess the potencies of ID11B, ID12B and ID43B relative to Medarex Mab124 (PCT Publication WO 2006/121422, in the name of University of Massachusetts and Medarex, Inc.) against TcdB produced by multiple *C. difficile* ribotypes.

The results show that ID11B, ID12B and ID43B are more potent than Mab124 against all ribotypes of TcdB tested here using the standard Vero cell assay. The difference in potencies between ID11B (Figure 12), ID12B (Figure 13) and ID43B (Figure 14) relative to Mab124 were particularly marked when tested against TcdB from hypervirulent ribotype 027.

Example 7: Construction and potency analysis of quadrahead constructs

Quadrahead constructs comprising two different anti-TcdA ICVDs (based on Q34A3 and B4F10) and two different anti-TcdB ICVDs (based on Q31B1 and B10F1) were produced in yeast using the methodology detailed in the Preparative Methods section above. Each ICVD in each quadrahead was connected by a [Gly₄Ser]₄ linker. The format of these quadraheads is summarised in Table 4 below:

52

Table 4

Name	Format	Production (mg/ml)	ICVD monomer order (N to C terminal)				Position of CDR3 R to H substitutions in each ICVD monomer from N to C terminal			
			1st	2nd	3rd	4th	1st	2nd	3rd	4th
ID1C	AA'BB'	0.93	B4F10	Q34A3	Q31B1	B10F1				
ID3C	BAA'B'	2.26	Q31B1	B4F10	Q34A3	B10F1				
ID5C	BB'AA'	1.27	Q31B1	B10F1	B4F10	Q34A3	107	105		
ID6C	ABB'A'	1.08	B4F10	Q31B1	B10F1	Q34A3		107	105	
ID7C	A'BB'A	1.22	Q34A3	Q31B1	B10F1	B4F10		107	105	
ID8C	BB'AA'	0.33* (batch 1) 0.72* (batch 2)	Q31B1	B10F1	B4F10	Q34A3	107	105, 108	100, 109, 110	
ID11C	A'BB'A	0.59*	Q34A3	Q31B1	B10F1	B4F10		107	105, 108	100, 109, 110

*These quadraheads had a lower production level than ID-1C to ID-7C. However, it is expected this is due to batch to batch variation and that these quadraheads would normally be produced at a similar level to ID-1C to ID-7C.

It can be seen from Table 4 above that the order of ICVDs in a quadrahead influences production level, with ID3C being produced at the highest level in yeast. ID-8C is ID5C with additional CDR3 R to H substitutions. ID11C is ID7C with additional CDR3 R to H substitutions.

The potency of these quadraheads against TcdA and TcdB from various *C.difficile* ribotypes was analysed using the cytotoxicity assay described in Example 4 above.

7.1 – Potency of ID-1C (AA'BB') and ID-3C (BAA'B')

ID1C and ID3C were found to potently neutralise TcdA from ribotypes 027 and 087 (Figure 15, Graph I) and TcdB from ribotypes 027 and 017 (Figure 15, Graph II). The most significant difference between these two quadraheads is the separation between the two anti-TcdB ICVDs. In ID1C, the separation is the [Gly₄Ser]₄ linker alone, whilst in ID3C the two anti-TcdB ICVDs are separated by [Gly₄Ser]₄, anti-TcdA ICVD, [Gly₄Ser]₄, anti-TcdA ICVD, [Gly₄Ser]₄. This may be the reason for ID1C having a slightly greater potency than ID3C against TcdA and TcdB from these ribotypes.

7.2 – Potency of ID-5C and ID-8C (BB'AA')

ID-5C was found to potently neutralise 027 TcdA (Figure 16, Graph I) and 027 TcdB (Figure 16, Graph II).

ID8C is effectively a combination of anti-TcdA bihead ID33A and anti-TcdB bihead ID43B. The neutralising potency of ID-8C was compared to that of: (a) constituent bihead ID33A against TcdA from five ribotypes of *C.difficile* (Figure 17, Graphs I to III) and (b) constituent bihead ID43B against TcdB from six ribotypes of *C.difficile* (Figure 18, Graphs I to V). ID8C demonstrated a similar or under certain circumstances even improved potency relative to its constituent biheads against both TcdA and TcdB from the various ribotypes tested.

7.3 – Potency of ID-6C (ABB'A')

ID6C was found to potently neutralise 027 TcdA (Figure 19, Graph I) and 027 TcdB (Figure 19, Graph II).

7.4 – Potency of ID-7C and ID-11C (A'BB'A')

ID7C is effectively a combination of anti-TcdA bihead ID17A and anti-TcdB bihead ID41B. The neutralising potency of ID8C was compared to that of: (a) constituent bihead ID17A against TcdA from five ribotypes of *C.difficile* (Figure 20) and (b) constituent bihead ID41B against TcdB from six ribotypes of *C.difficile* (Figure 21, Graphs I to III). ID7C demonstrated a similar potency relative to its constituent biheads against both TcdA and TcdB from the various ribotypes tested.

ID11C (ID7C with further CDR3 R to H modifications) also demonstrated potent neutralisation of 027 and 078 TcdA (Figure 22, Graph I) and 027 and 087 TcdB (Figure 22, Graph II).

5 Example 8: Analysis of TcdA and TcdB binding by ID-1C and ID-3C

An experiment was performed to investigate whether or not ID-1C and ID-3C bind both TcdA and TcdB simultaneously.

- 10 Two ELISA plates were set up to investigate the binding of ID-1C and two to investigate the binding of ID-3C. Columns 1 to 3 of each plate were coated with 50 µl anti TcdA mAb PCG4.1. Columns 4 to 9 were coated with 027 TcdB or 087 TcdB. Columns 10 to 12 were left uncoated. The plates were incubated at 4°C overnight, after which plates were washed, then all wells were blocked with 1% BSA in PBS for approximately 1.5 hours. Columns 6 to 12
- 15 were then incubated with 50ul of (a) ID-1C, (b) ID-3C or (c) both ID-1C and ID-3C, for 1 hour. The Plates were then washed and incubated with various concentrations (0-20ng/ml) of either 027 TcdA or 087 TcdA. After 2 hours of incubation, plates were washed, then incubated with rabbit anti-TcdA pAb or with rabbit polyclonal anti-ICVD for 1 hour. Plates were washed again and swine anti-rabbit-HRP was added to every well for 1 hour. After washing, plates were
- 20 incubated with 100µl TMB, allowed to develop, stopped with 50µl of 0.5M H₂SO₄, then read at 450nm in a plate reader.

- The results are shown in Figures 23 to 26, wherein ICVD binding in the form of quadraheads (open circle) is plotted on the right hand axis and amount of TcdA bound (closed symbols) is
- 25 plotted on the left hand axis. As expected, the plates show a smooth dose response for the anti-TcdA mAb wells with increasing toxin concentration (square symbol). The other wells without any ICVD present in the form of quadrahead show no significant signal (dotted line, triangle symbol) and the wells without any TcdB show no significant signal (dotted line, diamond symbol). Clearly absence of ICVD in the form of quadraheads or anti-TcdA mAb, or
- 30 absence of TcdB coating, completely negates TcdA binding) The signal from the rabbit polyclonal anti-ICVD wells demonstrated that the quadraheads had successfully bound to toxin (open circle). I

- The 027 (Figure 23) and 087 (Figure 24) ID-1C plates show smooth dose responses for ID-1C
- 35 with increasing toxin concentration (unbroken line, triangle symbol) and the 027 (Figure 25) and 087 (Figure 26) ID-3C plates show smooth dose responses for ID-3C with increasing toxin concentration (unbroken line, triangle symbol), demonstrating that both of these quadraheads successfully bind to the TcdB coatings and to the immobilised TcdA. It can be concluded therefore that quadraheads ID-1C and ID-3C simultaneously bind to TcdA and TcdB.

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Throughout the specification and the claims which follow, unless the context requires otherwise, the word 'comprise', and variations such as 'comprises' and 'comprising', will be

understood to imply the inclusion of a stated integer, step, group of integers or group of steps but not to the exclusion of any other integer, step, group of integers or group of steps. All patents and patent applications mentioned throughout the specification of the present invention are herein incorporated in their entirety by reference. The invention embraces all combinations of preferred and more preferred groups and suitable and more suitable groups and embodiments of groups recited above.

Claims

1. A polypeptide comprising an immunoglobulin chain variable domain which binds to *Clostridium difficile* toxin B, wherein the immunoglobulin chain variable domain comprises three complementarity determining regions (CDR1-CDR3) and four framework regions (FR1-FR4), wherein:

(a) CDR1 comprises a sequence sharing 40% or greater sequence identity with SEQ ID NO: 1, CDR2 comprises a sequence sharing 55% or greater sequence identity with SEQ ID NO: 2 and CDR3 comprises a sequence sharing 50% or greater sequence identity with SEQ ID NO: 3; or

(b) CDR1 comprises a sequence sharing 40% or greater sequence identity with SEQ ID NO: 4, CDR2 comprises a sequence sharing 55% or greater sequence identity with SEQ ID NO: 5 and CDR3 comprises a sequence sharing 60% or greater sequence identity with SEQ ID NO: 6.

2. The polypeptide according to claim 1, wherein:

in respect of part (a) of claim 1, CDR1 comprises a sequence sharing 60% or greater, such as 80% or greater sequence identity with SEQ ID NO: 1, CDR2 comprises a sequence sharing 60% or greater, such as 70% or greater, such as 75% or greater, such as 80% or greater, such as 85% or greater, such as 90% or greater sequence identity with SEQ ID NO: 2 and CDR3 comprises a sequence sharing 60% or greater, such as 65% or greater, such as 75% or greater, such as 80% or greater, such as 90% or greater sequence identity with SEQ ID NO: 3; or

in respect of part (b) of claim 1, CDR1 comprises a sequence sharing 60% or greater, such as 80% or greater sequence identity with SEQ ID NO: 4, CDR2 comprises a sequence sharing 60% or greater, such as 70% or greater, such as 75% or greater, such as 80% or greater, such as 85% or greater, such as 90% or greater sequence identity with SEQ ID NO: 5 and CDR3 comprises a sequence sharing 65% or greater, such as 70% or greater, such as 75% or greater, such as 85% or greater, such as 90% or greater sequence identity with SEQ ID NO: 6.

3. The polypeptide according to either claim 1 or 2, wherein:

in respect of part (a) of either claim 1 or 2, any residues of CDR1, CDR2 or CDR3 differing from their corresponding residues in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3, respectively, are conservative substitutions with respect to their corresponding residues; or

in respect of part (b) of either claim 1 or 2, any residues of CDR1, CDR2 or CDR3 differing from their corresponding residues in SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6, respectively, are conservative substitutions with respect to their corresponding residues.

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4. The polypeptide according to any one of claims 1 to 3, wherein CDR1, CDR2 and/or CDR3 are devoid of K or R.

10

5. The polypeptide according to any one of claims 1 to 4, wherein CDR1, CDR2 and/or CDR3 have been mutated to replace one or more R or K residues with an H residue.

6. The polypeptide according to claim 1, wherein:

15

in respect of part (a) of claim 1, CDR1 comprises SEQ ID NO: 1, CDR2 comprises SEQ ID NO: 2 and CDR3 comprises SEQ ID NO: 3; or

in respect of part (b) of claim 1, CDR1 comprises SEQ ID NO: 4, CDR2 comprises SEQ ID NO: 5 and CDR3 comprises SEQ ID NO: 6; or

20

in respect of part (b) of claim 1, CDR1 comprises SEQ ID NO: 7, CDR2 comprises SEQ ID NO: 8 and CDR3 comprises SEQ ID NO: 9.

7. The polypeptide according to claim 6, wherein:

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in respect of part (a) of claim 9, CDR1 consists of SEQ ID NO: 1, CDR2 consists of SEQ ID NO: 2 and CDR3 consists of SEQ ID NO: 3; or

in respect of part (b) of claim 9, CDR1 consists of SEQ ID NO: 4, CDR2 consists of SEQ ID NO: 5 and CDR3 consists of SEQ ID NO: 6; or

30

in respect of part (b) of claim 9, CDR1 consists of SEQ ID NO: 7, CDR2 consists of SEQ ID NO: 8 and CDR3 consists of SEQ ID NO: 9.

8. The polypeptide according to any one of claims 1 to 7, wherein:

35

in respect of part (a) of any one of claims 1 to 10, FR1, FR2, FR3 and FR4 each comprise a sequence sharing 40% or greater, such as 60% or greater, such as 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater sequence identity with FR1, FR2, FR3 and FR4 of SEQ ID NO 10, respectively; or

40

in respect of part (b) of any one of claims 1 to 10, FR1, FR2, FR3 and FR4 each comprise a sequence sharing 40% or greater, such as 60% or greater, such as 70% or greater, such as 80% or greater, such as 90% or greater, such as 95%

or greater sequence identity with FR1, FR2, FR3 and FR4 of SEQ ID NO 11, respectively; or

in respect of part (b) of any one of claims 1 to 10, FR1, FR2, FR3 and FR4 each comprise a sequence sharing 40% or greater, such as 60% or greater, such as 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater sequence identity with FR1, FR2, FR3 and FR4 of SEQ ID NO 12, respectively.

9. The polypeptide according to any one of claims 1 to 8, wherein residue 1 of FR1 is D, E or Q; and/or wherein residue 5 of FR1 is V.
10. The polypeptide according to claim 1 which comprises or consists of:
SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.
11. The polypeptide according to any one of claims 1 to 10, wherein the polypeptide is an antibody or an antibody fragment such as a VHH, a VH, a VL, a V-NAR, a Fab fragment or a F(ab')₂ fragment.
12. The polypeptide according to claim 11, wherein the polypeptide is a VHH.
13. The polypeptide according to claim 11, wherein the polypeptide is a VH.
14. The polypeptide according to any one of claims 1 to 13, wherein the polypeptide binds to the cell binding domain of *Clostridium difficile* toxin B.
15. A construct comprising two or more identical polypeptides according to any one of claims 1 to 14.
16. A construct comprising at least one polypeptide according to any one of claims 1 to 14 and at least one different polypeptide, wherein the different polypeptide binds to *Clostridium difficile* toxin B.
17. The construct according to claim 16, wherein the different polypeptide is a polypeptide according to any one of claims 1 to 14.
18. The construct according to claim 17, wherein the different polypeptide is selected from:
SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 27 SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

19. The construct according to claim 17, wherein the construct is selected from:

SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 24 or SEQ ID NO: 25.

20. A construct comprising at least one polypeptide according to any one of claims 1 to 14 and at least one different polypeptide, wherein the different polypeptide binds to a target other than *Clostridium difficile* toxin B.

21. The construct according to claim 20 wherein the different polypeptide binds to *Clostridium difficile* toxin A.

22. A construct comprising or consisting of four polypeptides, wherein at least one polypeptide is a polypeptide according to any one of claims 1 to 14 and the three further polypeptides each bind to a target selected from the list consisting of *Clostridium difficile* toxin A and *Clostridium difficile* toxin B.

23. The construct according to claim 22, wherein the construct comprises or consists of two polypeptides according to any one of claims 1 to 14 and two polypeptides which bind to *Clostridium difficile* toxin A.

24. The construct according to claim 23, wherein the construct is of the format, from N- to C- terminal, A-A-B-B, B-B-A-A, A-B-A-B, B-A-B-A, A-B-B-A or B-A-A-B, wherein A is a polypeptide which binds to *Clostridium difficile* toxin A and B is a polypeptide which binds to *Clostridium difficile* toxin B, wherein the polypeptides which bind to *Clostridium difficile* toxin A are identical or different and the polypeptides which bind to *Clostridium difficile* toxin B are identical or different.

25. The construct according to claim 24, wherein the polypeptides which bind to *Clostridium difficile* toxin A are identical and the polypeptides which bind to *Clostridium difficile* toxin B are identical.

26. The construct according to claim 24, wherein the construct is of the format A-A'-B-B', A-B-B'-A' or B-A-A'-B', wherein B is a polypeptide according to any one of claims 1 to 17, B' is a different polypeptide according to any one of claims 1 to 17, A is a polypeptide which binds to *Clostridium difficile* toxin A and A' is a different polypeptide which binds to *Clostridium difficile* toxin A.

27. The construct according to claim 26, wherein the construct is selected from: SEQ ID NOs: 41-47.

28. The construct according to any one of claims 15 to 27 wherein the polypeptides are all connected by linkers.
- 5 29. The construct according to claim 28, wherein the linkers are of the format (G₄S)_x wherein x is 1 to 10, such as 6.
- 10 30. The polypeptide or construct according to any one of claims 1 to 29, wherein the polypeptide or construct is capable of neutralising *Clostridium difficile* ribotypes 087 and 027.
31. The polypeptide or construct according to claim 30, wherein the polypeptide or construct is capable of neutralising *Clostridium difficile* ribotypes 087, 027, 078, 017, 106 and 001.
- 15 32. The polypeptide or construct according to any one of claims 1 to 31, wherein the polypeptide or construct neutralises TcdA cytotoxicity in the standard Vero cell assay with an EC₅₀ of 450000 pM or less, such as 350000 pM or less, such as 250000 pM or less, such as 200000 pM or less, such as 150000 pM or less, such as 100000 pM or less, such as 80000 pM or less, such as 60000 pM or less, such as 40000 pM or less, such as 20000 pM or less, such as 10000 pM or less, such as 5000 pM or less, such as 4000 pM or less, such as 3000 pM or less, such as 2000 pM or less, such as 1000 pM or less, such as 500 pM or less, such as 100 pM or less, such as 50 pM or less, such as 10 pM or less, such as 9 pM or less, such as 8 pM or less, such as 7 pM or less, such as 6 pM or less, such as 5 pM or less, such as 4 pM or less, such as 3 pM or less, such as 2 pM or less, such as 1 pM or less, such as 0.9 pM or less, such as 0.8 pM or less, such as 0.7 pM or less, such as 0.6 pM or less, such as 0.5 pM or less, such as 0.4 pM or less.
- 20 33. The polypeptide or construct according to any one of claims 1 to 32, which is substantially resistant to one or more proteases produced in the small or large intestine.
- 25 34. The polypeptide or construct according to claim 33, wherein the one or more proteases are selected from the group consisting of enteropeptidase, trypsin, chymotrypsin, *C. difficile*-specific proteases and inflammatory proteases.
- 30 35. A pharmaceutical composition comprising the polypeptide or construct according to any one of claims 1 to 34 and one or more pharmaceutically acceptable diluents or carriers.
- 35 36. The pharmaceutical composition according to claim 35 comprising at least one further active agent.
- 40 37. The pharmaceutical composition according to either claim 35 or claim 36, wherein the composition is presented in enterically coated form.

38. The polypeptide, pharmaceutical composition or construct according to any one of claims 1 to 37, for use as a functional or medicinal food.
- 5 39. The polypeptide, pharmaceutical composition or construct according to any one of claims 1 to 37, for use as a medicament.
40. The polypeptide, pharmaceutical composition or construct according to claim 39, for use in the treatment of *Clostridium difficile* infection.
- 10 41. The polypeptide, pharmaceutical composition or construct for use according to any one of claims 39 to 40, which is administered orally, intravenously, subcutaneously, intrarectally or by enema.
- 15 42. The polypeptide, pharmaceutical composition or construct for use according to claim 41, which is administered orally.
43. Use of the polypeptide, pharmaceutical composition or construct according to any one of claims 1 to 37 in the manufacture of a medicament for the treatment of *Clostridium difficile* infection.
- 20 44. A method of treating *Clostridium difficile* infection comprising administering to a person in need thereof a therapeutically effective amount of the polypeptide, pharmaceutical composition or construct according to any one of claims 1 to 37.
- 25 45. A polynucleotide encoding the polypeptide or construct according to any one of claims 1 to 34.
- 30 46. The polynucleotide according to claim 45, wherein the polynucleotide comprises or consists of a sequence sharing 70% or greater, such as 80% or greater, such as 90% or greater, such as 95% or greater, such as 99% or greater sequence identity with any one of SEQ ID NOs: 34-40.
- 35 47. The polynucleotide according to claim 46, wherein the polynucleotide comprises or consists of any one of SEQ ID NOs: 34-40.
48. A cDNA comprising the polynucleotide according to any one of claims 45 to 47.
49. A vector comprising the polynucleotide or cDNA according to any one of claims 45 to 48.
- 40 50. A host cell transformed with a vector according to claim 49 and which is capable of expressing the polypeptide or construct according to any one of claims 1 to 34, such as a mammalian cell, a plant cell, a yeast cell such as a yeast belonging to the genera

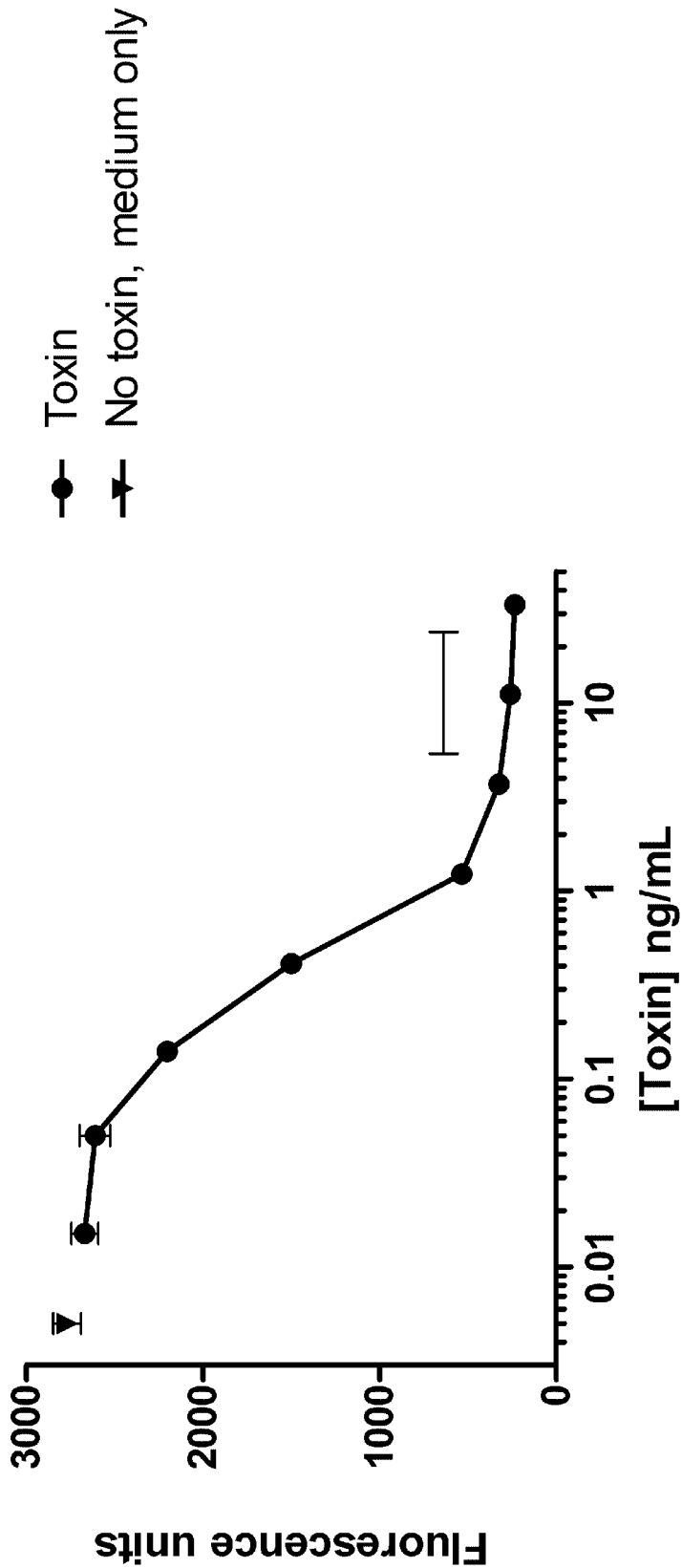
Aspergillus, *Saccharomyces*, *Kluyveromyces*, *Hansenula* or *Pichia*, such as *S. cerevisiae* or a bacterial cell such as *E. coli*.

51. A process for the preparation of the polypeptide or construct according to any one of
5 claims 1 to 37, comprising the following steps:
- i) cloning into a vector, such as a plasmid, the polynucleotide or cDNA according to any one of claims 45 to 48,
 - 10 ii) transforming a cell, such as a bacterial cell or a yeast cell capable of producing the polypeptide or construct according to any one of claims 1 to 37, with said vector in conditions allowing the production of the polypeptide or construct,
 - 15 iii) recovering the polypeptide or construct, such as by affinity chromatography.
52. A polypeptide which specifically binds to the epitope of *Clostridium difficile* toxin B bound by the polypeptide of any one of claims 1 to 14.
- 20 53. A VH or VHH which specifically binds to and has neutralising activity against *Clostridium difficile* toxin B.

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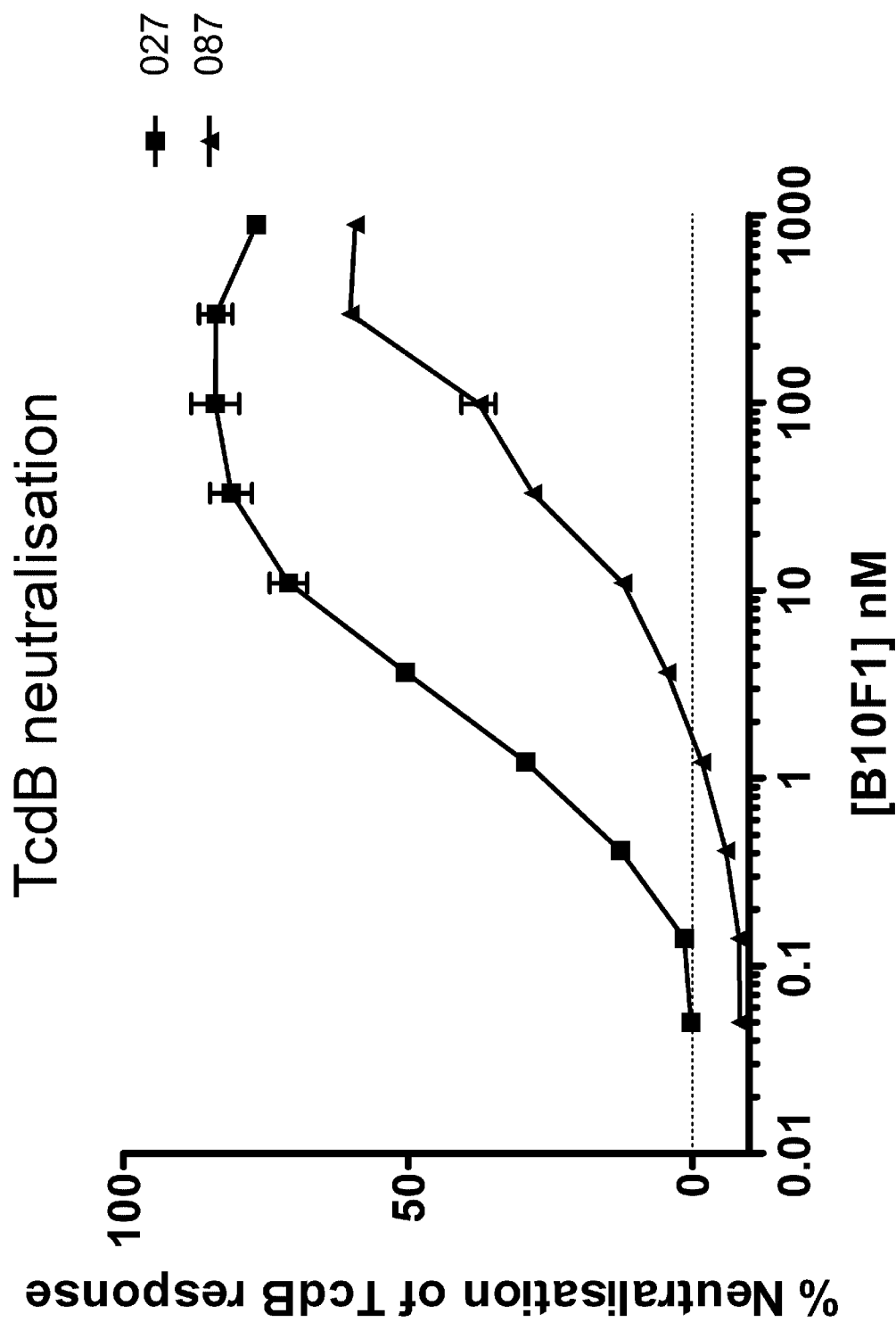
Figure 1

Reduction in vero cell viability in the presence of *C. difficile* toxin



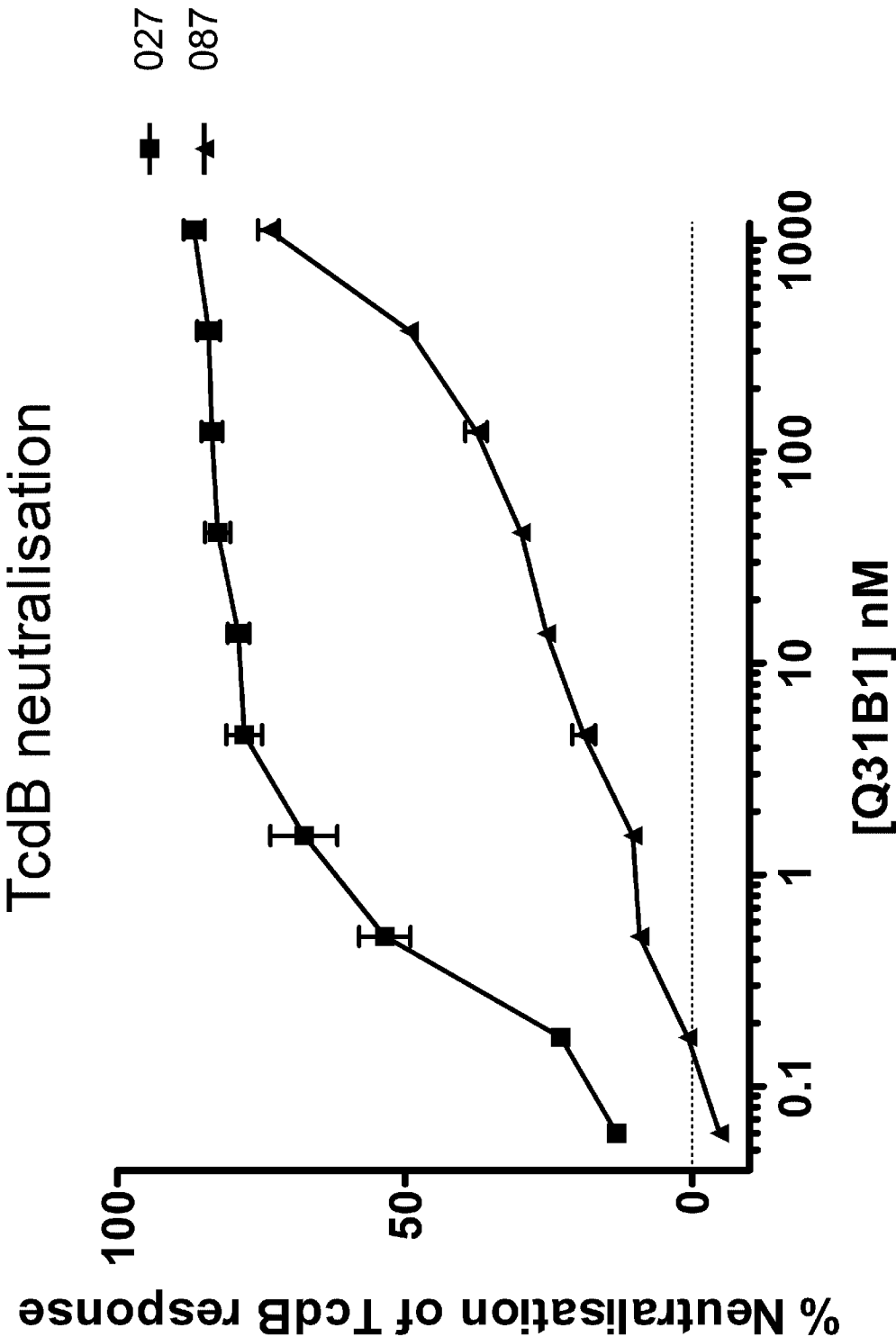
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Figure 2



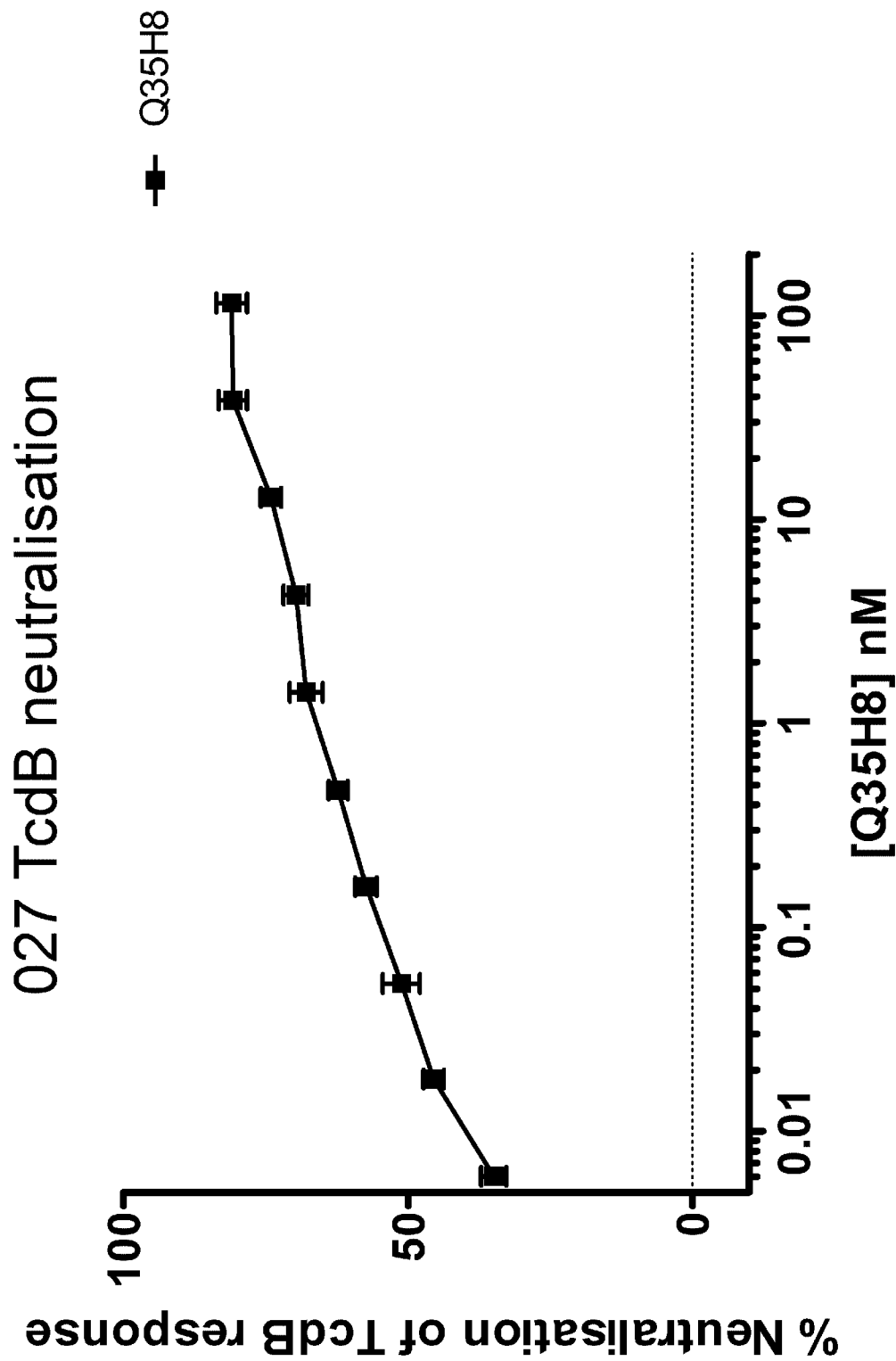
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Figure 3



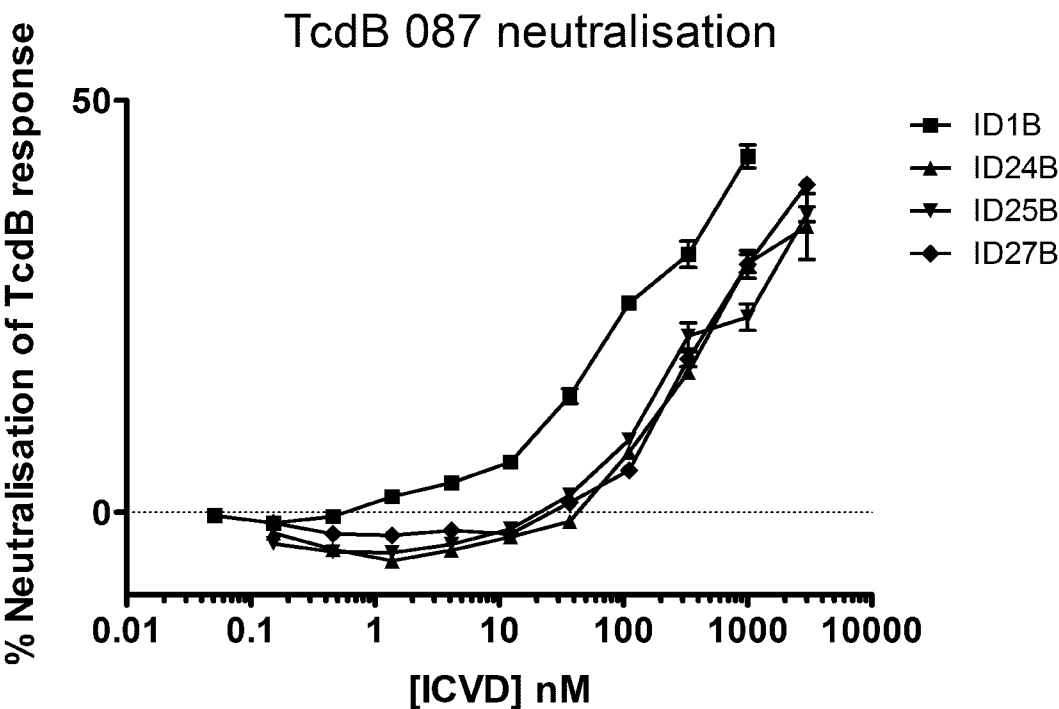
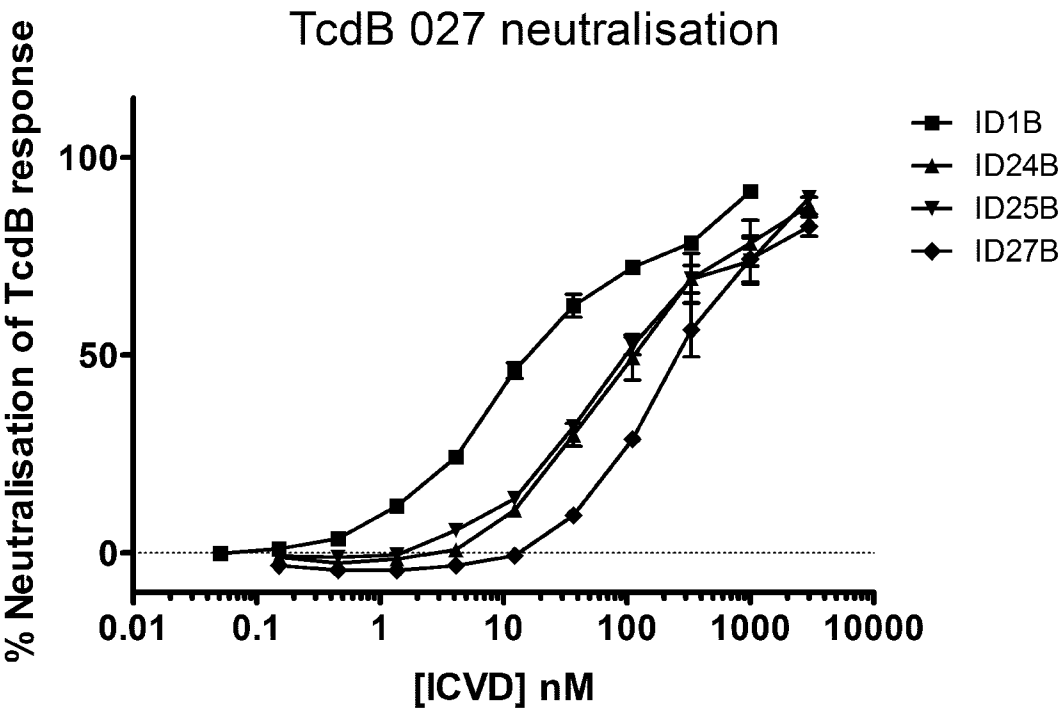
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Figure 4



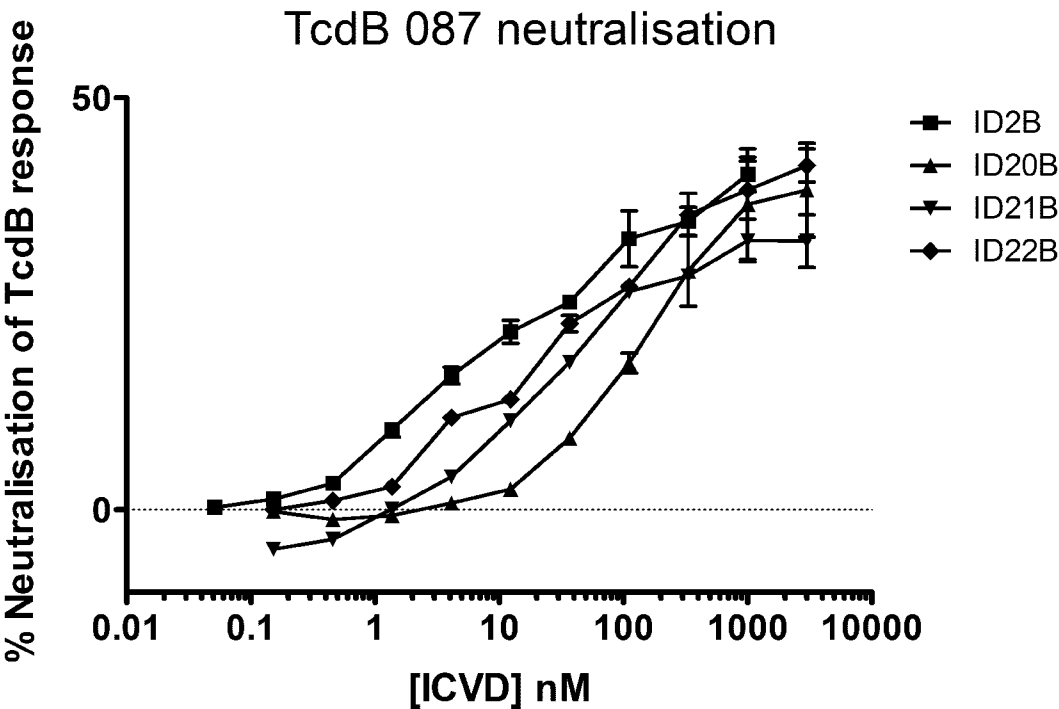
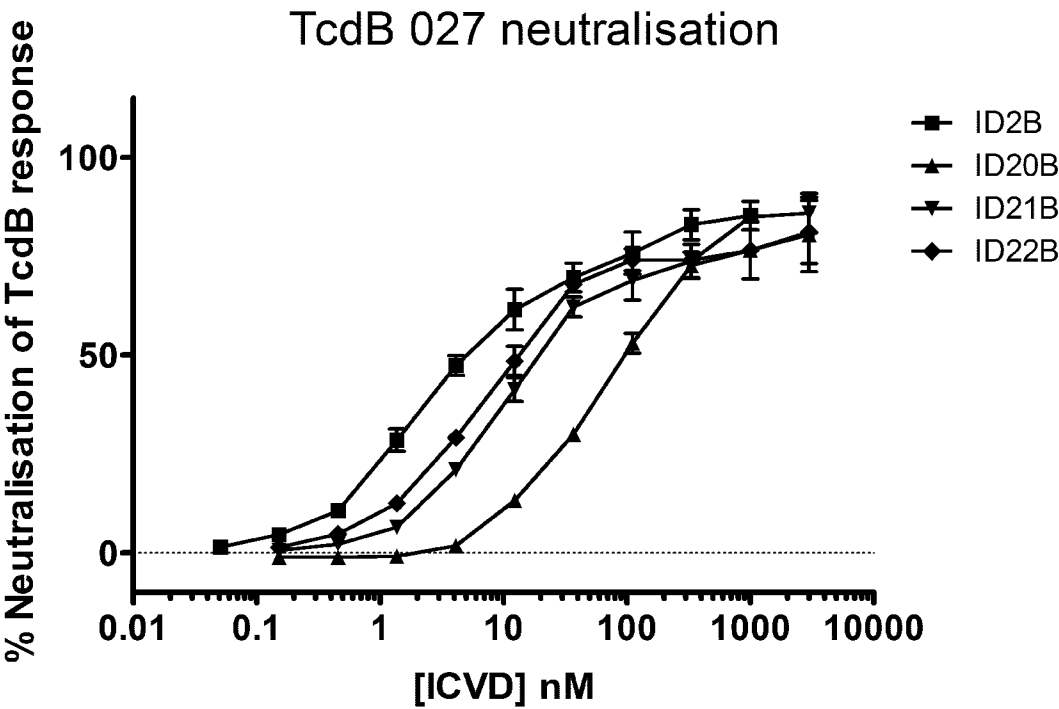
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Figure 5



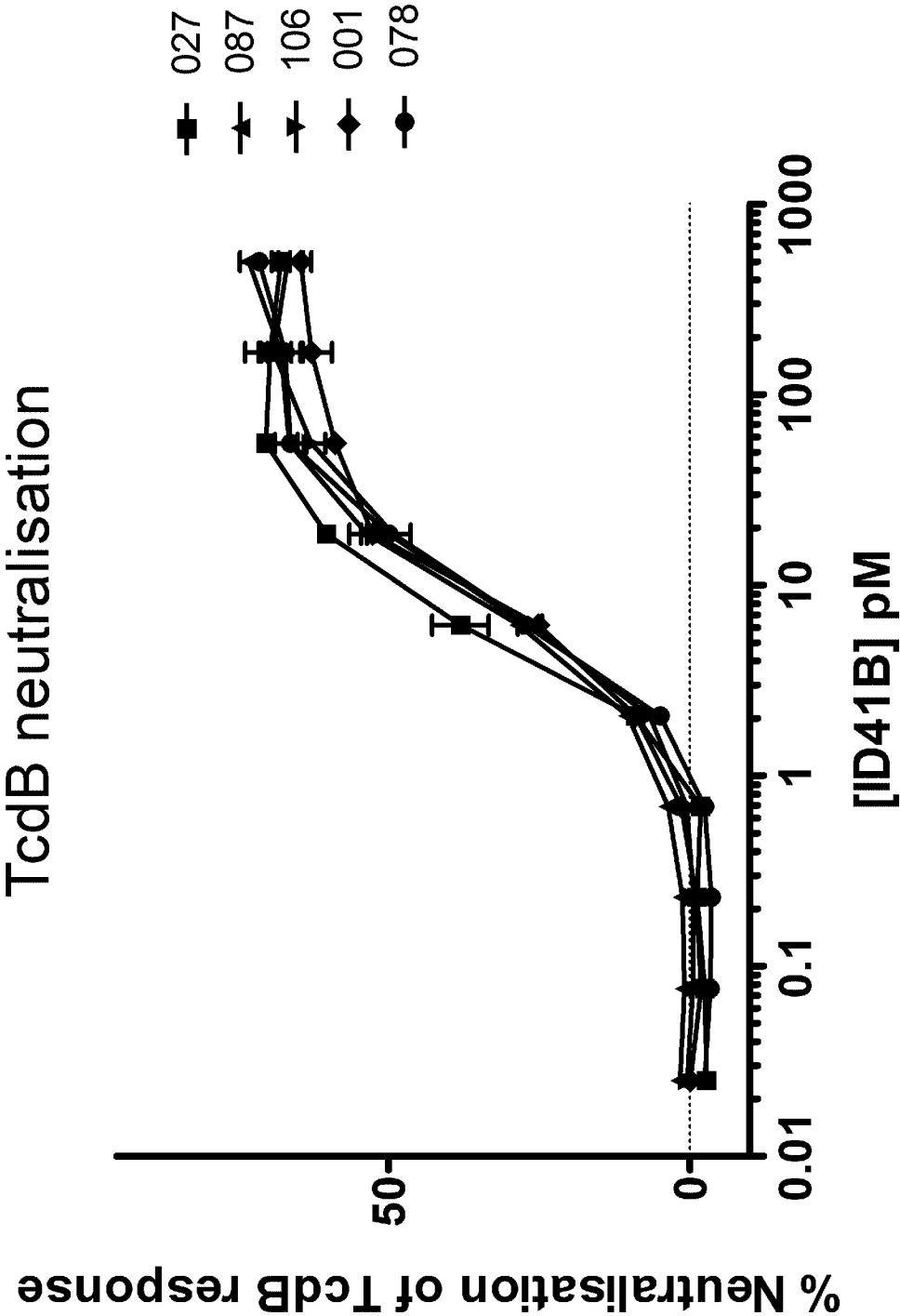
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Figure 6

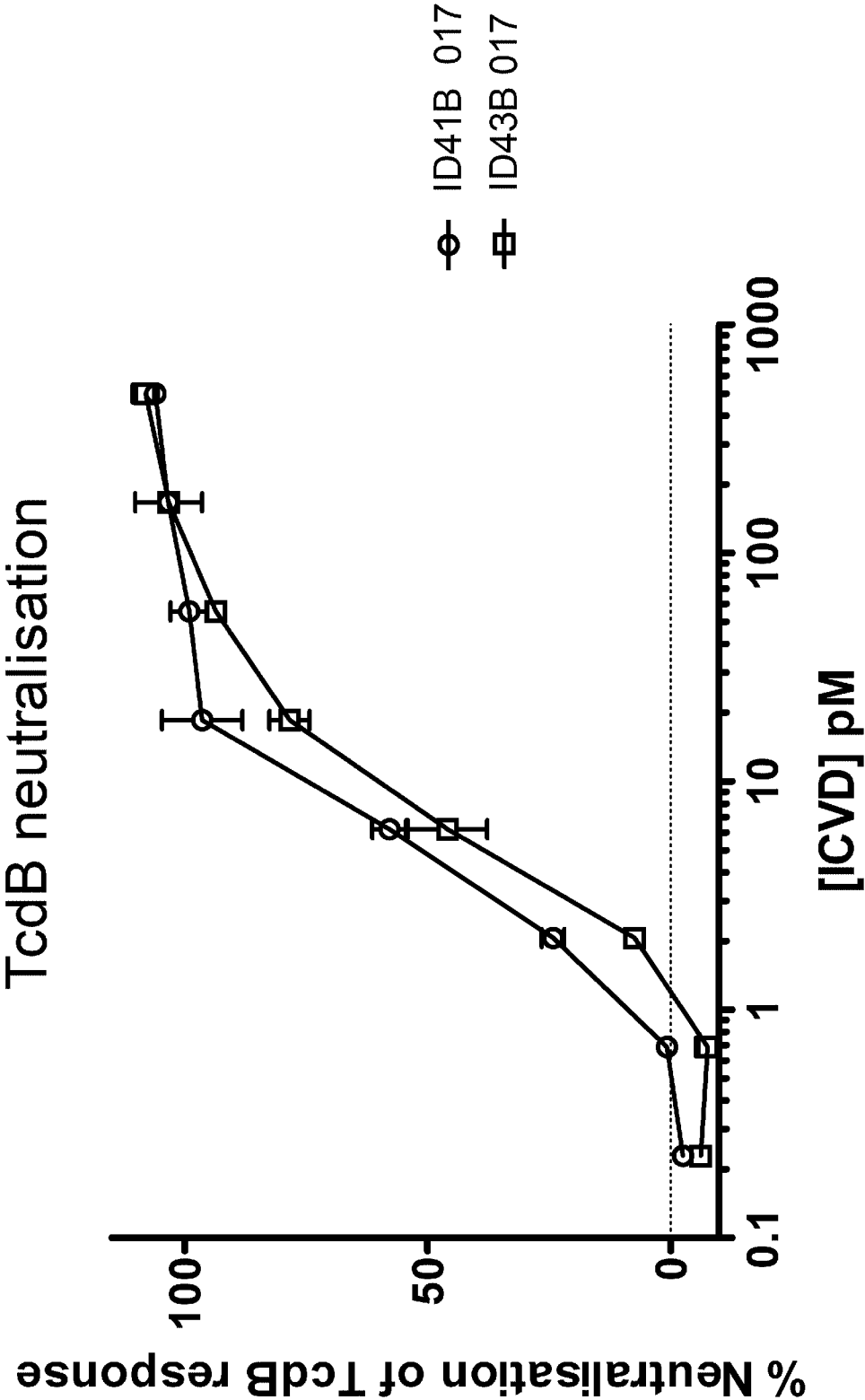


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Figure 7

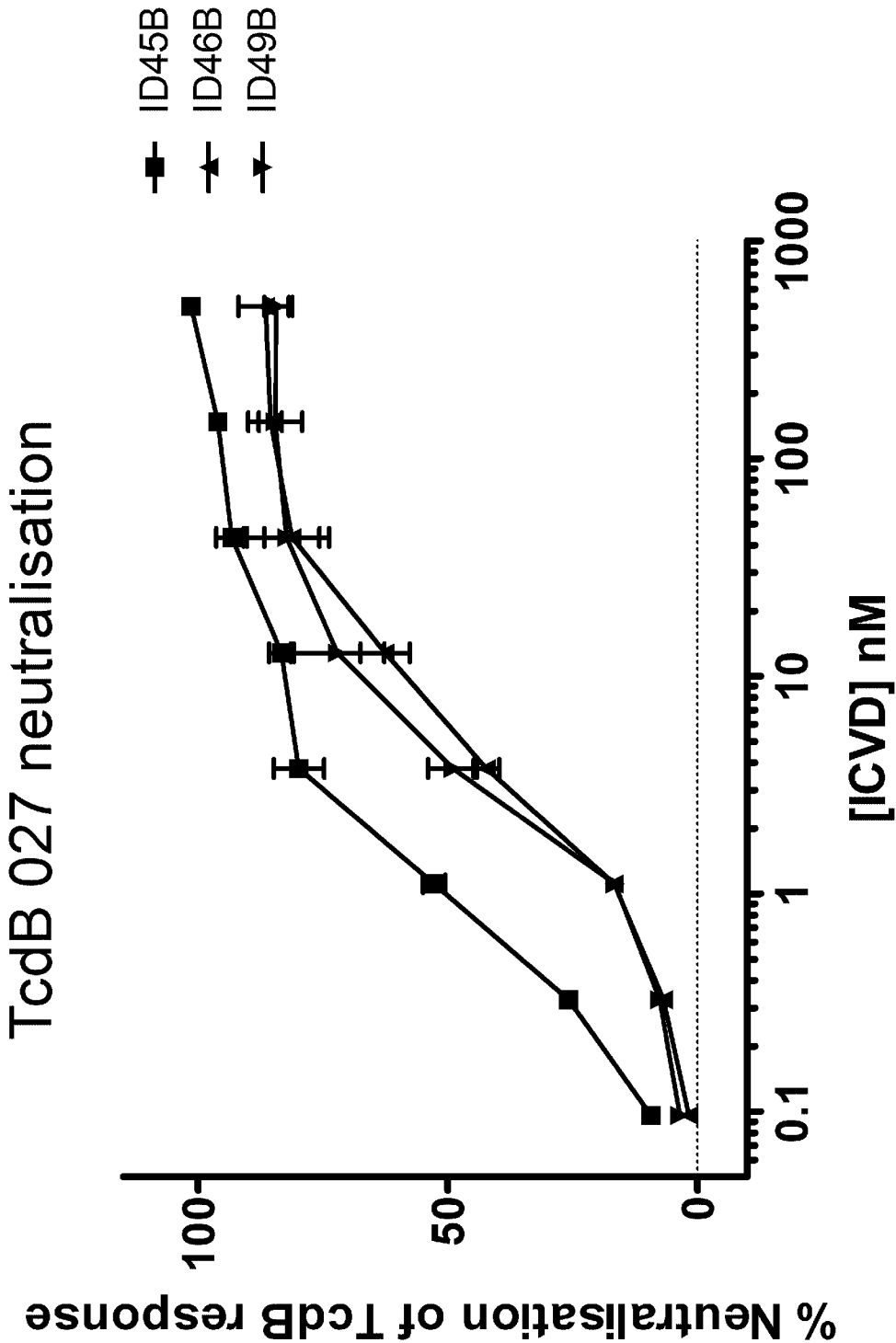


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Figure 8

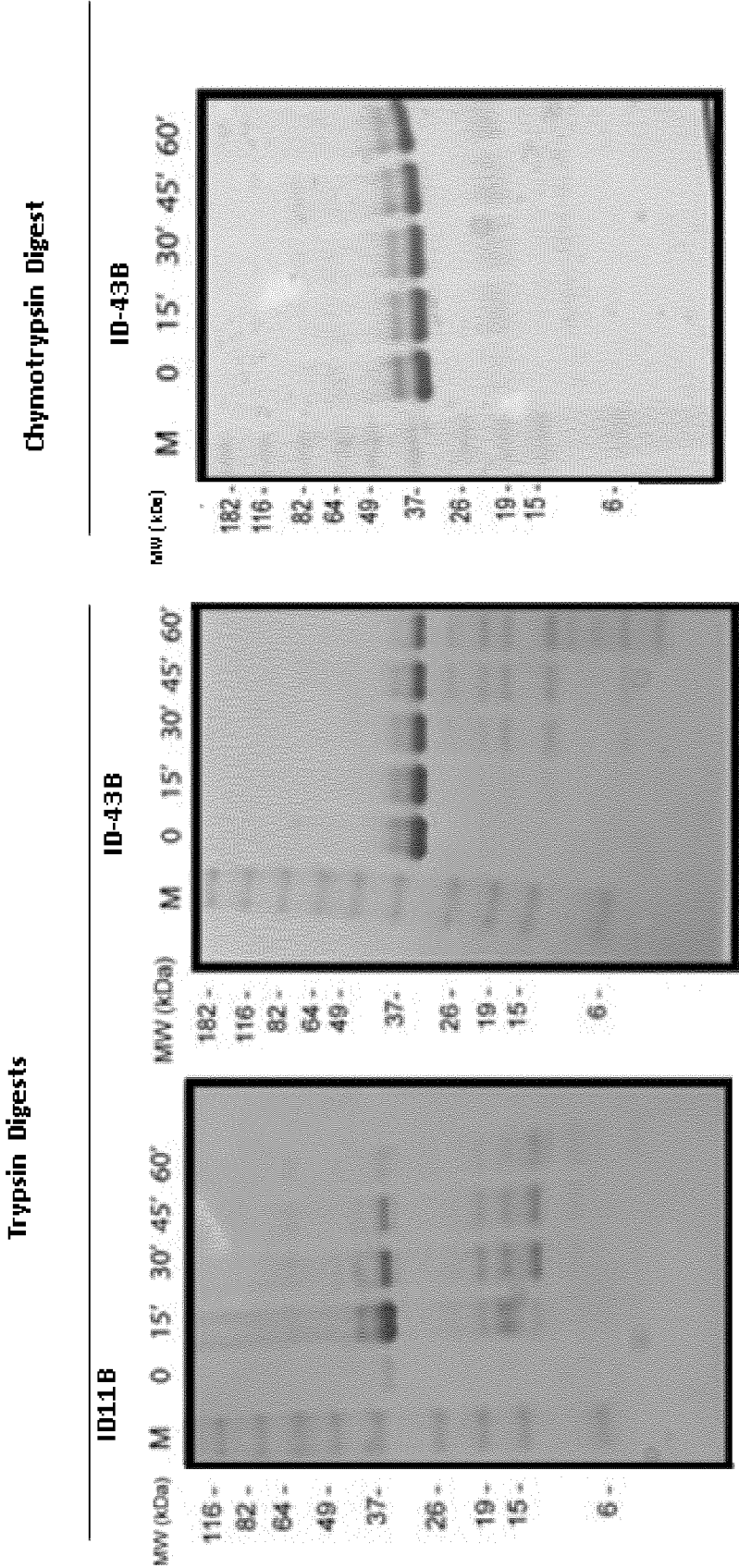


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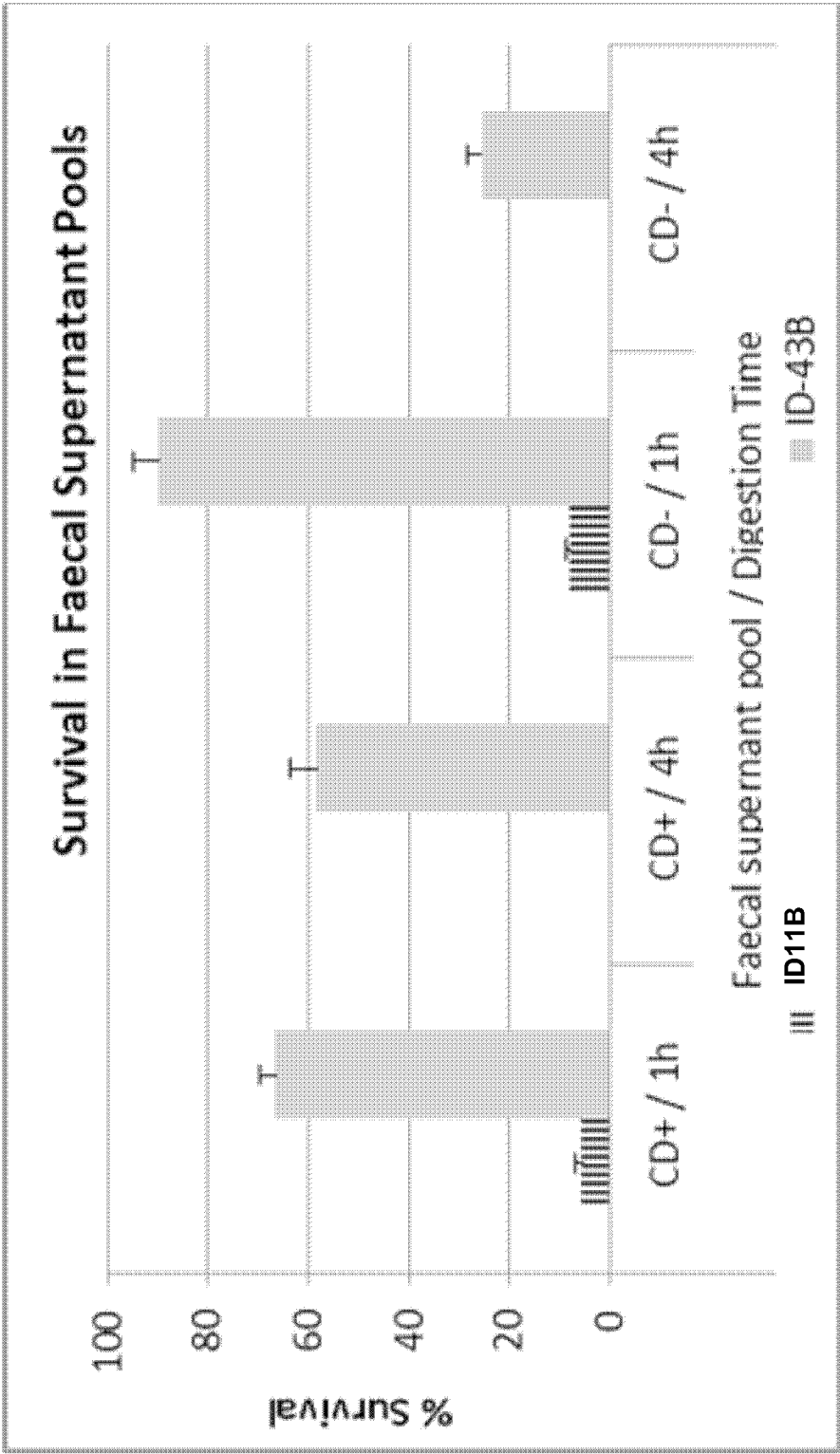
Figure 9



10/33
Figure 10



11/33
Figure 11



12/33
Figure 12

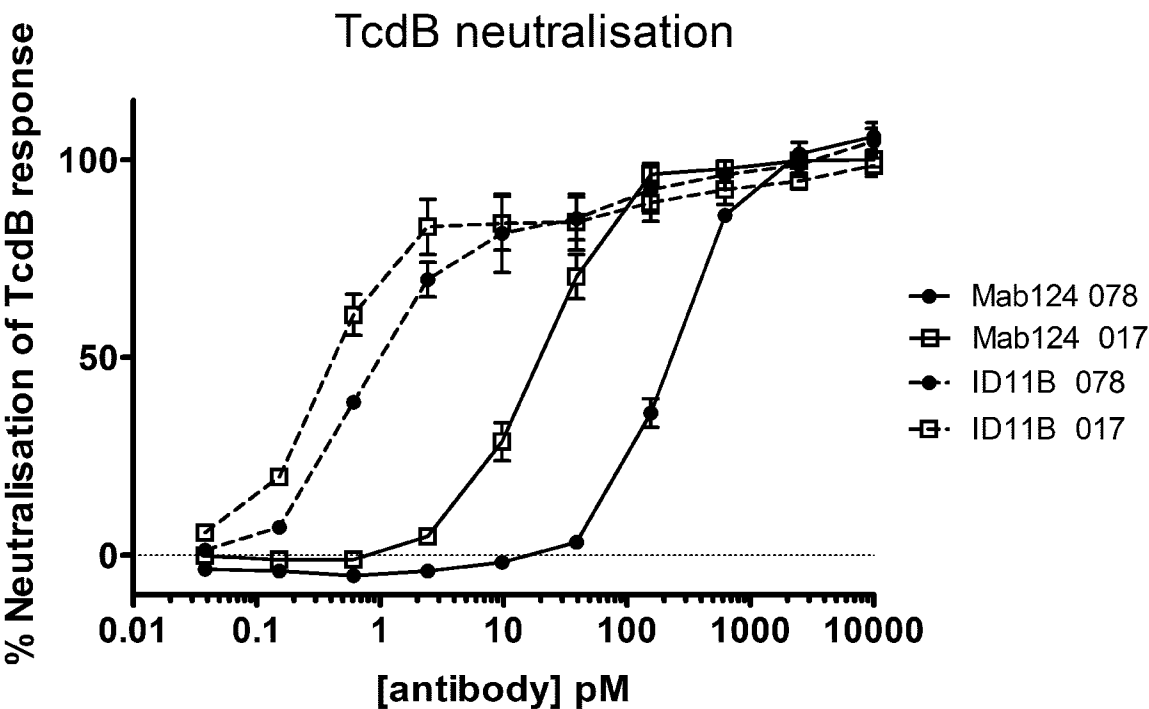
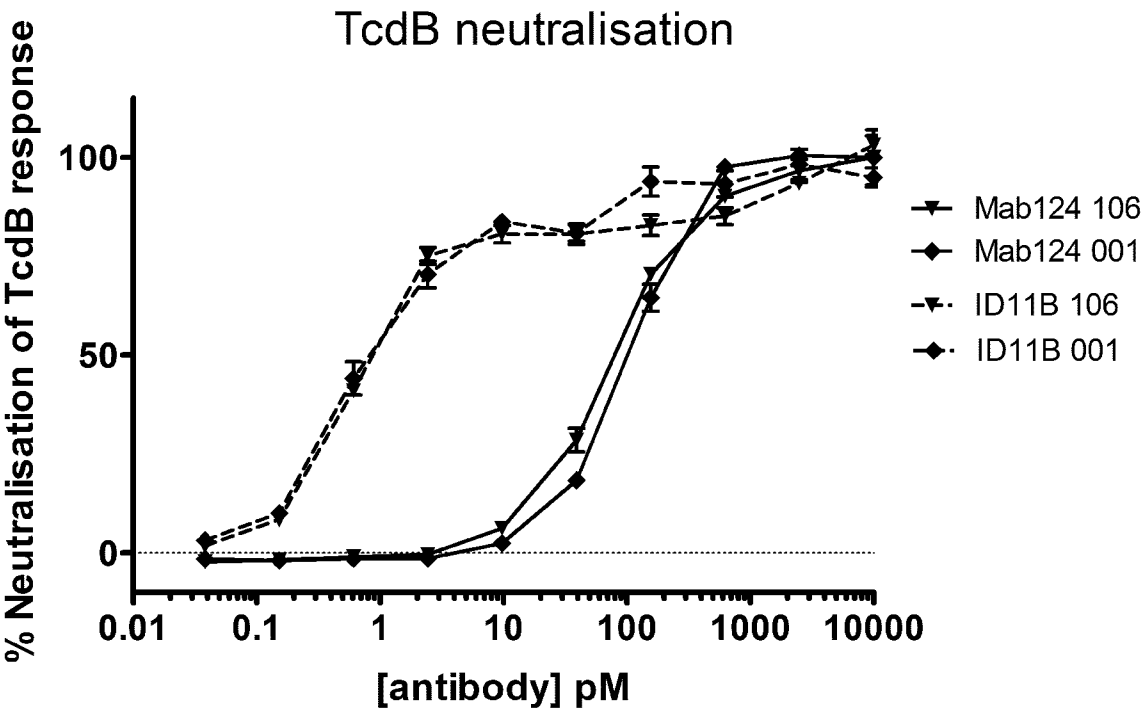
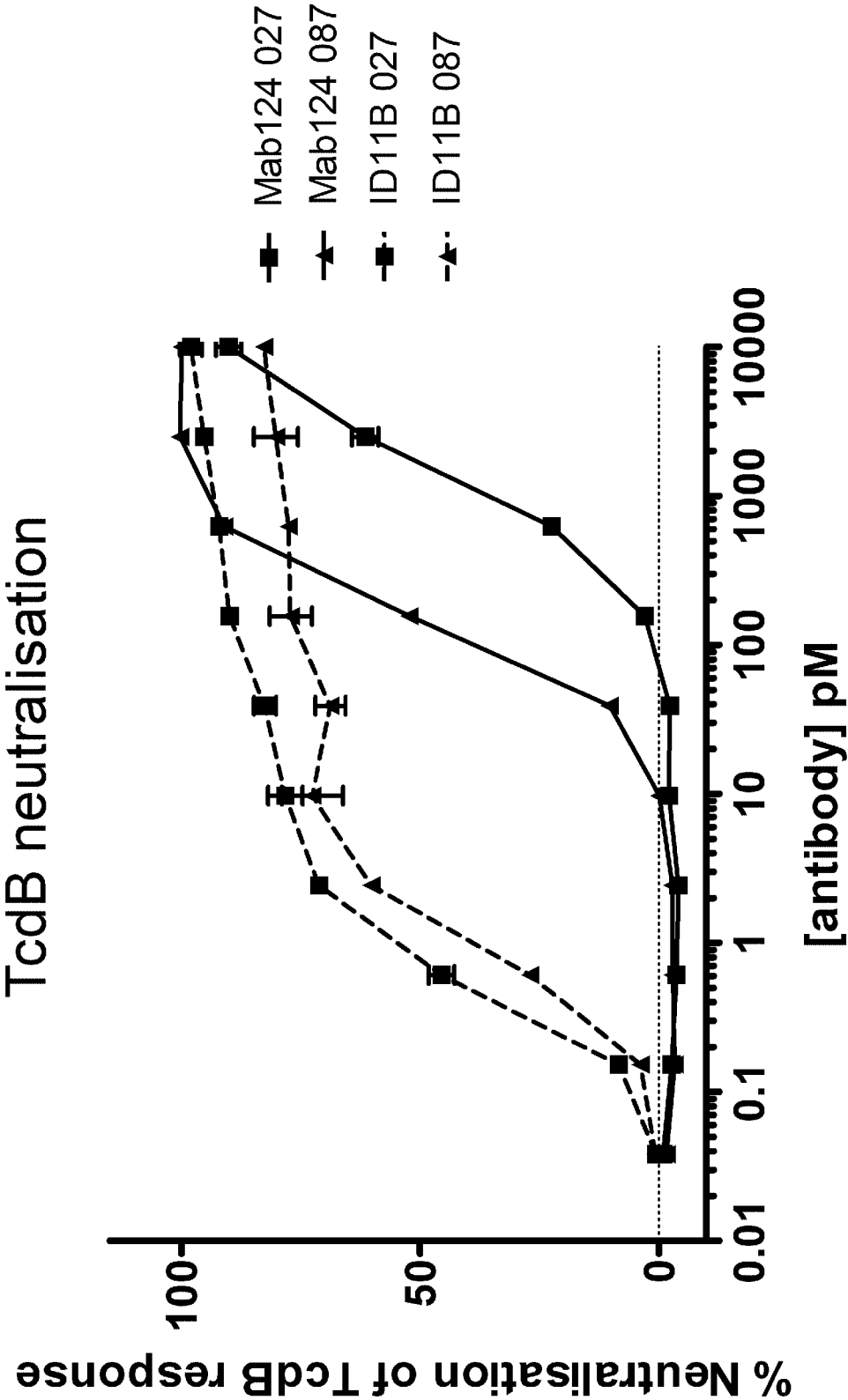


Figure 12 continued



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Figure 13

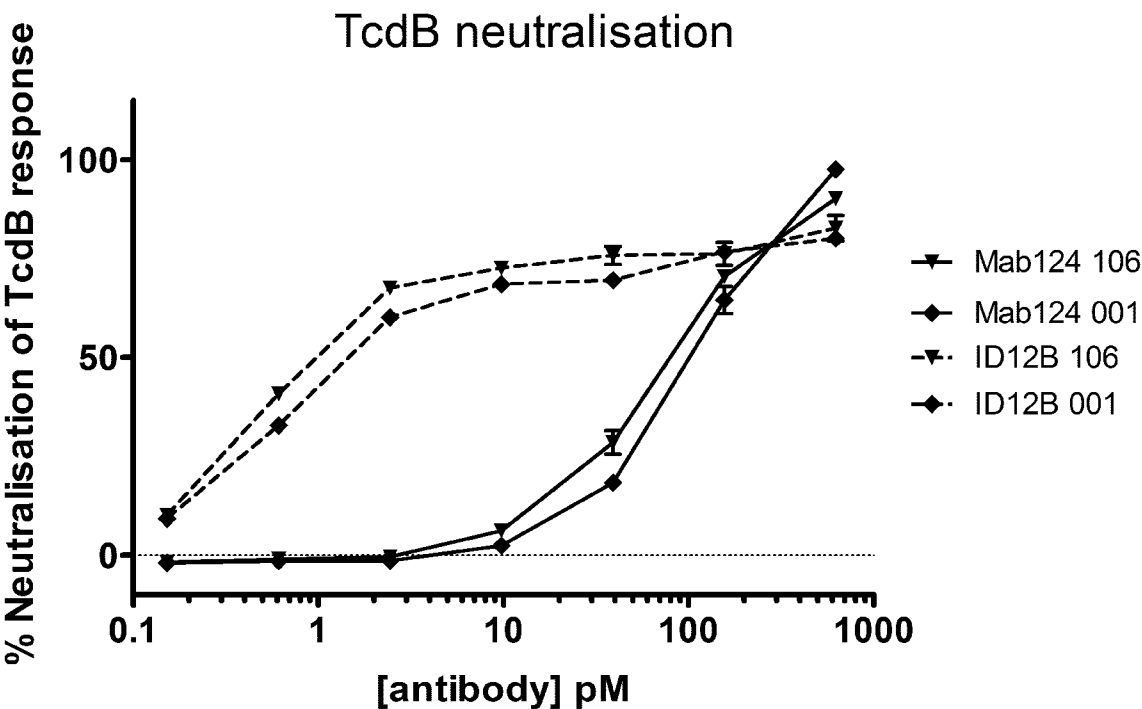
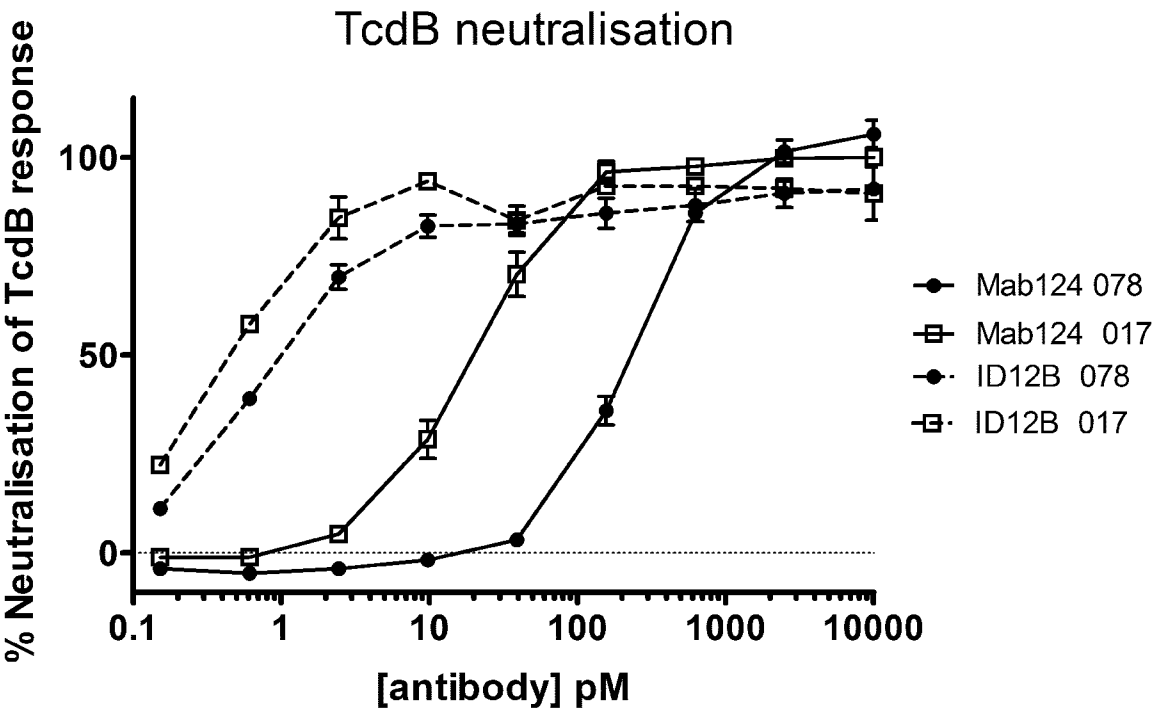
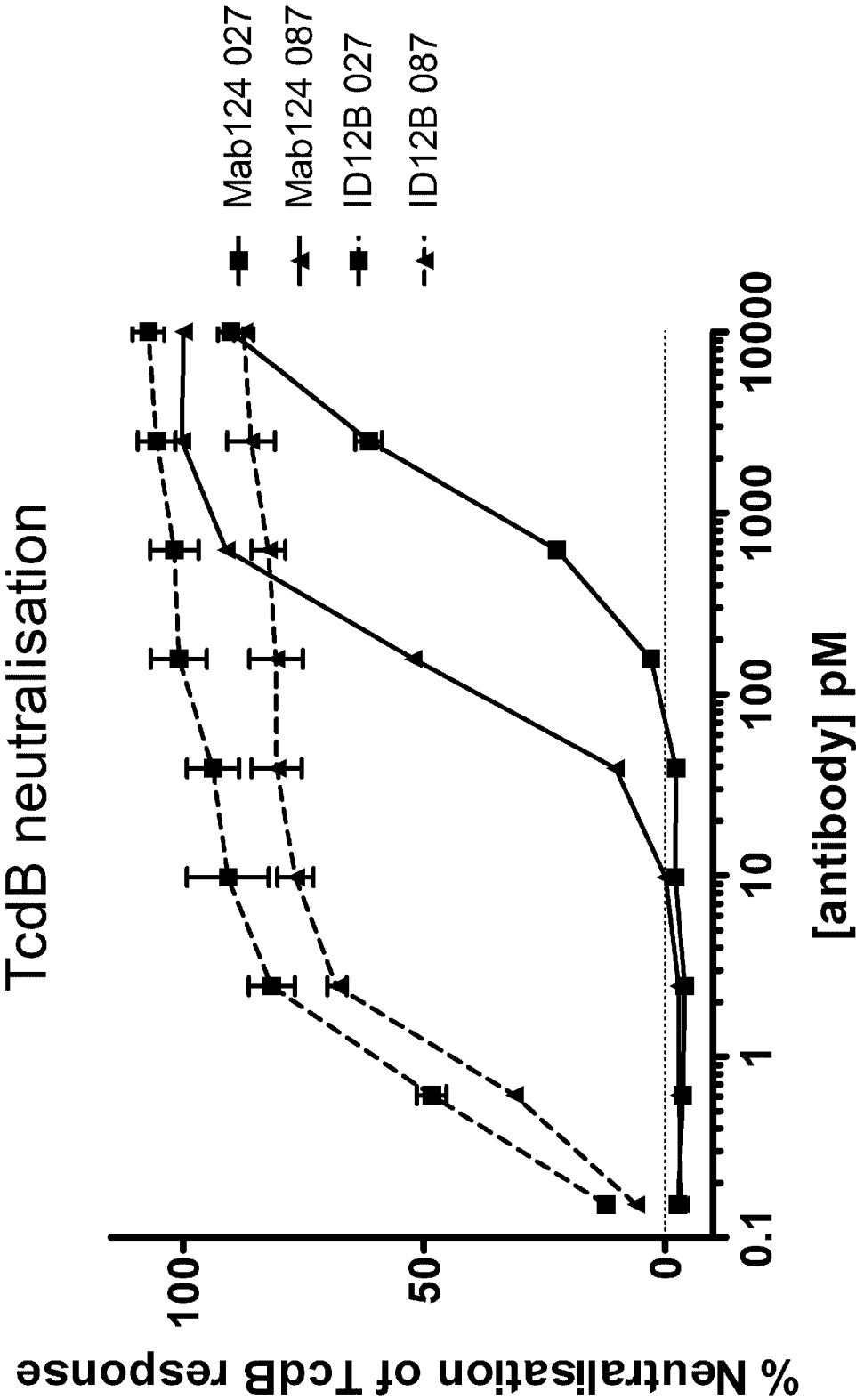


Figure 13 continued



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Figure 14

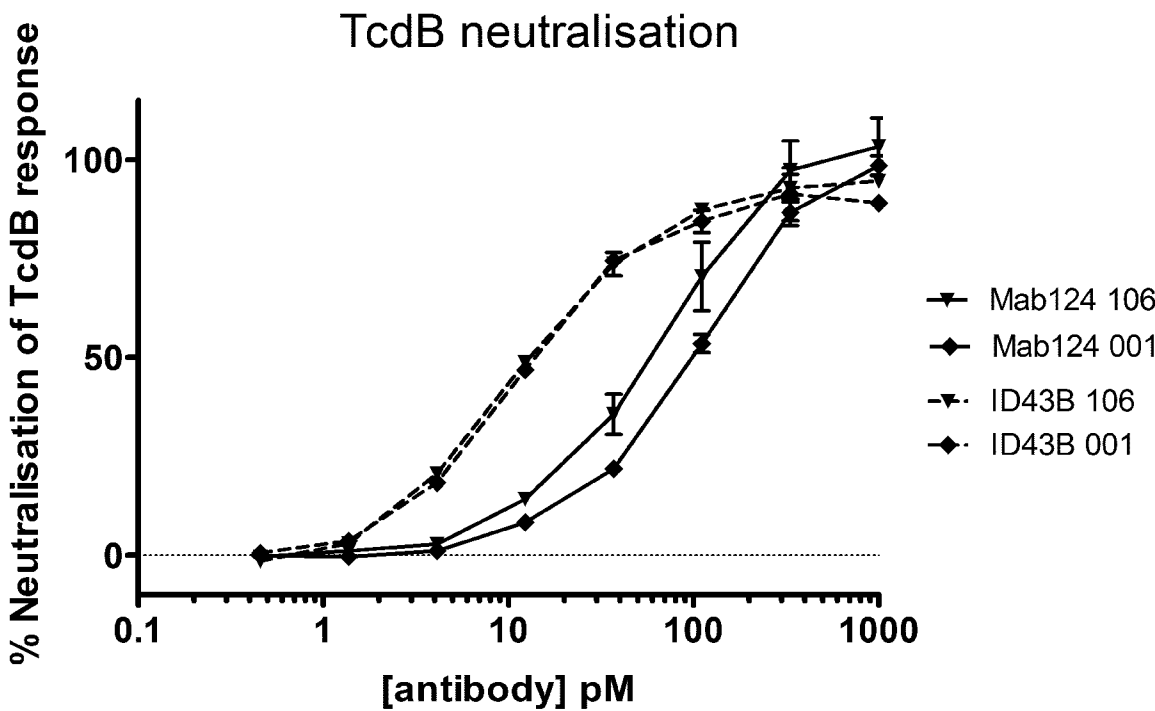
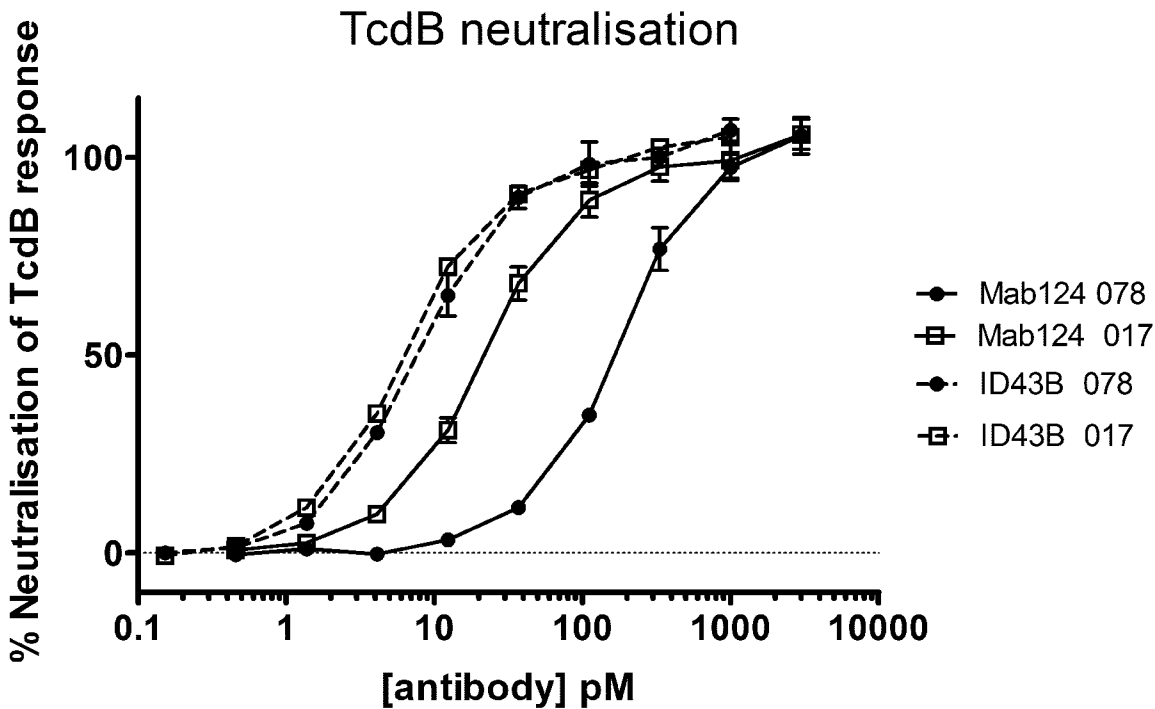
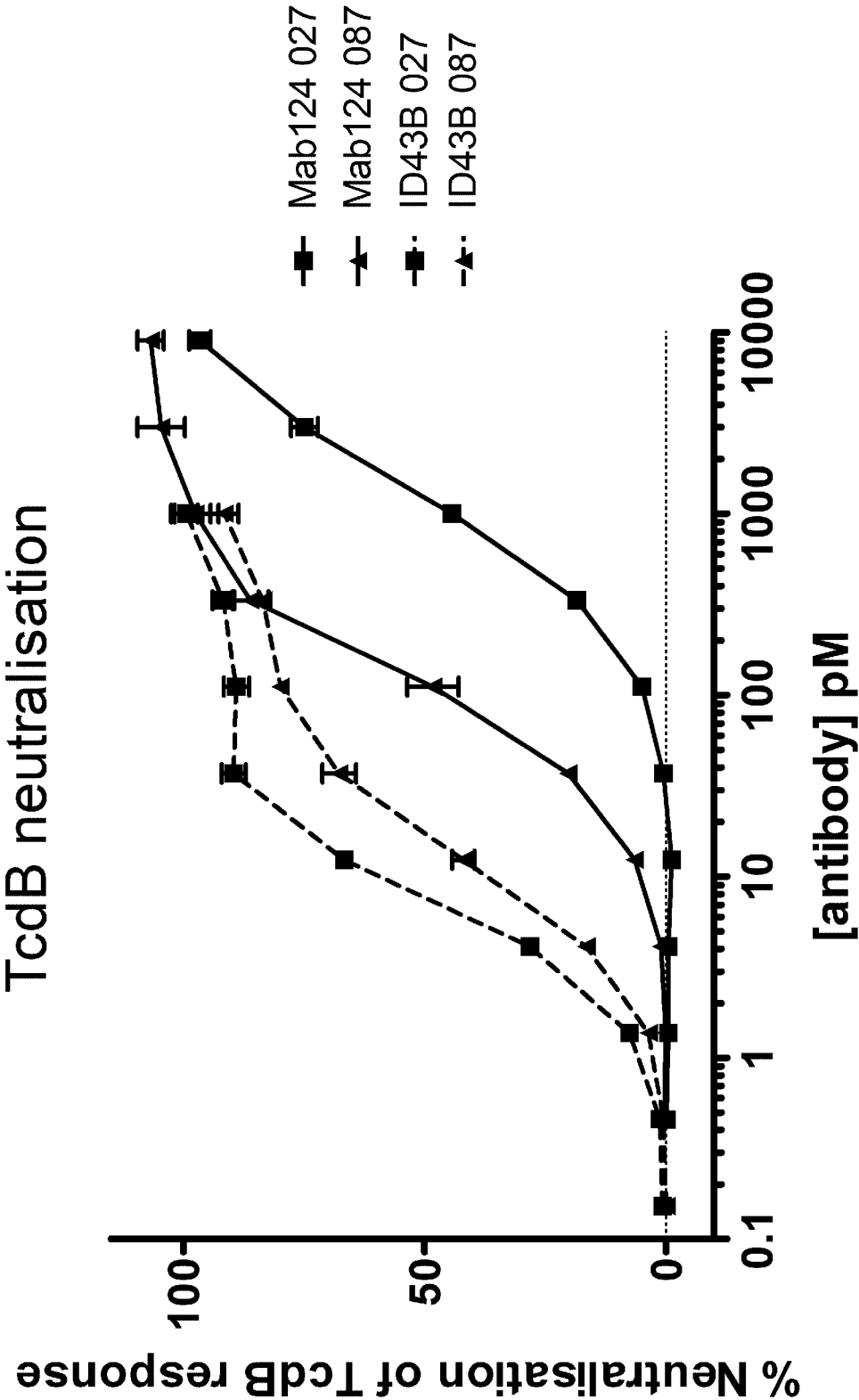


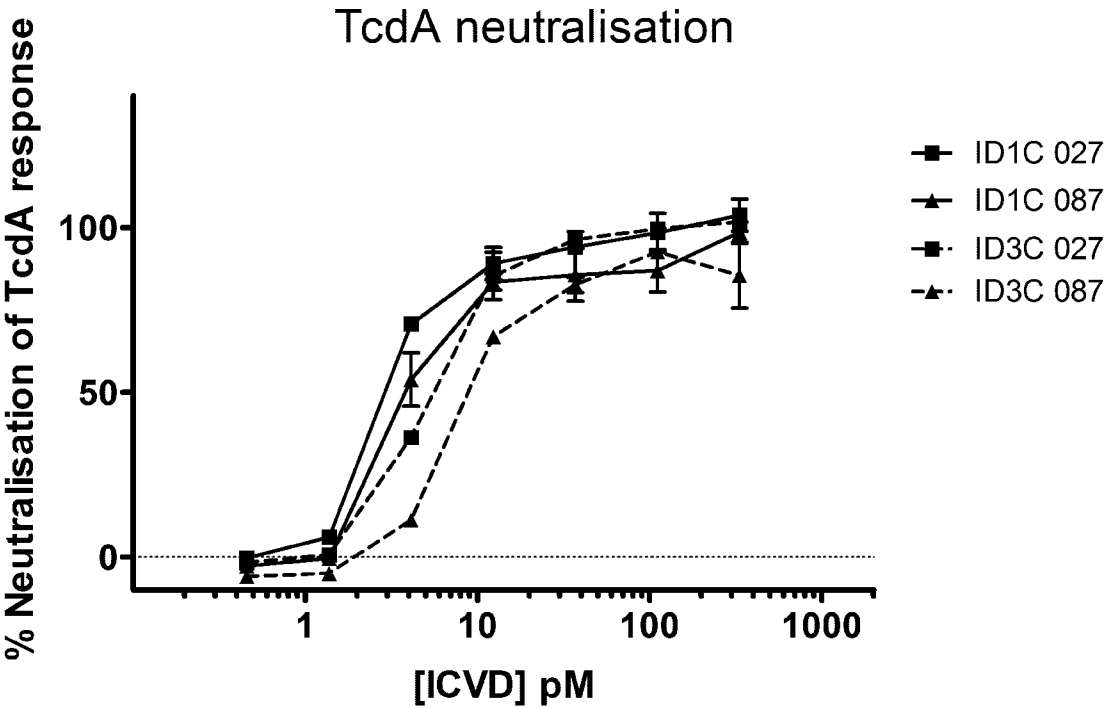
Figure 14 continued



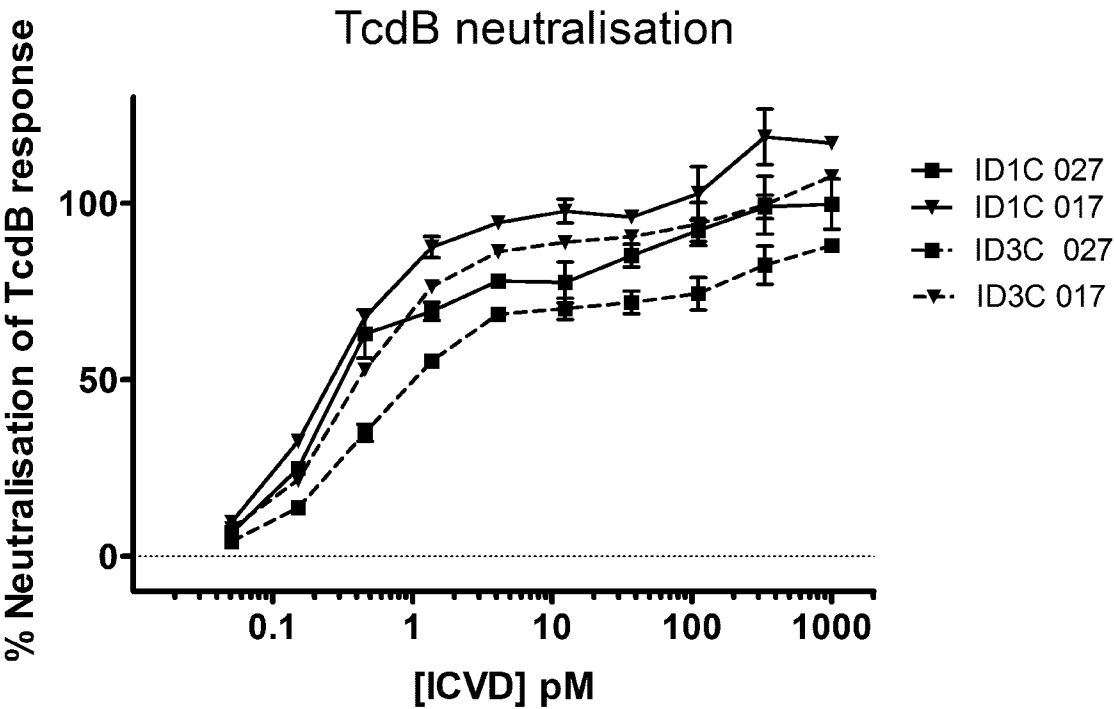
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Figure 15

I



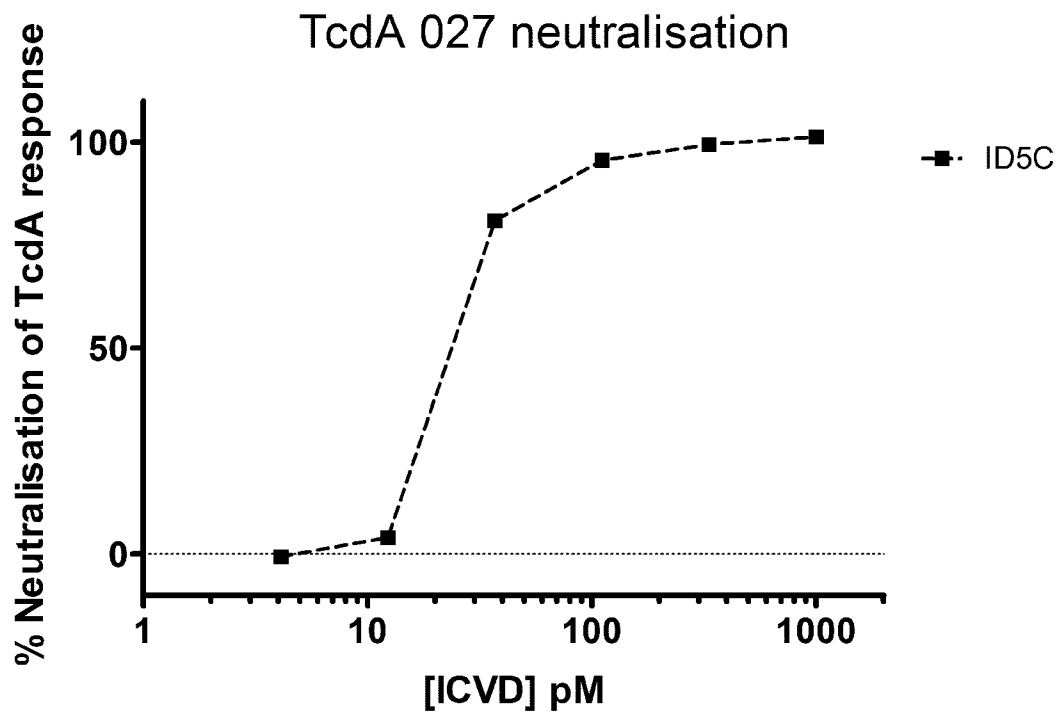
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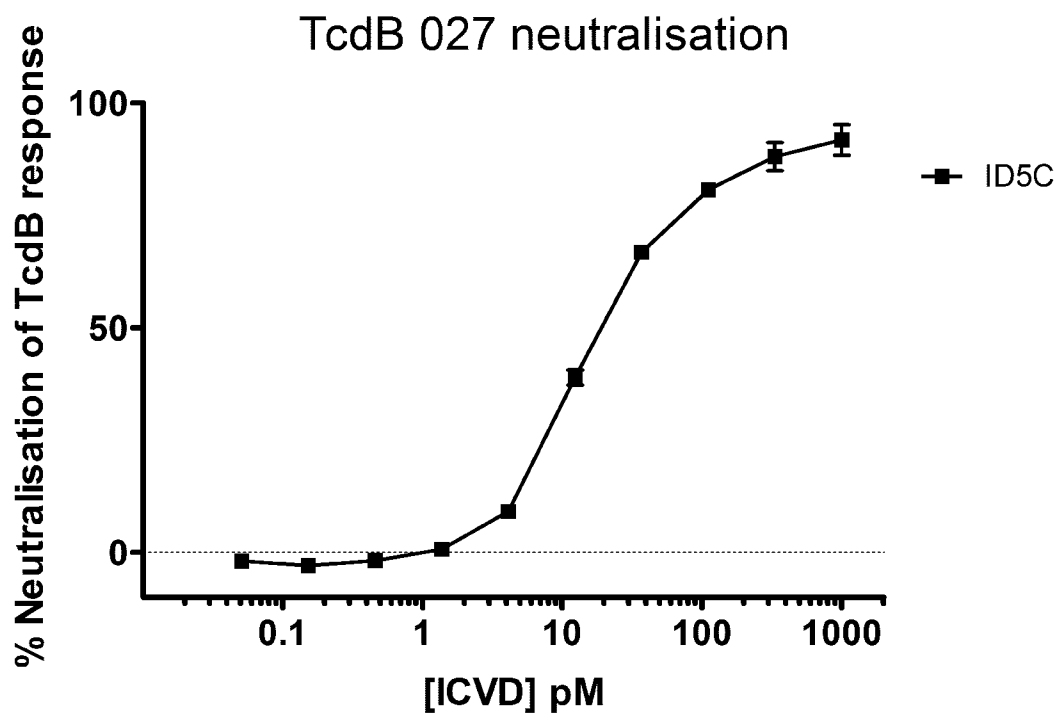
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Figure 16

I



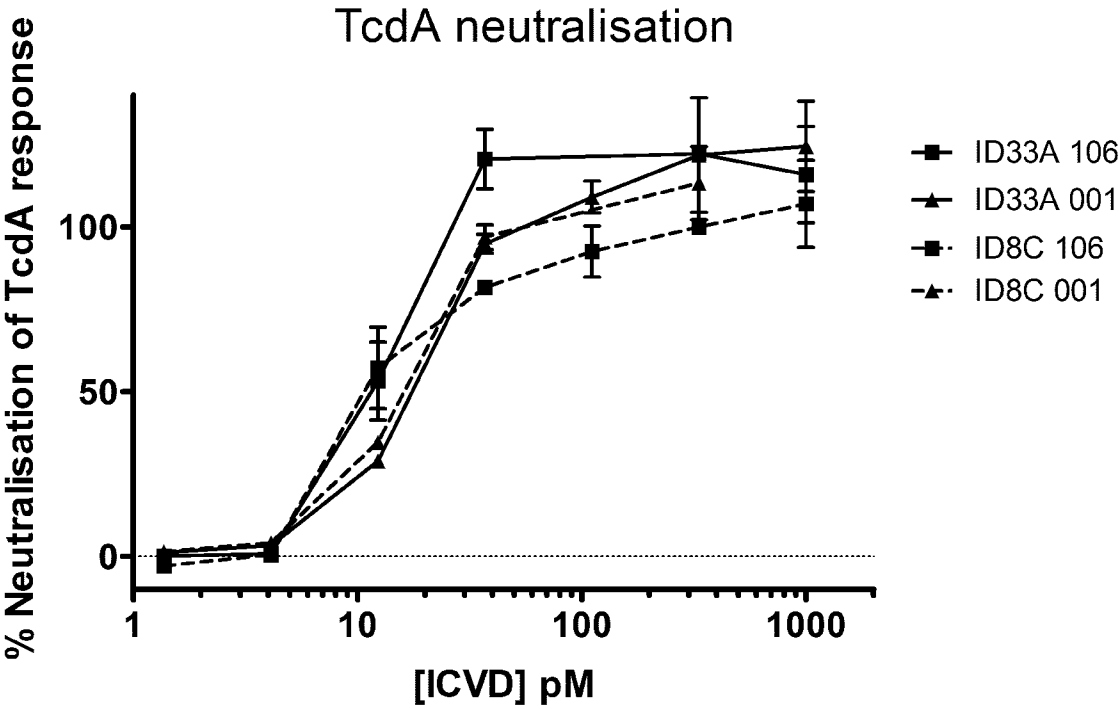
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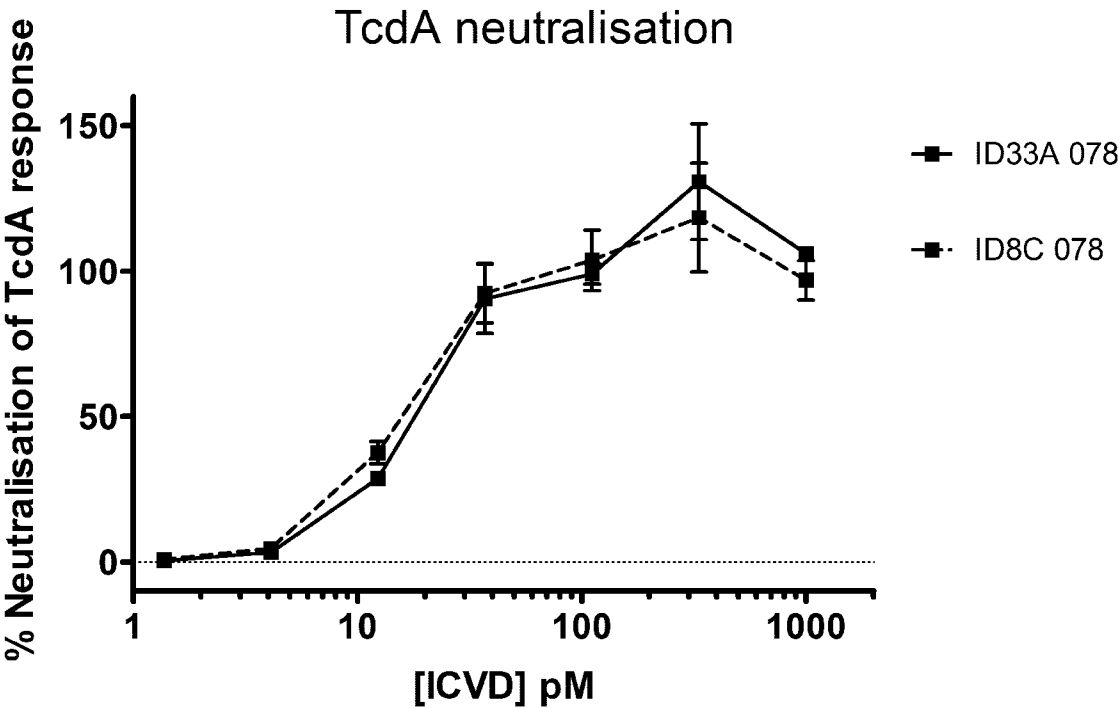
20/33

Figure 17

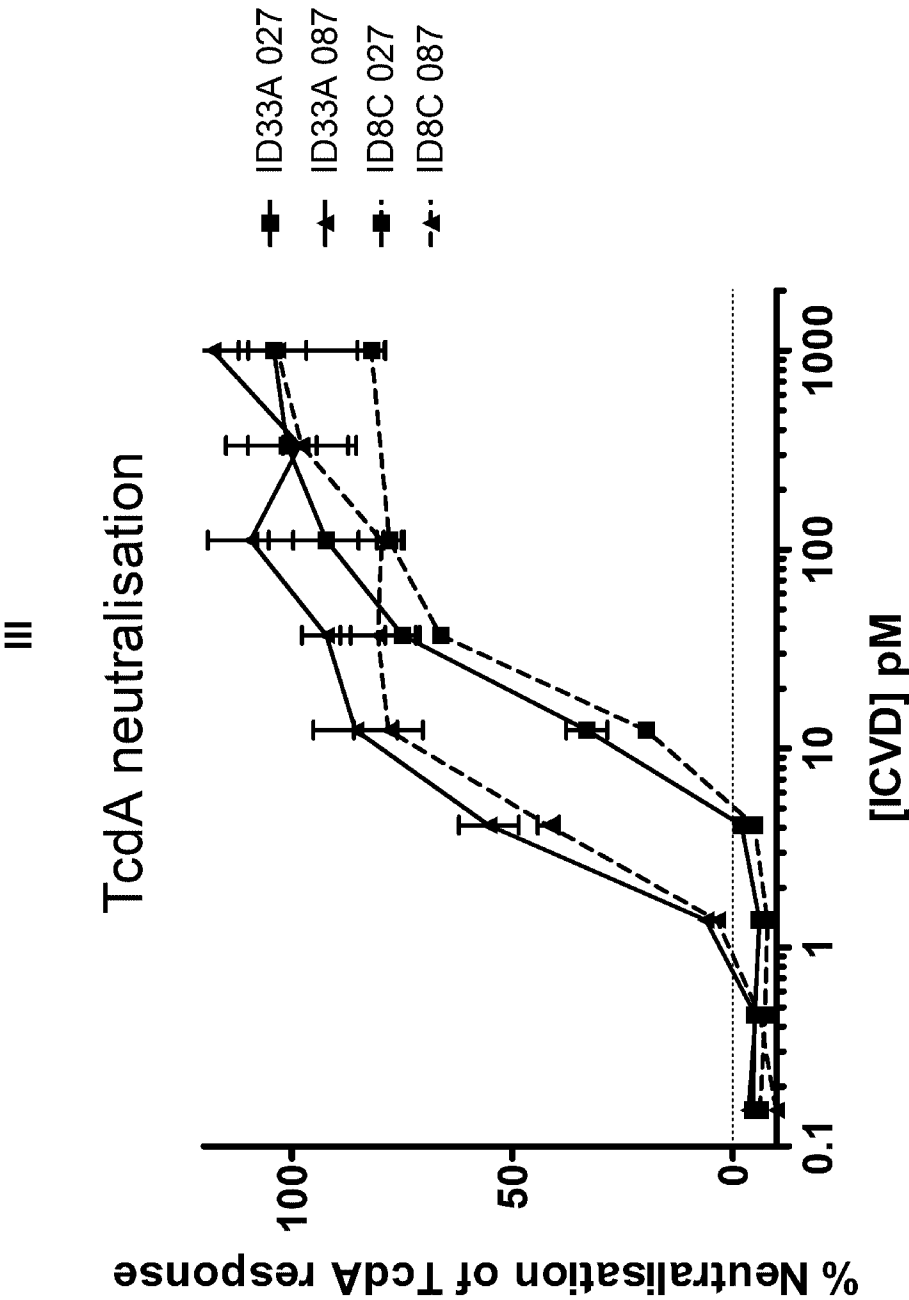
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II



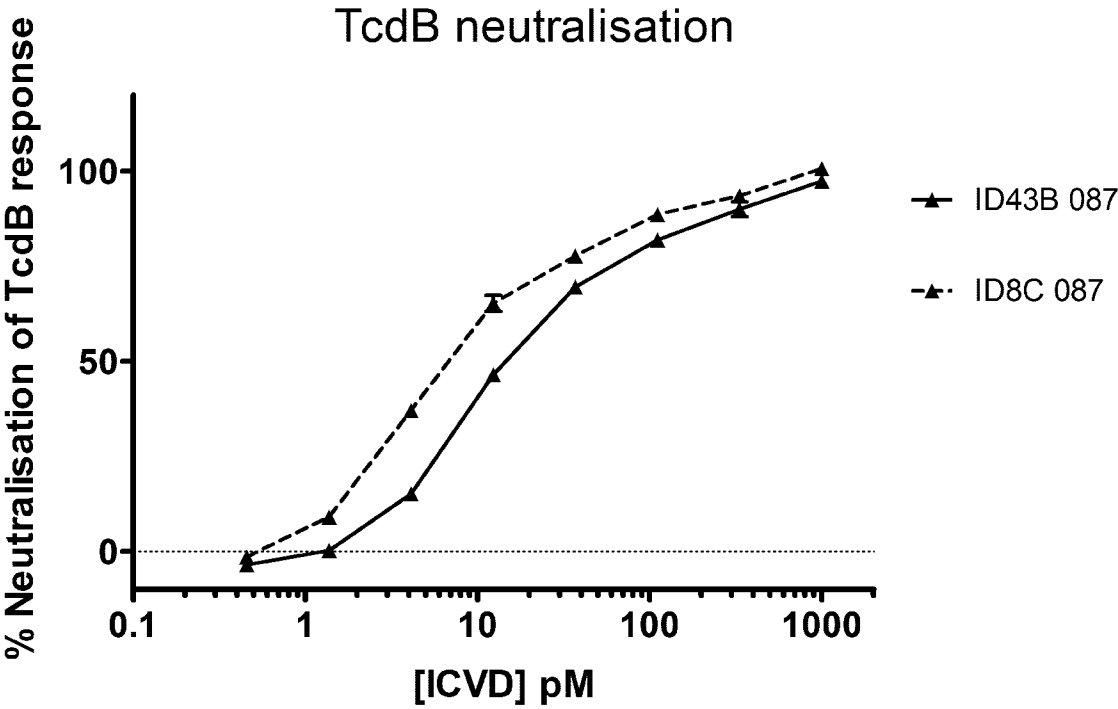
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Figure 17 continued



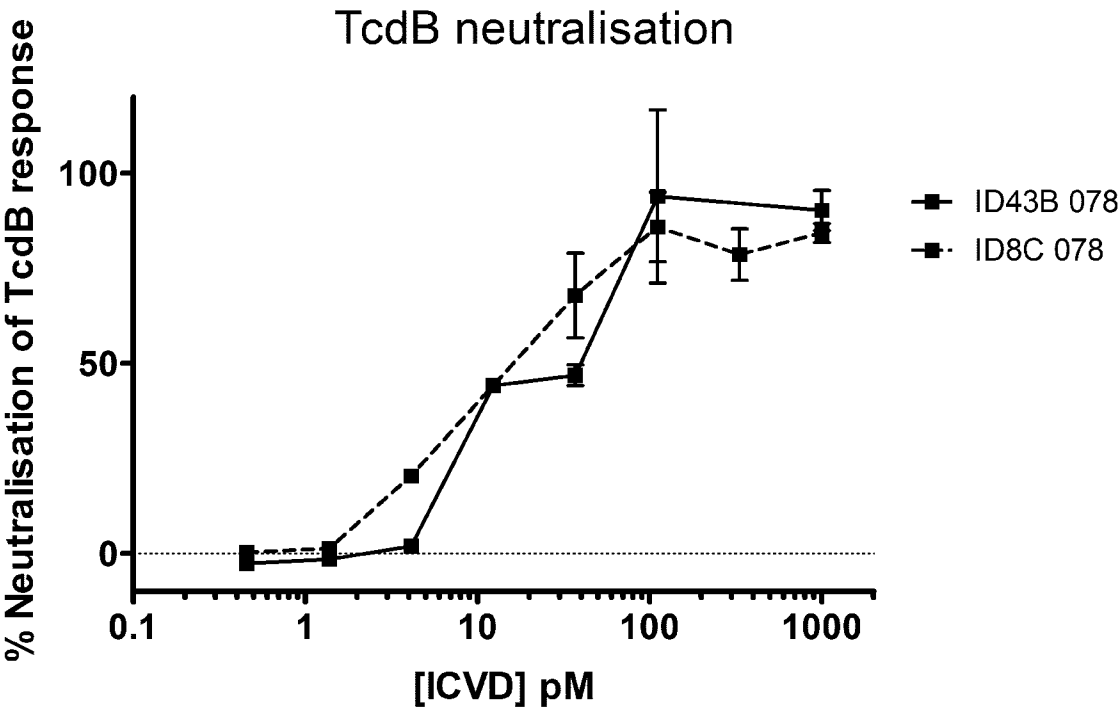
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Figure 18

I



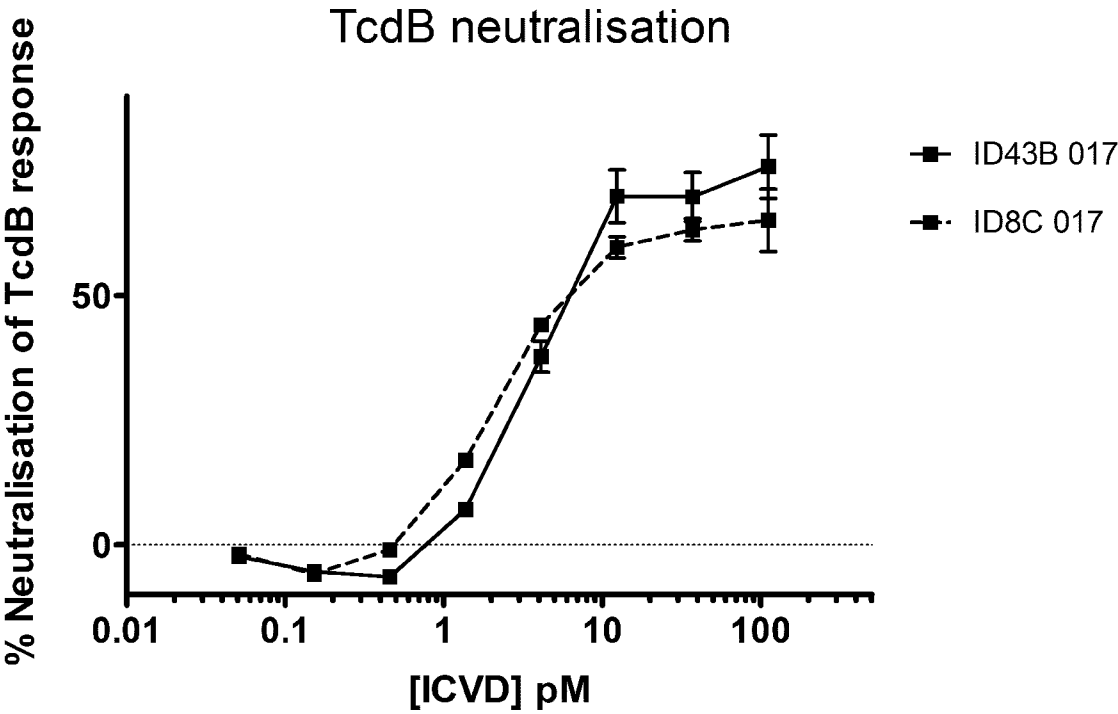
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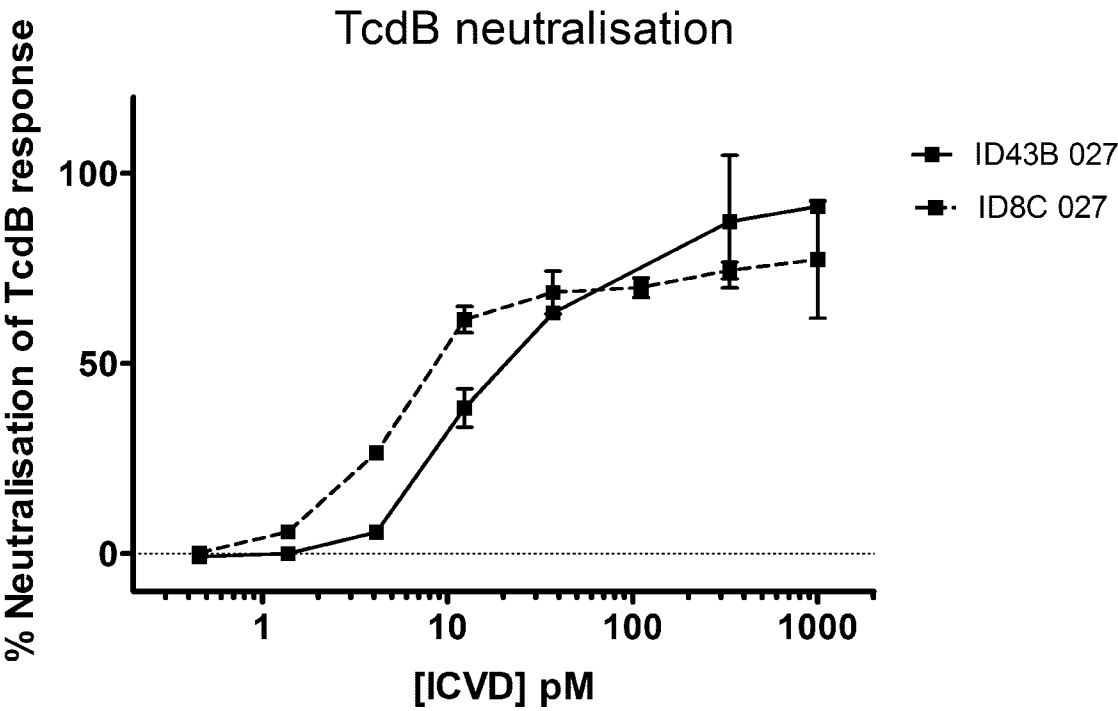
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Figure 18 continued

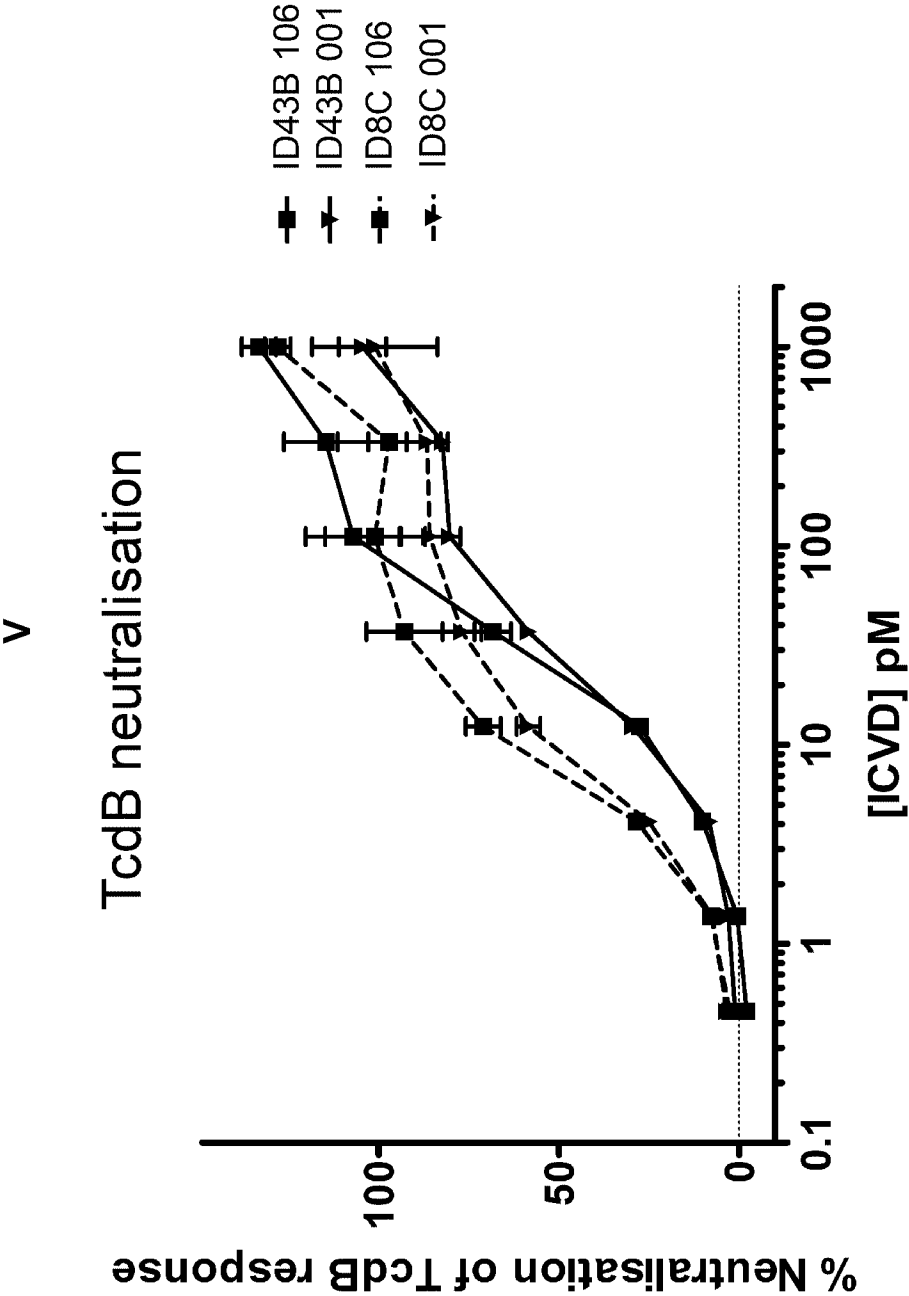
III



IV

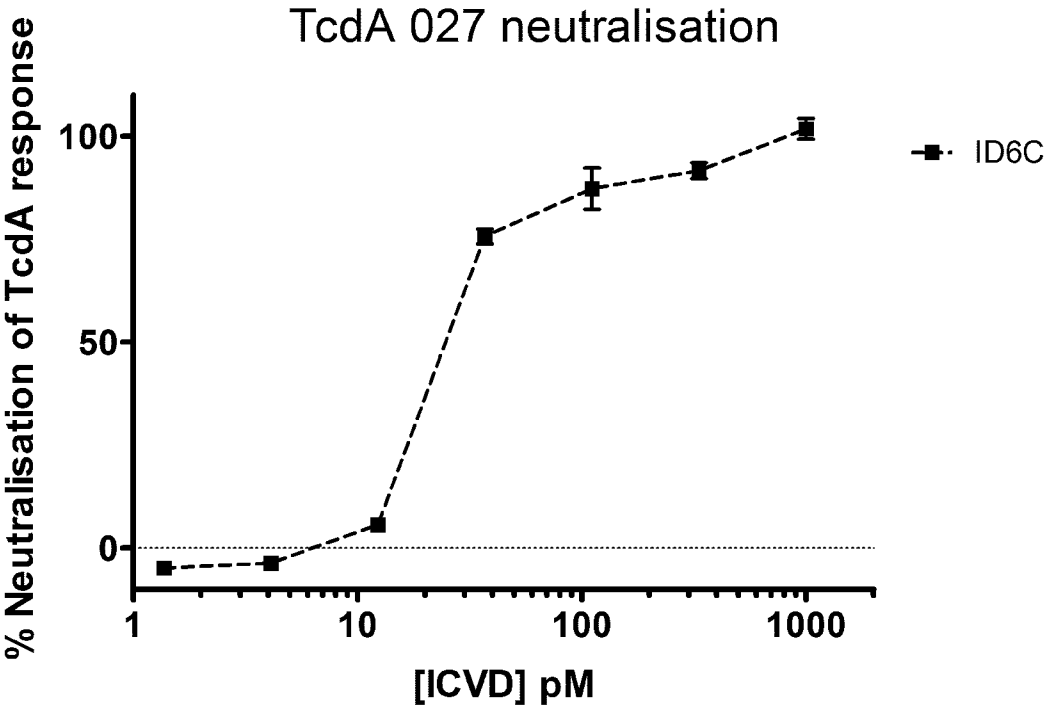


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Figure 18 continued

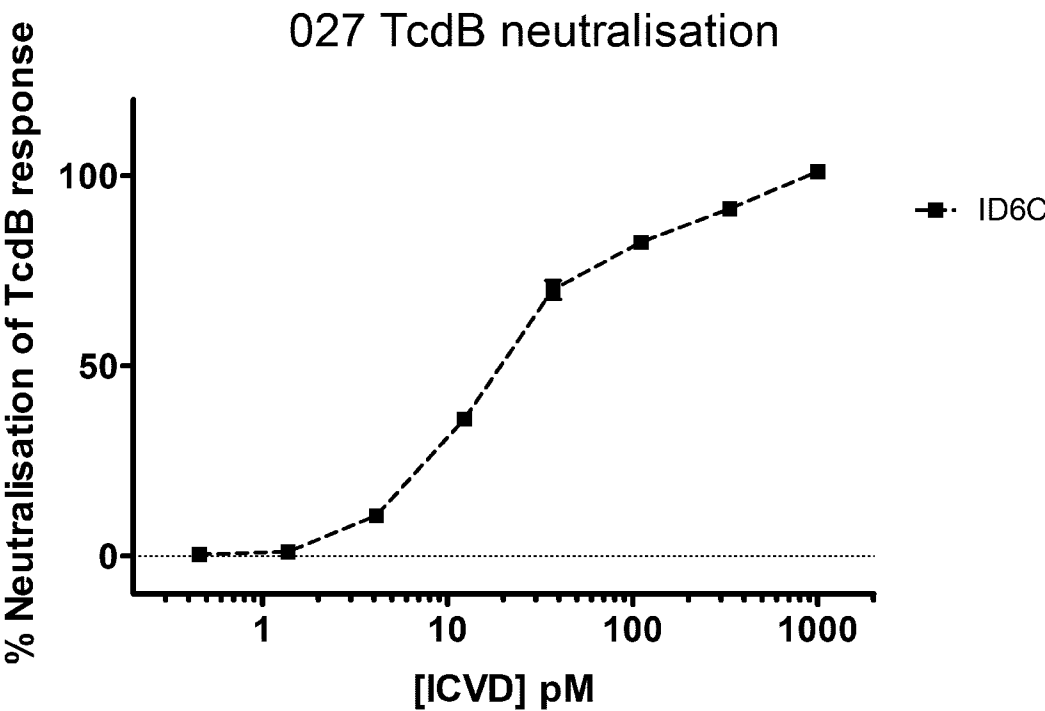


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Figure 19

I

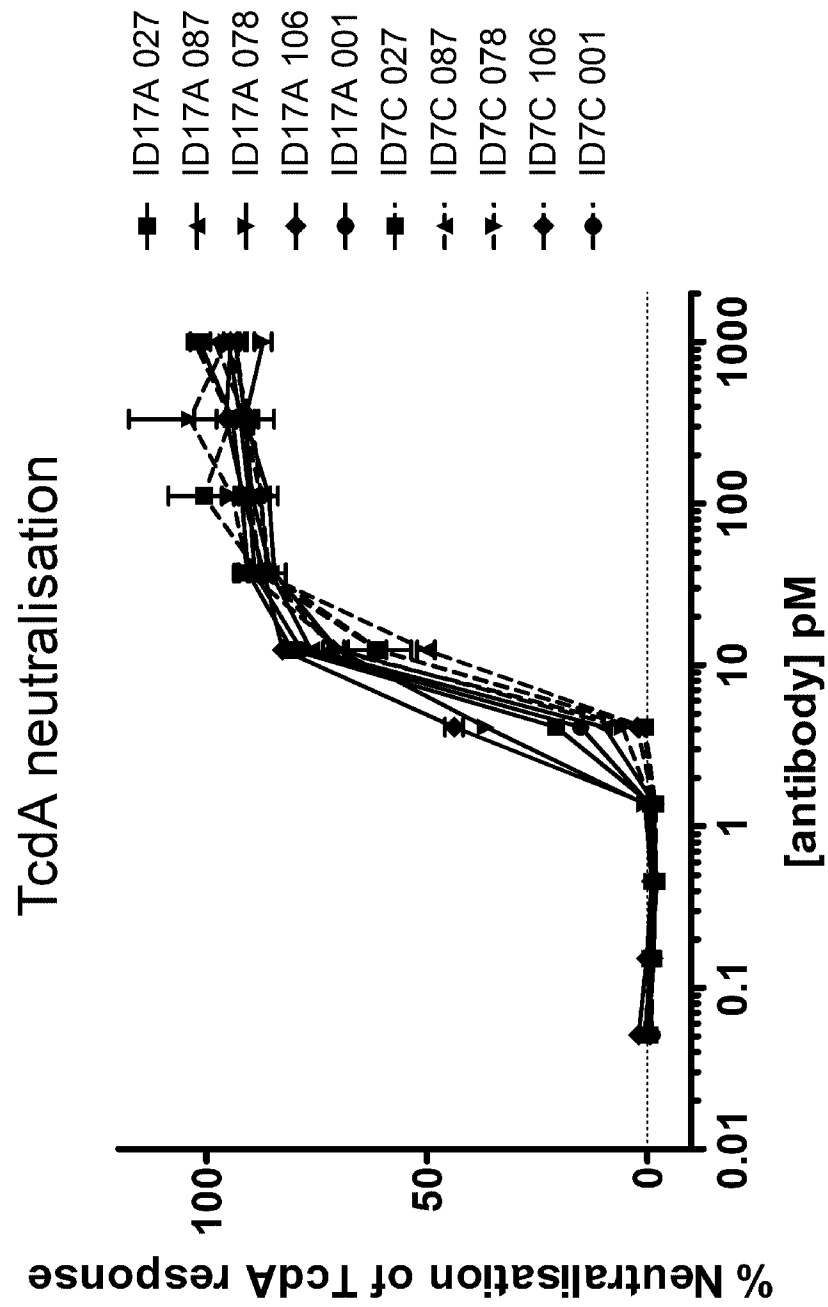


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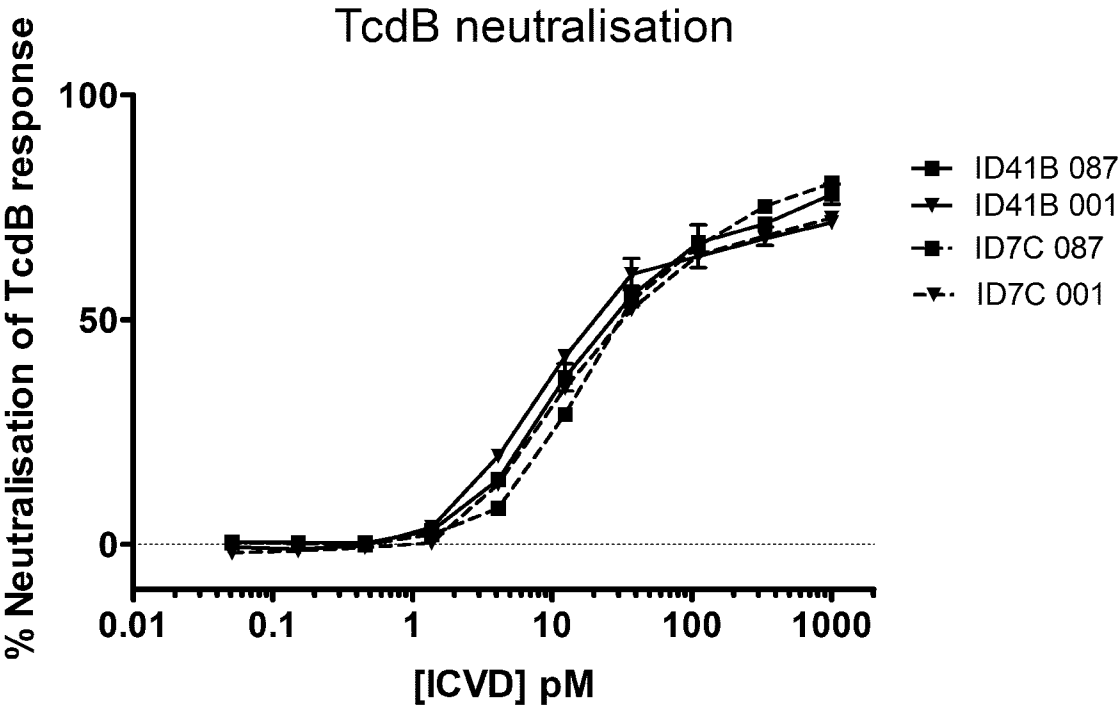
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Figure 20

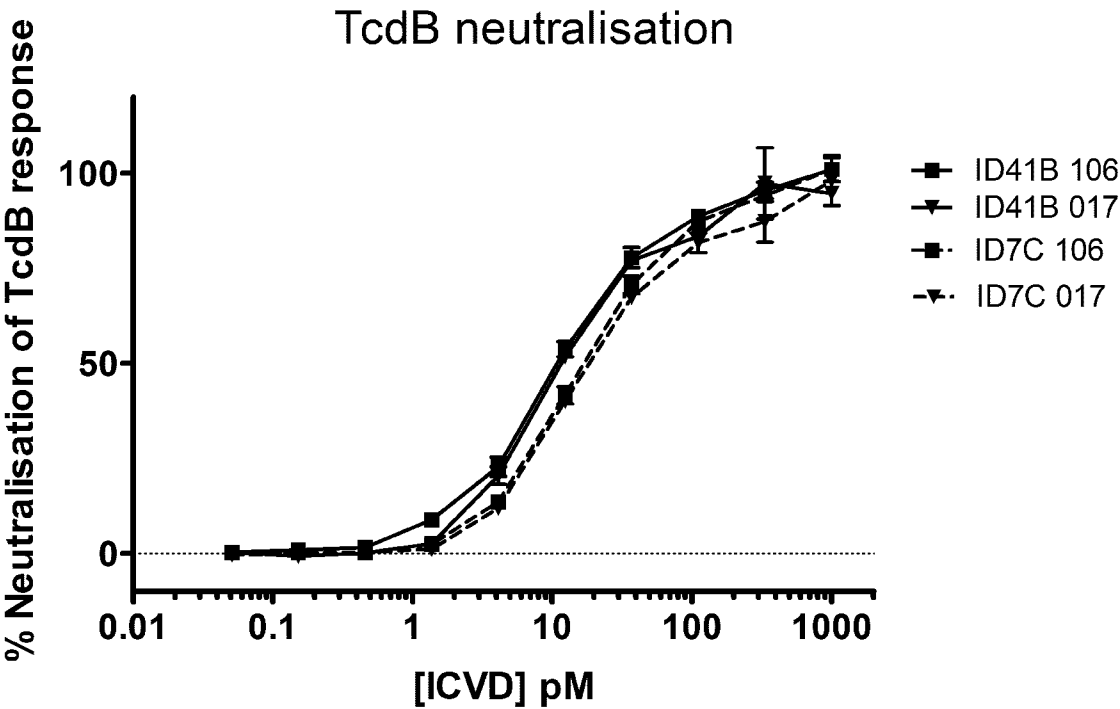


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Figure 21

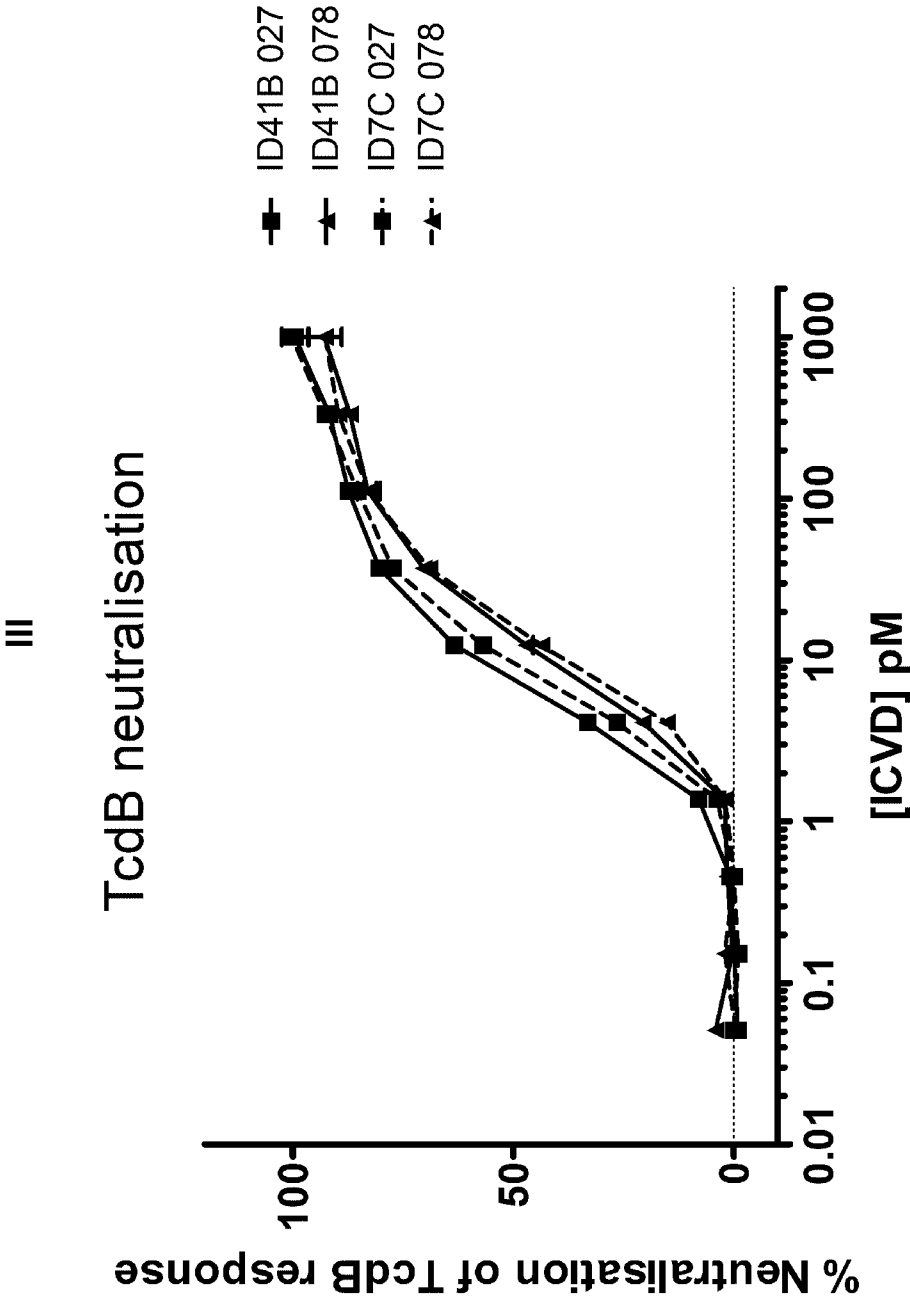
I



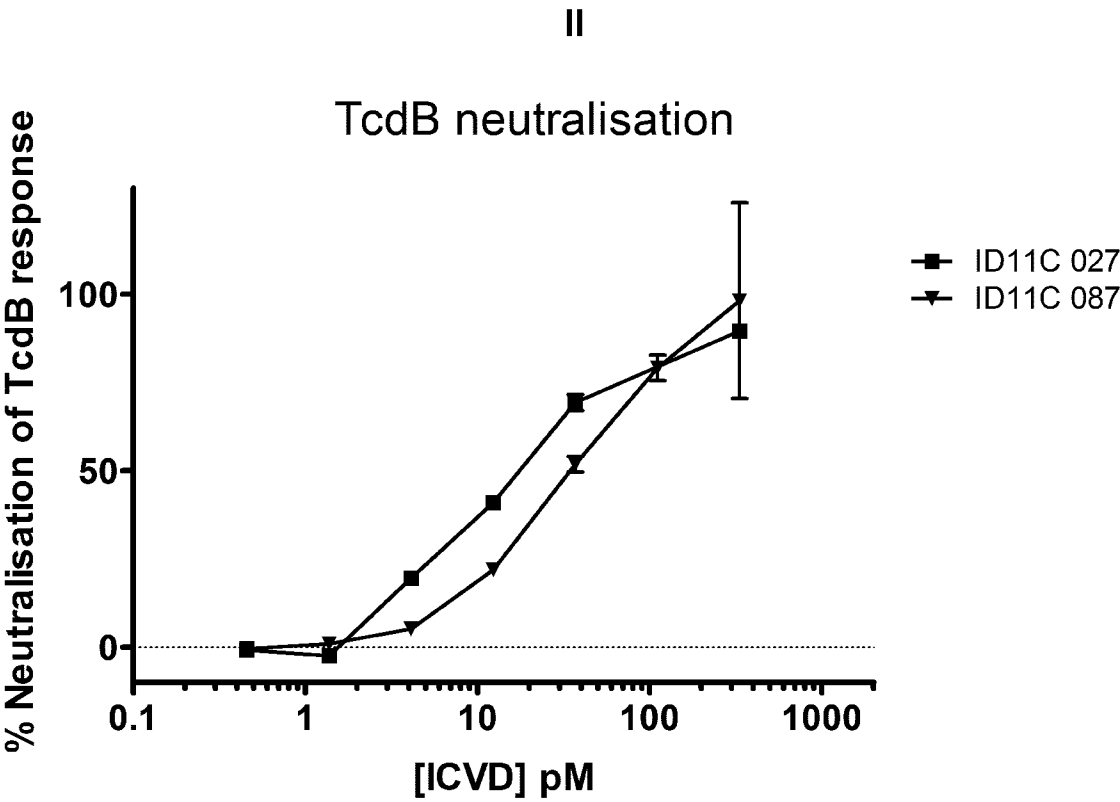
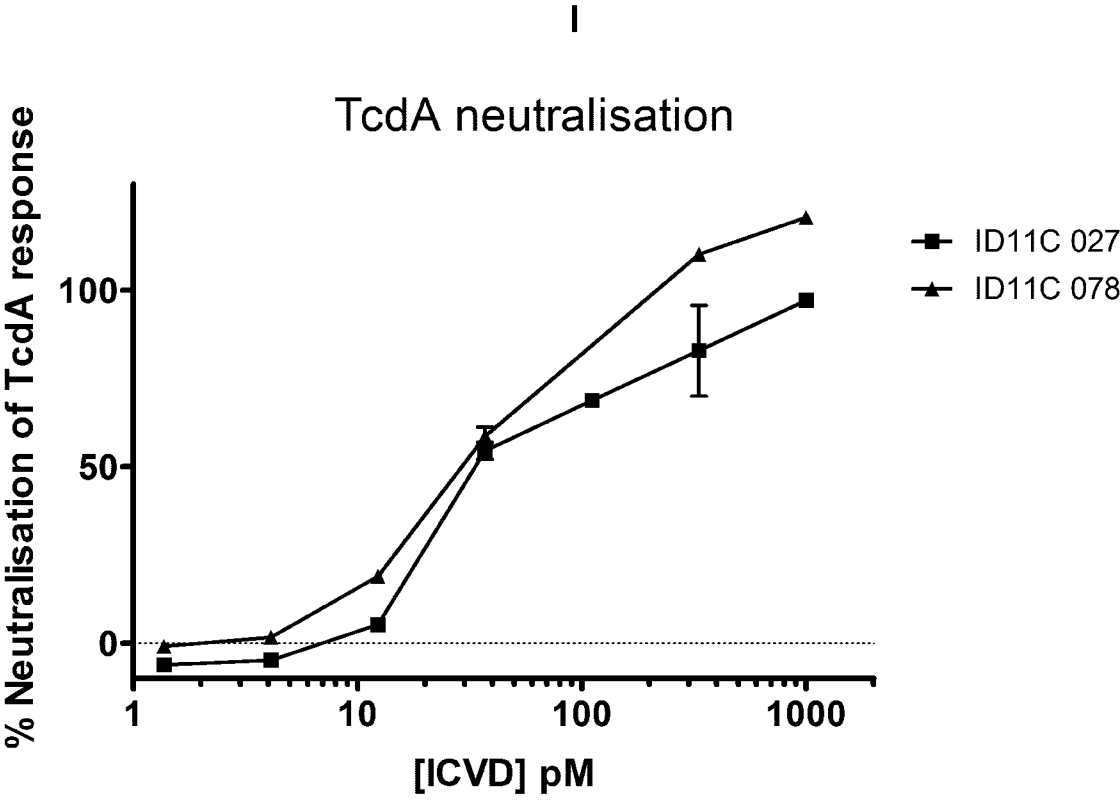
II



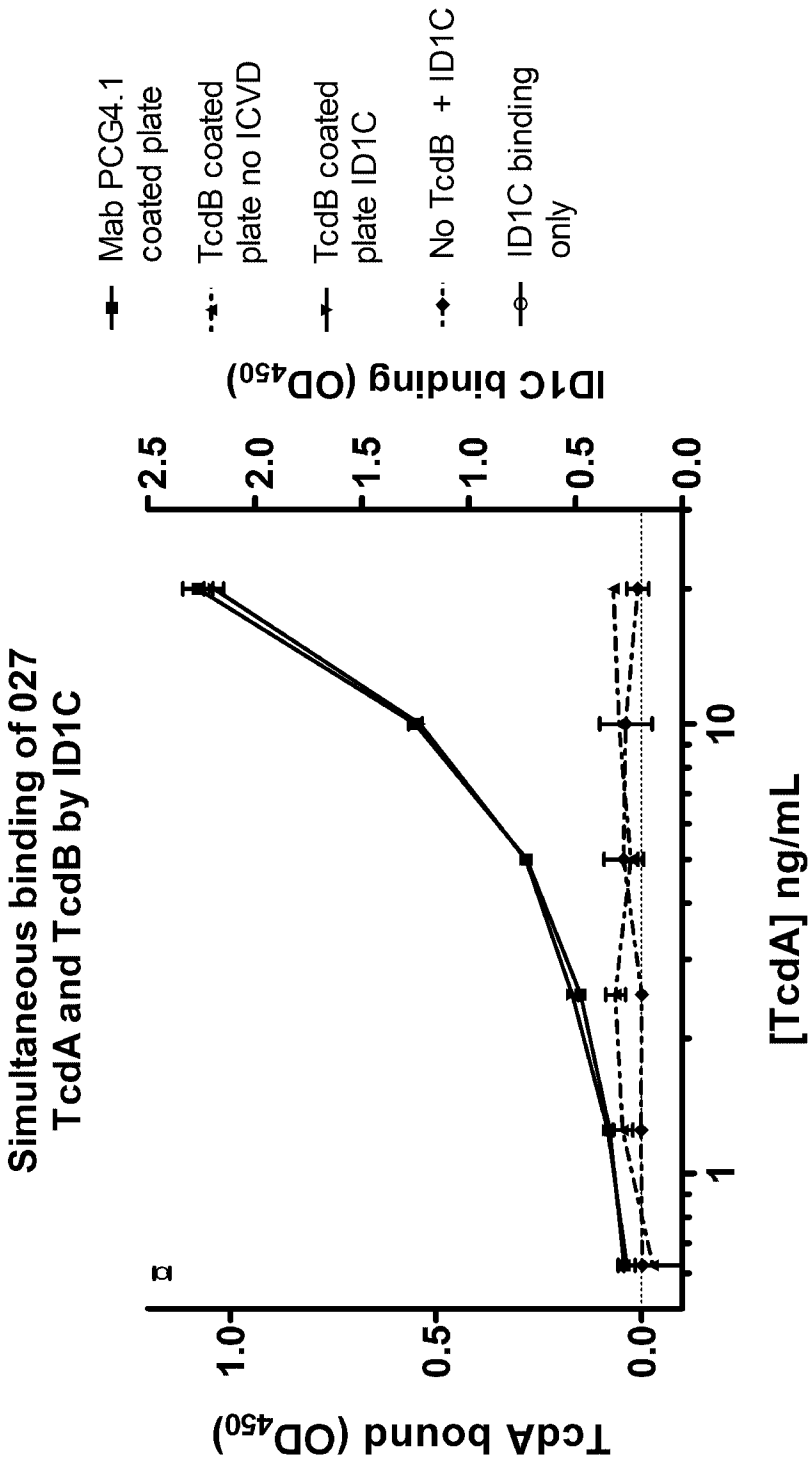
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Figure 21 continued



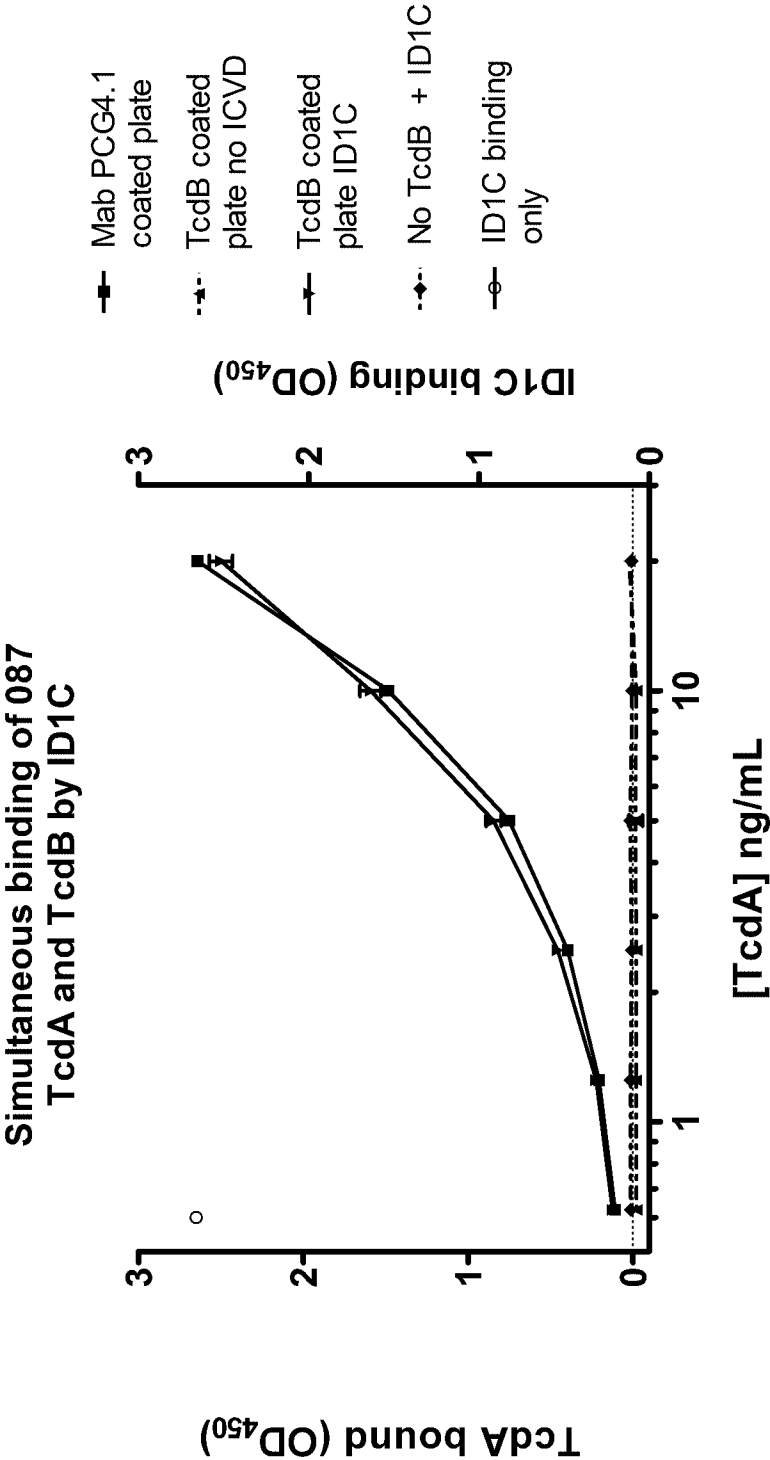
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Figure 22



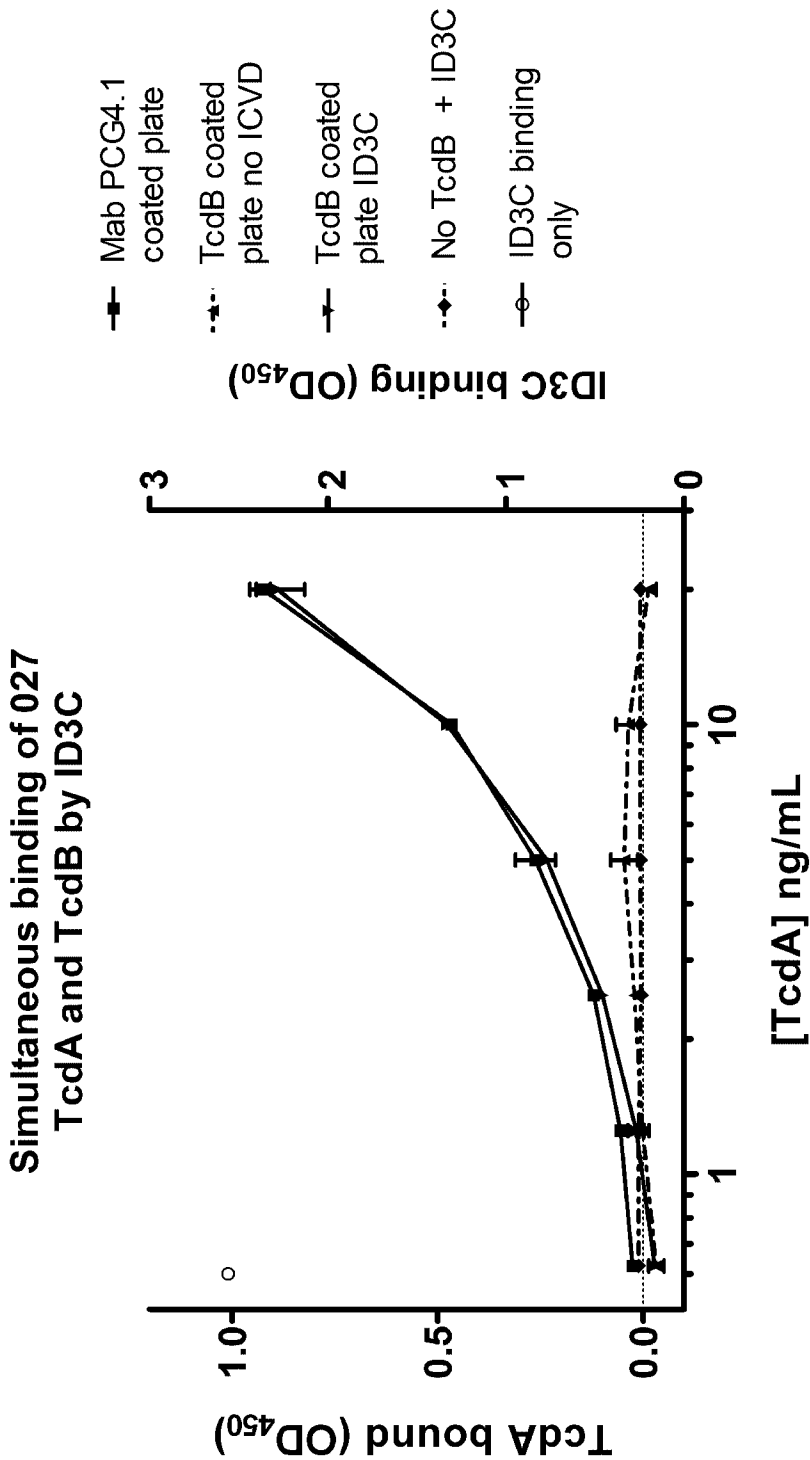
30/33
Figure 23



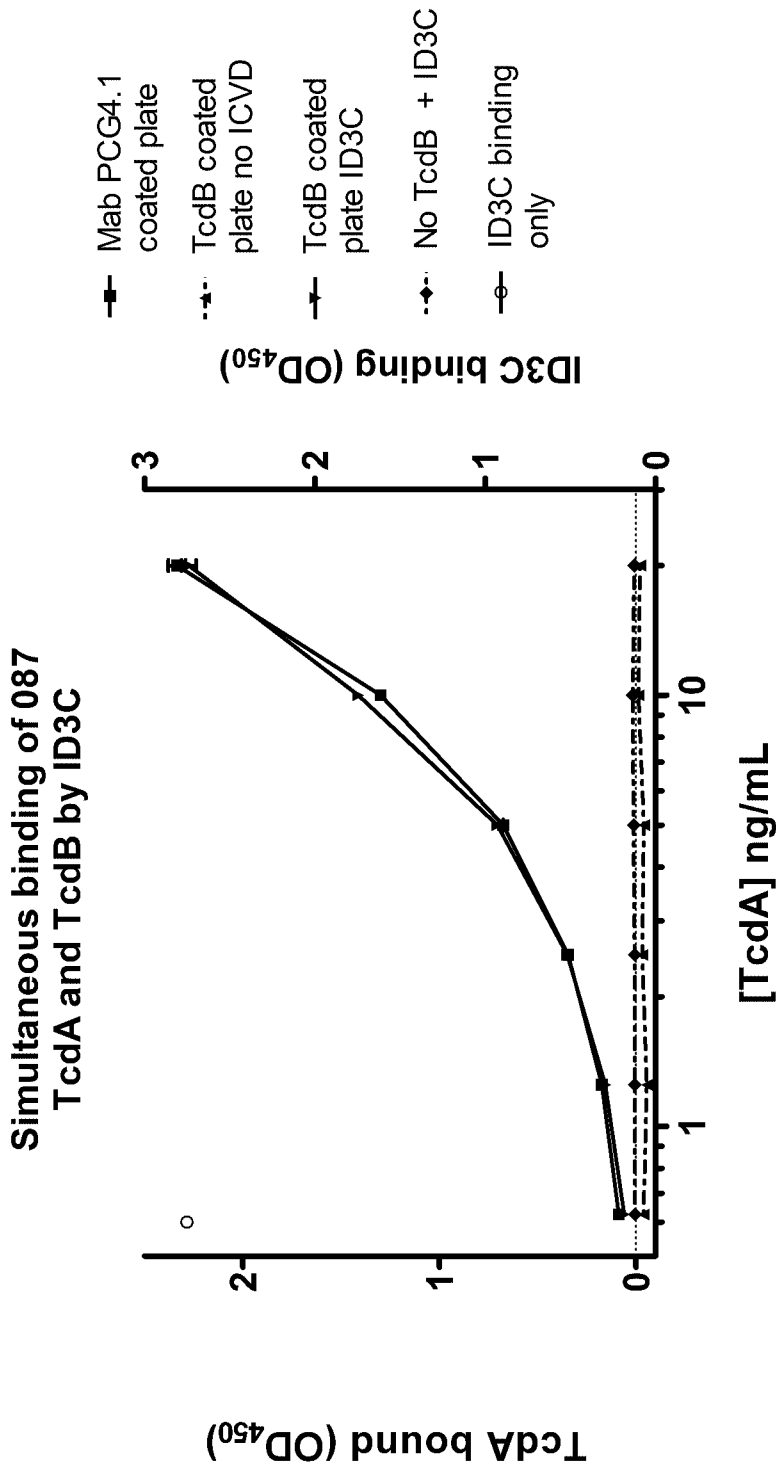
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Figure 24



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Figure 25



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Figure 26



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/057034

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K14/33 A61K39/40 C07K16/12 C07K16/40 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) C07K A61K		
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 practicable, search terms used) EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUSSACK G ET AL: "Neutralization of Clostridium difficile toxin A with single-domain antibodies targeting the cell receptor binding domain", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC, BETHESDA, MD, USA, vol. 286, no. 11, 18 March 2011 (2011-03-18), pages 8961-8976, XP002692187, ISSN: 1083-351X, DOI: 10.1074/JBC.M110.198754 [retrieved on 2011-01-07] the whole document ----- -/-	1-6,8,9, 11-17, 20-26, 28-46, 48-53
☒ Further documents are listed in the continuation of Box C. ☒ 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 application or patent 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	
1 June 2016	03/08/2016	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Madruga, Jaime	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/057034

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Greg Hussack: "Single-domain Antibody Inhibitors of Clostridium difficile Toxins", 8 November 2011 (2011-11-08), XP055053645, Retrieved from the Internet: URL: http://www.ruor.uottawa.ca/handle/10393/20362?locale=fr [retrieved on 2013-02-18] the whole document pages 88-93; table 4.2	1-6,8,9, 11-17, 20-26, 28-46, 48-53
X	-& HUSSACK GREG: "Single-domain Antibody Inhibitors of Clostridium difficile Toxins", INTERNET CITATION, 8 November 2011 (2011-11-08), pages 1-4, XP002692189, Retrieved from the Internet: URL: www.ruor.uottawa.ca/fr/handle/10393/20362 [retrieved on 2013-02-14] the whole document	1-6,8,9, 11-17, 20-26, 28-46, 48-53
A	----- GREG HUSSACK ET AL: "Engineered Single-Domain Antibodies with High Protease Resistance and Thermal Stability", PLOS ONE, vol. 6, no. 11, 30 November 2011 (2011-11-30), page e28218, XP055179537, DOI: 10.1371/journal.pone.0028218 the whole document	1-53
A	----- MANDY UNGER ET AL: "Selection of Nanobodies that Block the Enzymatic and Cytotoxic Activities of the Binary Clostridium Difficile Toxin CDT", SCIENTIFIC REPORTS, vol. 5, 19 January 2015 (2015-01-19), page 7850, XP055210742, DOI: 10.1038/srep07850 the whole document abstract pages 2,8,9; figures 3-4; table 1	1-53
X	----- WO 2013/087874 A1 (UNIVERSITAETSKLINIKUM HAMBURG [DE]) 20 June 2013 (2013-06-20) the whole document pages 27-28; figures 8,9; example 4 ----- -/--	1-6,8,9, 11-17, 20-26, 28-46, 48-53

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/057034

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/087857 A2 (UNIVERSITAETSKLINIKUM HAMBURG [DE]; NOLTE FRIEDRICH [DE]; UNGER MANDY) 20 June 2013 (2013-06-20) the whole document page 25 - page 26; figures 2,3,6 -----	1-6,8,9, 11-17, 20-26, 28-46, 48-53
X	WO 2012/055030 A1 (CA NAT RESEARCH COUNCIL [CA]; HUSSACK GREG [CA]; ARBABI-GHAHROUDI MEHD) 3 May 2012 (2012-05-03) the whole document claims; figure 2; examples -----	1-6,8,9, 11-17, 20-26, 28-46, 48-53
X	US 2014/294826 A1 (SHOEMAKER CHARLES B [US]) 2 October 2014 (2014-10-02) paragraphs [0009], [0208], [0223], [0074]; figures 11,12, 27-28, 30; examples 20-24; sequences the whole document -----	1-6,8,9, 11-17, 20-26, 28-46, 48-53
X	HUSSACK G ET AL: "Isolation and characterization of clostridium difficile toxin-specific single-domain antibodies", METHODS IN MOLECULAR BIOLOGY, HUMANA PRESS, INC, US, vol. 911, 1 January 2012 (2012-01-01), pages 211-239, XP008180179, ISSN: 1064-3745, DOI: 10.1007/978-1-61779-968-6_14 the whole document -----	1-6,8,9, 11-17, 20-26, 28-46, 48-53
A	WO 2009/147248 A2 (ABLYNX NV [BE]; HULTBERG ANNA [NL]; MAASSEN BRAM [NL]; VANLANDSCHOOT P) 10 December 2009 (2009-12-10) page 250 -----	1-53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/057034

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-53(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-53(partially)

Polypeptides comprising an immunoglobulin chain variable domain which binds to Clostridium difficile toxin B, having CDR1-CDR3 and FR1-FR4; constructs comprising one, two or more such polypeptides, pharmaceutical compositions comprising said polypeptides, uses of these products as medicaments, especially to treat Clostridium difficile infections, polynucleotides encoding said polypeptides, cDNA encoding said polypeptides, vectors comprising such polynucleotides, host cells comprising said vectors, process for preparing such polypeptides or constructs, all where the CDR1-3 of the polypeptide have the sequence of SEQ ID NO: 1-3, or variants thereof having certain % identity with these sequences, respectively. This relates to the ICVD referred to as B10F1.

2. claims: 1-53(partially)

As invention 1, but wherein the CDR1-3 of the polypeptide have the sequence of SEQ ID NO: 4-6, or variants thereof having certain % identity with these sequences, respectively. This relates to the ICVD referred to as Q31B1.

3. claims: 1-53(partially)

As invention 1, but wherein the CDR1-3 of the polypeptide have the sequence of SEQ ID NO: 7-9, or variants thereof having certain % identity with these sequences, respectively. This relates to the ICVD referred to as Q35H8.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/057034

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013087874 A1	20-06-2013	DE 102011121237 A1 WO 2013087874 A1	20-06-2013 20-06-2013
WO 2013087857 A2	20-06-2013	DE 102011121238 A1 WO 2013087857 A2	20-06-2013 20-06-2013
WO 2012055030 A1	03-05-2012	CA 2815888 A1 EP 2632949 A1 US 2013230537 A1 WO 2012055030 A1	03-05-2012 04-09-2013 05-09-2013 03-05-2012
US 2014294826 A1	02-10-2014	NONE	
WO 2009147248 A2	10-12-2009	AU 2009254501 A1 CA 2726652 A1 CN 102112155 A EP 2285408 A2 JP 5809557 B2 JP 2011521662 A JP 2015156867 A US 2011182897 A1 US 2016152693 A1 WO 2009147248 A2 ZA 201009135 B	10-12-2009 10-12-2009 29-06-2011 23-02-2011 11-11-2015 28-07-2011 03-09-2015 28-07-2011 02-06-2016 10-12-2009 27-06-2012