NEUTRALISING ANTIBODIES TO THE MAJOR EXOTOXINS TCDA AND TCDB OF CLOSTRIDIUM DIFFICILE

The present invention relates to antibodies to exotoxins of *Clostridium difficile*, for example TcdA and TcdB, pharmaceutical compositions comprising the same, processes of producing said antibodies and compositions and use of the antibodies and compositions in treatment and/or prophylaxis, in particular treatment or prophylaxis of *Clostridium difficile* infection, pseudomembranous colitis, fulminant colitis and/or toxic mega colon.

The two major exotoxins TcdA and TcdB have been established as the major pathogenicity determinants of *Clostridium difficile* in a large number of *in vitro* and *in vivo* studies. Non-toxigenic strains are not pathogenic to animals and man (1, 2). To date a clear understanding of the role of binary toxin has yet to be established (3).

Both toxins are entero- and cyto-toxic, but the balance of evidence suggests that TcdA is a more powerful enterotoxin than TcdB, whilst TcdB is typically observed to be ~1000x more cytotoxic than TcdA (4). Whilst both toxins are capable of inducing an inflammatory response, TcdA appears to aid the migration of the more inflammatory TcdB deeper into the gut mucosa (5).

In toto, a large collection of data generated for over 30 years support a model where both toxins are likely to be important in the human disease process. It is probable that TcdA initiates early (i.e. before TcdB) and rapid (i.e. 1-3 hours) gut damage through loss of tight junctions and destruction of villi tips and hence diarrhoea, probably through albumin driven fluid loss. This damage to the integrity of the gut lining enables TcdB to exert its superior molar potency (TcdB is typically cited as being 1000x more cytotoxic than TcdA) more rapidly and effectively (i.e. deeper into tissue, alternative cellular targets and damaging systemically accessed organs). Either toxin can be effective alone *in vitro* on human or animals cells and tissues. Either toxin can be effective alone *in vivo* in animals depending upon other eliciting factors such as mechanical damage, barrier overload and host specific sensitivities. It is now clear that in hamsters at least either TcdA or TcdB alone delivered by a *Clostridium difficile* gut infection can cause death (1). It is well established that A-B+ strains are capable of causing symptoms and death in humans (6,7). However, the majority (~95%) of clinical strains are A+B+ hence drugs aimed at treating *Clostridium difficile* infections (CDI) must be capable of neutralising the activities of and clearing both toxins effectively.

CDI is most typically a nosocomial infection of older patients or those with complicating co-morbidities. However, an increase in community acquired infections has been noted. Infection is almost always associated with or induced by use of broad spectrum

antibiotics. Healthcare associated costs are estimated to be in excess of $1bn per annum in the US alone. These costs are primarily due to patients having longer hospitals stays. Current therapies involve the use of antibiotics such as clindamycin, vancomycin or fidaxomicin which kill the *Clostridium difficile* cells within the gut. Current therapies address the bacterial infection but do not deal with or prevent directly the significant pathogenesis caused by TcdA and TcdB which are major contributors to CDI symptoms and mortality.

CDI symptoms in humans include mild to severe diarrhoea, pseudomembranous colitis (PMC) and fulminant colitis or so called toxic mega colon. Death results in 5-15% of patients receiving current best care. Thus at the present time there is no specific therapy available to patients to prevent the damage and injury caused by *C. difficile* toxins after infection.

Raising an antibody response through vaccination and parenteral administration of polyclonal and monoclonal antibodies have all been shown to be capable of protecting animals from symptoms of diarrhoea and death (8-15). Early studies in hamsters suggested that antibodies against TcdA alone were all that was necessary for protection. However, use of strains functionally deleted for TcdA or TcdB demonstrate that either toxin is capable of causing disease in hamsters, but that both toxins together are more effective (1).

For therapeutic applications, monoclonal antibodies (Mabs) can offer efficacy, safety, manufacturing and regulatory advantages over serum derived polyclonal antibodies or serum derived hyper-immune sera. For these reasons Mabs are usually the preferred option for therapeutic products.

There have been a number of attempts to generate protective Mabs against TcdA and TcdB. The most advanced of these in the clinic is a mixture of 2 IgG1 Mabs, one against each TcdA and TcdB originally called CDA1 and MDX1388 developed by MBL and Medarex. They were demonstrated to be unable to fully protect hamsters in models of acute or relapse infections (15). This Mab combination is now being developed as MK3415A by Merck Inc. In a human phase II trial MK3415A resulted in a statistically significant reduction in disease recurrence ($p = 0.006$) (see also Lowy *et al.*, NEJM (2010) 362: 197-205) but did not affect the duration / severity of diarrhoea or death rates (16). This may mean that these antibodies may only be useful for preventing recurrence of infection. Recurrence of infection results in approximately 25% of patients. Thus there likely to be a significant patient population in which these antibodies are not effective.

In order to be able to have a positive influence upon diarrhoea (for example as a result of acute damage to gut tight junctions due to TcdA) and death (for example resulting from prolonged poor nutritional status, dehydration stress and initiation of an inflammatory cascade, widespread anatomical damage to the gut lining and possibly damage to distant organs due to

systemic toxin TcdB more so than TcdA) Mabs are required with superior affinity, toxin neutralisation, superior prevention of loss of TEER (trans-epithelial electrical resistance), antigen decoration and antigen immune clearance.

Summary of the Present Invention

The present invention provide a Mab(s) with a very high level of potency *in vitro* and *in vivo* which have the potential to have an impact upon duration and severity of diarrhoea and death rate in humans suffering from *Clostridium difficile* infection (CDI).

In one embodiment there is provided a monoclonal antibody specific to antigen TcdA or TcdB, wherein the antibody has high affinity for the target antigen and is suitable for reducing the duration and/or severity of diarrhoea and morbidity in a patient with *Clostridium difficile* infection or at risk of said infection.

In one embodiment there is provided a Mab specific to TcdA or TcdB, or a population of at least two Mabs at least one of which is specific to TcdA and at least one of which is specific to TcdB, wherein the EC_{50} of the or each antibody or the combination of antibodies is 200ng/ml or less, for example 150ng/ml or less such as 100ng/ml.

The antibodies of the present disclosure are useful because they are likely to provide a means of treating the severity and duration of symptoms of a primary infection such as diarrhoea in a patient or preventing death and not just prevent the reoccurrence of disease symptoms.

In at least some embodiments the antibodies according to the present disclosure show no reduction in potency in the presence of high concentrations of toxin.

Detailed Description of the Present Invention

Specific as employed herein is intended to refer to an antibody that only recognises the antigen to which it is specific or an antibody that has significantly higher binding affinity to the antigen to which is specific compared to binding to antigens to which it is non-specific, for example 5, 6, 7, 8, 9, 10 times higher binding affinity.

Binding affinity may be measured by standard assays such as surface plasmon resonance, such as BIAcore.

In one embodiment the EC_{50} is less than 75, 70, 60, 65, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.5 ng/ml *Clostridium difficile* infection in cell culture assays and the patient. This is significantly lower (more potent) than known antibodies and is thought to be a major factor as to why the antibodies of the present disclosure have a significant and positive impact on survival of subjects receiving treatment.

As employed herein potency is the ability of the antibody to elicit an appropriate biological response, for example neutralisation of the deleterious toxin effects, at a given dose

or concentration. Examples of potency include the percent maximal neutralisation of toxin activity (extent of protection), the lowest relative concentration of Mab to antigen (e.g. EC_{50}), the speed and durability of neutralisation activity.

In cell culture assays neutralisation might be observed as one or more of the following: prevention of binding of toxin to cells, immunoprecipitation of toxin from solution, prevention of loss of cell form and shape, prevention of loss of cytoskeletal structures, prevention of loss of cell monolayer tight junctions and trans-epithelial electrical resistance, prevention of cell death, apoptosis and production of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and MIP1α.

In tissue section and explant assays neutralisation may, for example be observed as prevention of necrosis and/or oedematous fluid accumulation.

In *in vivo* assays neutralisation may be observed as one or more of the following: prevention of fluid accumulation in ligated ileal loops and prevention of gut tissue necrosis, diarrhoea, pseudo-membrane formation of death of animals,

Thus in one embodiment there is provided an antibody (for example an anti-toxin A antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

QASQSISNALA	SEQ ID NO: 1
SASSLAS	SEQ ID NO: 2
QYTHYSHTSKNP	SEQ ID NO: 3
GFTISSYYMS	SEQ ID NO: 4
IISSGGHFTWYANWAKG	SEQ ID NO: 5
AYVSGSSFNGYAL	SEQ ID NO: 6

In one embodiment sequences 1 to 3 are in a light chain of the antibody.

In one embodiment sequences 4 to 6 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 1 is CDR L1, SEQ ID NO: 2 is CDR L2 and SEQ ID NO; 3 is CDR L3.

In one embodiment SEQ ID NO: 4 is CDR H1, SEQ ID NO: 5 is CDR H2 and SEQ ID NO; 6 is CDR H3.

In one embodiment SEQ ID NO: 1 is CDR L1, SEQ ID NO: 2 is CDR L2, SEQ ID NO; 3 is CDR L3, SEQ ID NO: 4 is CDR H1, SEQ ID NO: 5 is CDR H2 and SEQ ID NO; 6 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 922 anti-toxin A antibody;Light chain Variable region sequence) SEQ ID NO: 7:

DPVMTQSPSTLSASVGDRVTITCQASQSISNALAWYQQKPGKAPKLLIYSASSLASGVPSRFK
GSGSGTEFTLTISSLQPDDFATYYCQYTHYSHTSKNPFGGGTKVEIK

wherein the CDRs are underlined and construct is referred to herein as 922.g1 VK (gL1).

The polynucleotide sequence encoding SEQ ID NO: 7 is shown in Figure 1 and SEQ ID NO: 8 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 922 anti-toxin A antibody heavy chain variable region sequence) SEQ ID NO: 9:

EVQLVESGGGLVQPGGSLRLSCAASGFTISSYYMSWVRQAPGKGLEWIGIISSGGHFTWYANW
AKGRFTISSDSTTVYLQMNSLRDEDTATYFCARAYVSGSSFNGYALWGQGTLVTVS

wherein the CDRs are underlined and construct is referred to herein as 922.g1 VH (gH1)

The polynucleotide sequence encoding SEQ ID NO: 9 is shown in Figure 1 and SEQ ID NO: 10 therein.

In one embodiment the antibody comprises the variable regions shown in SEQ ID NO: 7 and 9.

Thus in one embodiment there is provided an antibody (for example an anti-toxin A antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

QASQSISNYLA	SEQ ID NO: 11
SASTLAS	SEQ ID NO: 12
QYSHYGTGVFGA	SEQ ID NO: 13
AFSLSNYYMS	SEQ ID NO: 14
IISSGSNALKWYASWPKG	SEQ ID NO: 15
NYVGSGSYYGMDL	SEQ ID NO: 16

In one embodiment sequences 11 to 13 are in a light chain of the antibody.

In one embodiment sequences 14 to 16 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 11 is CDR L1, SEQ ID NO: 12 is CDR L2 and SEQ ID NO: 13 is CDR L3.

In one embodiment SEQ ID NO: 14 is CDR H1, SEQ ID NO: 15 is CDR H2 and SEQ ID NO; 16 is CDR H3.

In one embodiment SEQ ID NO: 11 is CDR L1, SEQ ID NO: 12 is CDR L2, SEQ ID NO: 13 is CDR L3, SEQ ID NO: 14 is CDR H1, SEQ ID NO: 15 is CDR H2 and SEQ ID NO; 16 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 923 anti-toxin A antibody; Light chain Variable region sequence) SEQ ID NO: 17:

DVVMTQSPSSLSASVGDRVTITCQASQSISNYLAWYQQKPGKVPKLLIYSASTLASGVPSRFK
GSGSGTQFTLTISSLQPEDVATYYCQCTYSHYGTGVFGAFGGGTKVEIK

wherein the CDRs are underlined and construct is referred to herein as CA923.g1 gL1

The polynucleotide sequence encoding SEQ ID NO: 17 is shown in Figure 1 and SEQ ID NO: 18 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 923 anti-toxin A antibody heavy chain variable region sequence) SEQ ID NO: 19:

EVQLVESGGGLVQPGGSLRLSCAASAFSLSNYYMSWVRQAPGKGLEWIGIISSGSNALKWYAS
WPKGRFTISKDSTTVYLQMNSLRAEDTATYFCARNYVGSGSYYGMDLWGQGTLVTVS

wherein the CDRs are underlined and construct is referred to herein as CA923.g1 gH1

The polynucleotide sequence encoding SEQ ID NO: 19 is shown in Figure 2 and SEQ ID NO: 20 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO 17: and SEQ ID NO: 19.

In one embodiment there is provided an antibody (for example an anti-toxin A antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

QASQSISSYFS	SEQ ID NO: 21
GASTLAS	SEQ ID NO: 22
QCTDYSGIYFGG	SEQ ID NO: 23
GFSLSSYYMS	SEQ ID NO: 24
IISSGSSTTFTWYASWAKG	SEQ ID NO: 25
AYVGSSSYYGFDP	SEQ ID NO: 26

In one embodiment sequences 21 to 23 are in a light chain of the antibody.

In one embodiment sequences 24 to 26 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 21 is CDR L1, SEQ ID NO: 22 is CDR L2 and SEQ ID NO; 23 is CDR L3.

In one embodiment SEQ ID NO: 24 is CDR H1, SEQ ID NO: 25 is CDR H2 and SEQ ID NO; 26 is CDR H3.

In one embodiment SEQ ID NO: 21 is CDR L1, SEQ ID NO: 22 is CDR L2, SEQ ID NO; 23 is CDR L3, SEQ ID NO: 24 is CDR H1, SEQ ID NO: 25 is CDR H2 and SEQ ID NO; 26 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 993 anti-toxin A antibody; Light chain Variable region sequence) SEQ ID NO: 27:

DVVMTQSPSTLSASVGDRVTITCQASQSISSYFSWYQQKPGKAPQLLIYGASTLASGVPSRFK
GSGSGTELTLTISSLQPDDFATYYCQCTDYSGIYFGGFGGGTKVEIK

wherein the CDRs are underlined and construct is referred to herein as CA993.g1 gL1

The polynucleotide sequence encoding SEQ ID NO: 27 is shown in Figure 2 and SEQ ID NO: 28 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 993 anti-toxin A antibody heavy chain variable region sequence) SEQ ID NO: 29:

EVQLVESGGGLVQPGGSLKLSCTASGFSLSSYYMSWVRQAPGKGLEWIGIISSGSSTTFTWYA
SWAKGRFTISKTSTTVYLQMNSLKTEDTATYFCARAYVGSSSYYGFDPWGQGTLVTVS

wherein the CDRs are underlined and construct is referred to herein as CA993.g1 gH1

The polynucleotide sequence encoding SEQ ID NO: 29 is shown in Figure 2 and SEQ ID NO: 30 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 27 and SEQ ID NO: 29.

In one embodiment there is provided an antibody (for example an anti-toxin A antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

QASQSINNYFS	SEQ ID NO: 31
GAANLAS	SEQ ID NO: 32
QNNYGVHIYGAA	SEQ ID NO: 33
GFSLSNYDMI	SEQ ID NO: 34
FINTGGITYYASWAKG	SEQ ID NO: 35
VDDYIGAWGAGL	SEQ ID NO: 36

In one embodiment sequences 31 to 33 are in a light chain of the antibody.

In one embodiment sequences 34 to 36 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 31 is CDR L1, SEQ ID NO: 32 is CDR L2 and SEQ ID NO; 33 is CDR L3.

In one embodiment SEQ ID NO: 34 is CDR H1, SEQ ID NO: 35 is CDR H2 and SEQ ID NO: 36 is CDR H3.

In one embodiment SEQ ID NO: 31 is CDR L1, SEQ ID NO: 32 is CDR L2, SEQ ID NO; 33 is CDR L3, SEQ ID NO: 34 is CDR H1, SEQ ID NO: 35 is CDR H2 and SEQ ID NO; 36 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 995 anti-toxin A antibody; Light chain Variable region sequence) SEQ ID NO: 37:

DVVMTQSPSTLSASVGDRVTITCQASQSINNYFSWYQQKPGKAPKLLIYGAANLASGVPSRFK
GSGSGTEYTLTISSLQPDDFATYSCQNNYGVHIYGAAFGGGTKVEIK

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 37 is shown in Figure 3 and SEQ ID NO: 38 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 995 anti-toxin A antibody heavy chain variable region sequence) SEQ ID NO: 39

EVQLVESGGGLVQPGGSLRLSCTASGFSLSNYDMIWVRQAPGKGLEYIGFINTGGITYYASWA
KGRFTISRDSSTVYLQMNSLRAEDTATYFCARVDDYIGAWGAGLWGQGTLVTVS

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 39 is shown in Figure 3 and SEQ ID NO: 40 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 37 and SEQ ID NO: 39.

In one embodiment there is provided an antibody (for example an anti-toxin A antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

QASQSISSYLS	SEQ ID NO: 41
RASTLAS	SEQ ID NO: 42
LGVYGYSNDDGIA	SEQ ID NO: 43
GIDLSSHHMC	SEQ ID NO: 44
VIYHFGSTYYANWATG	SEQ ID NO: 45
ASIAGYSAFDP	SEQ ID NO: 46

In one embodiment sequences 41 to 43 are in a light chain of the antibody.
In one embodiment sequences 44 to 46 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 41 is CDR L1, SEQ ID NO: 42 is CDR L2 and SEQ ID NO; 43 is CDR L3.

In one embodiment SEQ ID NO: 44 is CDR H1, SEQ ID NO: 45 is CDR H2 and SEQ ID NO: 46 is CDR H3.

In one embodiment SEQ ID NO: 41 is CDR L1, SEQ ID NO: 42 is CDR L2, SEQ ID NO; 43 is CDR L3, SEQ ID NO: 44 is CDR H1, SEQ ID NO: 45 is CDR H2 and SEQ ID NO; 46 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 997 anti-toxin A antibody; Light chain Variable region sequence) SEQ ID NO: 47:

ALVMTQSPSSFSASTGDRVTITCQASQSISSYLSWYQQKPGKAPKLLIYRASTLASGVPSRFS
GSGSGTEYTLTISCLQSEDFATYYCLGVYGYSNDDGIAFGGGTKVEIK

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 47 is shown in Figure 3 and SEQ ID NO: 48 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 997 anti-toxin A antibody heavy chain variable region sequence) SEQ ID NO: 49:

EVQLVESGGGLVQPGGSLRLSCTVSGIDLSSHHMCWVRQAPGKGLEYIGVIYHFGSTYYANWA
TGRFTISKDSTTVYLQMNSLRAEDTATYFCARASIAGYSAFDPWGQGTLVTVS

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 49 is shown in Figure 4 and SEQ ID NO: 50 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 47 and SEQ ID NO: 49.

In one embodiment there is provided an antibody (for example an anti-toxin A antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

QASQSIYSYLA	SEQ ID NO: 51
DASTLAS	SEQ ID NO: 52
QGNAYTSNSHDNA	SEQ ID NO: 53
GIDLSSDAVG	SEQ ID NO: 54
IIATFDSTYYASWAKG	SEQ ID NO: 55
TGSWYYISGWGSYYYGMDL	SEQ ID NO: 56

In one embodiment sequences 51 to 53 are in a light chain of the antibody.

In one embodiment sequences 54 to 56 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 51 is CDR L1, SEQ ID NO: 52 is CDR L2 and SEQ ID NO: 53 is CDR L3.

In one embodiment SEQ ID NO: 54 is CDR H1, SEQ ID NO: 55 is CDR H2 and SEQ ID NO; 56 is CDR H3.

In one embodiment SEQ ID NO: 51 is CDR L1, SEQ ID NO: 52 is CDR L2, SEQ ID NO; 53 is CDR L3, SEQ ID NO: 54 is CDR H1, SEQ ID NO: 55 is CDR H2 and SEQ ID NO: 56 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1000 anti-toxin A antibody; Light chain Variable region sequence) SEQ ID NO: 57:

EIVMTQSPSTLSASVGDRVTITCQASQSIYSYLAWYQQKPGKAPKLLIYDASTLASGVPSRFK
GSGSGTEFTLTISSLQPDDFATYYCQGNAYTSNSHDNAFGGGTKVEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 57 is shown in Figure 4 and SEQ ID NO: 58 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1000 anti-toxin A antibody heavy chain variable region sequence) SEQ ID NO: 59:

EVQLVESGGGLIQPGGSLRLSCTVSGIDLSSDAVGWVRQAPGKGLEYIGIIATFDSTYYASWA
KGRFTISKASSTTVYLQMNSLRAEDTATYFCARTGSWYYISGWGSYYYGMDLWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 59 is shown in Figure 4 and SEQ ID NO: 60 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 57 and SEQ ID NO: 59.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

RASKSVSTLMH	SEQ ID NO: 61
LASNLES	SEQ ID NO: 62
QQTWNDPWT	SEQ ID NO: 63
GFTFSNYGMA	SEQ ID NO: 64
SISSSGGSTYYRDSVKG	SEQ ID NO: 65
VIRGYVMDA	SEQ ID NO: 66

In one embodiment sequences 61 to 63 are in a light chain of the antibody.

In one embodiment sequences 64 to 66 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 61 is CDR L1, SEQ ID NO: 62 is CDR L2 and SEQ ID NO: 63 is CDR L3.

In one embodiment SEQ ID NO: 64 is CDR H1, SEQ ID NO: 65 is CDR H2 and SEQ ID NO: 66 is CDR H3.

In one embodiment SEQ ID NO: 61 is CDR L1, SEQ ID NO: 62 is CDR L2, SEQ ID NO; 63 is CDR L3, SEQ ID NO: 64 is CDR H1, SEQ ID NO: 65 is CDR H2 and SEQ ID NO: 66 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 926 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 67:

DTVLTQSPATLSLSPGERATLSCRASKSVSTLMHWFQQKPGQAPKLLIYLASNLESGVPARFS
GSGSGTDFTLTISSLEPEDFAVYYCQQTWNDPWTFGGGTKVEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 67 is shown in Figure 5 and SEQ ID NO: 68 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 926 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 69:

EVELLESGGGLVQPGGSLRLSCEASGFTFSNYGMAWVRQAPTKGLEWVTSISSSGGSTYYRDS
VKGRFTISRDNAKSSLYLQMNSLRAEDTATYYCTTVIRGYVMDAWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 69 is shown in Figure 5 and SEQ ID NO: 70 therein.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

RASGSVSTLMH	SEQ ID NO: 71
KASNLAS	SEQ ID NO: 72
HQSWNSDT	SEQ ID NO: 73
GFTFSNYGMA	SEQ ID NO: 74
TINYDGRTTHYRDSVKG	SEQ ID NO: 75
ISRSHYFDC	SEQ ID NO: 76

In one embodiment sequences 71 to 73 are in a light chain of the antibody.

In one embodiment sequences 74 to 76 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 71 is CDR L1, SEQ ID NO: 72 is CDR L2 and SEQ ID NO: 73 is CDR L3.

In one embodiment SEQ ID NO: 74 is CDR H1, SEQ ID NO: 75 is CDR H2 and SEQ ID NO: 76 is CDR H3.

In one embodiment SEQ ID NO: 71 is CDR L1, SEQ ID NO: 72 is CDR L2, SEQ ID NO; 73 is CDR L3, SEQ ID NO: 74 is CDR H1, SEQ ID NO: 75 is CDR H2 and SEQ ID NO: 76 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 927 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 77:

DTQMTQSPSTLSASVGDRVTITCRASGSVSTLMHWYQQKPGKAPKLLIYKASNLASGVPSRFS
GSGSGTEFTLTISSLQPDDFATYYCHQSWNSDTFGQGTRLEIK

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 77 is shown in Figure 5 and SEQ ID NO: 78 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 927 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 79:

EVQLVESGGGVVQPGRSLRLSCAASGFTFSNYGMAWVRQAPGKGLEWVATINYDGRTTHYRDS
VKGRFTISRDNSKSTLYLQMNSLRAEDTAVYYCTSISRSHYFDCWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 79 is shown in Figure 5 and SEQ ID NO: 80 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 77 and SEQ ID NO: 79.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

KASKSISNHLA	SEQ ID NO: 81
SGSTLQS	SEQ ID NO: 82
QQYDEYPYT	SEQ ID NO: 83
GFSLQSYTIS	SEQ ID NO: 84
AISGGGSTYYNLPLKS	SEQ ID NO: 85
PRWYPRSYFDY	SEQ ID NO: 86

In one embodiment sequences 81 to 83 are in a light chain of the antibody.

In one embodiment sequences 84 to 86 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 81 is CDR L1, SEQ ID NO: 82 is CDR L2 and SEQ ID NO: 83 is CDR L3.

In one embodiment SEQ ID NO: 84 is CDR H1, SEQ ID NO: 85 is CDR H2 and SEQ ID NO: 86 is CDR H3.

In one embodiment SEQ ID NO: 81 is CDR L1, SEQ ID NO: 82 is CDR L2, SEQ ID NO; 83 is CDR L3, SEQ ID NO: 84 is CDR H1, SEQ ID NO: 85 is CDR H2 and SEQ ID NO: 86 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1099 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 87:

DVQLTQSPSFLSASVGDRVTITCKASKSISNHLAWYQEKPGKANKLLIHSGSTLQSGTPSRFS
GSGSGTEFTLTISSLQPEDFATYYCQQYDEYPYTFGQGTRLEIKRT

wherein the CDRs are underlined.

In one embodiment the last two amino acids (RT) of SEQ ID NO: 87 are omitted.

The polynucleotide sequence encoding SEQ ID NO: 87 is shown in Figure 6 and SEQ ID NO: 88 therein. In one embodiment the codons encoding the last two amino acids (RT) are omitted.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1099 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 89:

EVQLQESGPGLVKPSETLSLTCTVSGFSLQSYTISWVRQPPGKGLEWIAAISGGGSTYYNLPL
KSRVTISRDTSKSQVSLKLSSVTAADTAVYYCTRPRWYPRSYFDYWGRGTLVTVS

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 89 is shown in Figure 6 and SEQ ID NO: 90 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO 87: and SEQ ID NO: 89.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

RASQRISTSIH	SEQ ID NO: 91
YASQSIS	SEQ ID NO: 92
QQSYSSLYT	SEQ ID NO: 93
GFTFSDSYMA	SEQ ID NO: 94
SISYGGTIIQYGDSVKG	SEQ ID NO: 95
RQGTYARYLDF	SEQ ID NO: 96

In one embodiment sequences 91 to93 are in a light chain of the antibody.

In one embodiment sequences 94 to 96 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 91 is CDR L1, SEQ ID NO: 92 is CDR L2 and SEQ ID NO; 93 is CDR L3.

In one embodiment SEQ ID NO: 94 is CDR H1, SEQ ID NO: 95 is CDR H2 and SEQ ID NO: 96 is CDR H3.

In one embodiment SEQ ID NO: 91 is CDR L1, SEQ ID NO: 92 is CDR L2, SEQ ID NO; 93 is CDR L3, SEQ ID NO: 94 is CDR H1, SEQ ID NO: 95 is CDR H2 and SEQ ID NO: 96 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1102 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 97:

NIVLTQSPATLSLSPGERATLSCRASQRISTSIHWYQQKPGQAPRLLIKYASQSISGIPARFS GSGSGTDFTLTISSLEPEDFAVYYCQQSYSSLYTFGQGTKLEIK

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 97 is shown in Figure 6 and SEQ ID NO: 98 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1102 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 99:

EVQLVESGGGLVQPGGSLRLSCAVSGFTFSDSYMAWVRQAPGKGLEWIASISYGGTIIQYGDS VKGRFTISRDNAKSSLYLQMNSLRAEDTAVYYCARRQGTYARYLDFWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 99 is shown in Figure 7 and SEQ ID NO: 100 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO 97: and SEQ ID NO: 99.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

RASESVSTLLH	SEQ ID NO: 101
KASNLAS	SEQ ID NO: 102
HQSWNSPPT	SEQ ID NO: 103
GFTFSNYGMA	SEQ ID NO: 104
IINYDASTTHYRDSVKG	SEQ ID NO: 105
YGRSHYFDY	SEQ ID NO: 106

In one embodiment sequences 101 to 103 are in a light chain of the antibody.

In one embodiment sequences 104 to 106 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 101 is CDR L1, SEQ ID NO: 102 is CDR L2 and SEQ ID NO: 103 is CDR L3.

In one embodiment SEQ ID NO: 104 is CDR H1, SEQ ID NO: 105 is CDR H2 and SEQ ID NO: 106 is CDR H3.

In one embodiment SEQ ID NO: 101 is CDR L1, SEQ ID NO: 102 is CDR L2, SEQ ID NO; 103 is CDR L3, SEQ ID NO: 104 is CDR H1, SEQ ID NO: 105 is CDR H2 and SEQ ID NO; 106 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1114 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 107:

ATQMTQSPSSLSASVGDRVTITCRASESVSTLLHWYQQKPGKAPKLLIYKASNLASGVPSRFS GSGSGTDFTLTISSLQPEDFATYYCHQSWNSPPTFGQGTKLEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 107 is shown in Figure 7 and SEQ ID NO: 108 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1114 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 109:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYGMAWVRQAPGKGLEWVAIINYDASTTHYRDS VKGRFTISRDNAKSSLYLQMNSLRAEDTAVYYCTRYGRSHYFDYWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 109 is shown in Figure 7 and SEQ ID NO: 110 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 107 and SEQ ID NO: 109.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

RASESVSTLLH	SEQ ID NO: 111
KASNLAS	SEQ ID NO: 112
HQSWNSPPT	SEQ ID NO: 113
GFTFSNYGMA	SEQ ID NO: 114
IINYDASTTHYRDSVK	SEQ ID NO: 115
YGRSHYFDY	SEQ ID NO: 116

In one embodiment sequences 111 to 113 are in a light chain of the antibody.

In one embodiment sequences 114 to 116 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 111 is CDR L1, SEQ ID NO: 112 is CDR L2 and SEQ ID NO: 113 is CDR L3.

In one embodiment SEQ ID NO: 114 is CDR H1, SEQ ID NO: 115 is CDR H2 and SEQ ID NO: 116 is CDR H3.

In one embodiment SEQ ID NO: 111 is CDR L1, SEQ ID NO: 112 is CDR L2, SEQ ID NO; 113 is CDR L3, SEQ ID NO: 114 is CDR H1, SEQ ID NO: 115 is CDR H2 and SEQ ID NO: 116 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1114 graft 8 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 117:

DTVLTQSPSSLSASVGDRVTITCRASESVSTLLHWYQQKPGKAPKLLIYKASNLASGVPSRFS
GSGSGTDFTLTISSLQPEDFATYYCHQSWNSPPTFGQGTKLEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 117 is shown in Figure 8 and SEQ ID NO: 118 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1114 graft 8 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 119:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYGMAWVRQAPGKGLEWVAIINYDASTTHYRDS
VKGRFTISRDNAKSSLYLQMNSLRAEDTAVYYCTRYGRSHYFDYWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 119 is shown in Figure 8 and SEQ ID NO: 120 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 117 and SEQ ID NO: 119.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

KASQNIYMYLN	SEQ ID NO: 121
NTNKLHT	SEQ ID NO: 122
LQHKSFPYT	SEQ ID NO: 123
GFTFRDSFMA	SEQ ID NO: 124
SISYEGDKTYYGDSVKG	SEQ ID NO: 125
LTITTSGDS	SEQ ID NO: 126

In one embodiment sequences 121 to 123 are in a light chain of the antibody.

In one embodiment sequences 124 to 126 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 121 is CDR L1, SEQ ID NO: 122 is CDR L2 and SEQ ID NO: 123 is CDR L3.

In one embodiment SEQ ID NO: 124 is CDR H1, SEQ ID NO: 125 is CDR H2 and SEQ ID NO: 126 is CDR H3.

In one embodiment SEQ ID NO: 121 is CDR L1, SEQ ID NO: 122 is CDR L2, SEQ ID NO: 123 is CDR L3, SEQ ID NO: 124 is CDR H1, SEQ ID NO: 125 is CDR H2 and SEQ ID NO: 126 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1125 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 127:

DIQMTQSPSSLSASVGDRVTITCKASQNIYMYLNWYQQKPGKAPKRLIYNTNKLHTGVPSRFS
GSGSGTEYTLTISSLQPEDFATYYCLQHKSFPYTFGQGTKLEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 127 is shown in Figure 8 and SEQ ID NO: 128 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1125 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 129:

EVQLVESGGGLVQPGGSLRLSCAASGFTFRDSFMAWVRQAPGKGLEWVASISYEGDKTYYGDS
VKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARLTITTSGDSWGQGTMVTVSS

wherein the CDRs are underlined.

In one embodiment the last amino acid (S) of SEQ ID NO: 129 is omitted.

The polynucleotide sequence encoding SEQ ID NO: 129 is shown in Figure 9 and SEQ ID NO: 130 therein. In one embodiment the codon AGC encoding the last amino acid S is omitted.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 127 and SEQ ID NO: 129.

In one embodiment there is provided antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

KASQHVGTNVD	SEQ ID NO: 131
GASIRYT	SEQ ID NO: 132
LQYNYNPYT	SEQ ID NO: 133
GFIFSNFGMS	SEQ ID NO: 134
SISPSGGNAYYRDSVKG	SEQ ID NO: 135
RAYSSPFAF	SEQ ID NO: 136

In one embodiment sequences 131 to 133 are in a light chain of the antibody.

In one embodiment sequences 134 to 136 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 131 is CDR L1, SEQ ID NO: 132 is CDR L2 and SEQ ID NO: 133 is CDR L3.

In one embodiment SEQ ID NO: 134 is CDR H1, SEQ ID NO: 135 is CDR H2 and SEQ ID NO: 136 is CDR H3.

In one embodiment SEQ ID NO: 131 is CDR L1, SEQ ID NO: 132 is CDR L2, SEQ ID NO: 133 is CDR L3, SEQ ID NO: 134 is CDR H1, SEQ ID NO: 135 is CDR H2 and SEQ ID NO: 136 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1129 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 137:

DTQMTQSPSSLSASVGDRVTITCKASQHVGTNVDWYQQKPGKVPKLLIYGASIRYTGVPDRFT GSGSGTDFTLTISSLQPEDVATYYCLQYNYNPYTFGQGTKLEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 137 is shown in Figure 8 and SEQ ID NO: 138 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1129 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 139:

EVQLVESGGGVVQPGRSLRLSCATSGFIFSNFGMSWVRQAPGKGLEWVASISPSGGNAYYRDS VKGRFTISRDNSKTTLYLQMNSLRAEDTAVYYCTRRAYSSPFAFWGQGTLVTVSS

wherein the CDRs are underlined.

In one embodiment the last amino acid (S) of SEQ ID NO: 139 is omitted.

The polynucleotide sequence encoding SEQ ID NO: 139 is shown in Figure 8 and SEQ ID NO: 140 therein. In one embodiment the codon AGC encoding the last amino acid S is omitted.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 137 and SEQ ID NO: 139.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

KASKSISNHLA	SEQ ID NO: 141
SGSTLQP	SEQ ID NO: 142
QQYDEYPYT	SEQ ID NO: 143
GFSLNSYTIT	SEQ ID NO: 144
AISGGGSTYFNSALKS	SEQ ID NO: 145
PRWYPRSYFDY	SEQ ID NO: 146

In one embodiment sequences 141 to 143 are in a light chain of the antibody.

In one embodiment sequences 144 to 146 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 141 is CDR L1, SEQ ID NO: 142 is CDR L2 and SEQ ID NO: 143 is CDR L3.

In one embodiment SEQ ID NO: 144 is CDR H1, SEQ ID NO: 145 is CDR H2 and SEQ ID NO: 146 is CDR H3.

In one embodiment SEQ ID NO: 141 is CDR L1, SEQ ID NO: 142 is CDR L2, SEQ ID NO: 143 is CDR L3, SEQ ID NO: 144 is CDR H1, SEQ ID NO: 145 is CDR H2 and SEQ ID NO: 146 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1134 anti-toxin B antibody; Light chain Variable region sequence):

DVQLTQSPSFLSASVGDRVTITCKASKSISNHLAWYQEKPGKANKLLIHSGSTLQPGT PSRFSGSGSGTEFTLTISSLQPEDFATYYCQQYDEYPYTFGQGTRLEIK

SEQ ID NO: 147

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 147 is shown in Figure 9 and SEQ ID NO: 148 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1134 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 149:

EVQLQESGPGLVKPSETLSLTCTVSGFSLNSYTITWVRQPPGKGLEWIAAISGGGSTYFNSAL KSRVTISRDTSKSQVSLKLSSVTAADTAVYYCTRPRWYPRSYFDYWGRGTLVTVS

wherein the CDRs are underlined

The polynucleotide sequence encoding SEQ ID NO: 149 is shown in Figure 9 and SEQ ID NO: 150 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO 147: and SEQ ID NO: 149.

In one embodiment there is provided antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

KASQNVGNNVA	SEQ ID NO: 151
YASNRFT	SEQ ID NO: 152
QRVYQSTWT	SEQ ID NO: 153
GFSLTSYYVH	SEQ ID NO: 154
CIRTGGNTEYQSEFKS	SEQ ID NO: 155
GNYGFAY	SEQ ID NO: 156

In one embodiment sequences 151 to 153 are in a light chain of the antibody.

In one embodiment sequences 154 to 156 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 151 is CDR L1, SEQ ID NO: 152 is CDR L2 and SEQ ID NO: 153 is CDR L3.

In one embodiment SEQ ID NO: 154 is CDR H1, SEQ ID NO: 155 is CDR H2 and SEQ ID NO: 156 is CDR H3.

In one embodiment SEQ ID NO: 151 is CDR L1, SEQ ID NO: 152 is CDR L2, SEQ ID NO; 153 is CDR L3, SEQ ID NO: 154 is CDR H1, SEQ ID NO: 155 is CDR H2 and SEQ ID NO; 156 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1151 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 157:

AIQMTQSPSSLSASVGDRVTITCKASQNVGNNVAWYQHKPGKAPKLLIYYASNRFTGVPSRFT
GGGYGTDFTLTISSLQPEDFATYYCQRVYQSTWTFGQGTKVEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 157 is shown in Figure 9 and SEQ ID NO: 158 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1151 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 159:

EVQLQESGPGLVKPSETLSLTCTVSGFSLTSYYVHWVRQPPGKGLEWMGCIRTGGNTEYQSEF
KSRVTISRDTSKNQVSLKLSSVTAADTAVYYCARGNYGFAYWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 159 is shown in Figure 9 and SEQ ID NO: 160 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 157 and SEQ ID NO: 159.

In one embodiment there is provided an antibody (for example an anti-toxin B antibody) comprising a CDR, such as 1, 2, 3, 4, 5 or 6 CDRs, selected from:

KASQNINKYLD	SEQ ID NO: 161
NIQSLHT	SEQ ID NO: 162
FQHNSGW	SEQ ID NO: 163
GFTFTQAAMF	SEQ ID NO: 164
RISTKSNNFATYYPDSVKG	SEQ ID NO: 165
PAYYYDGTVPFAY	SEQ ID NO: 166

In one embodiment sequences 161 to 163 are in a light chain of the antibody.

In one embodiment sequences 164 to 166 are in a heavy chain of the antibody.

In one embodiment SEQ ID NO: 161 is CDR L1, SEQ ID NO: 162 is CDR L2 and SEQ ID NO: 163 is CDR L3.

In one embodiment SEQ ID NO: 164 is CDR H1, SEQ ID NO: 165 is CDR H2 and SEQ ID NO: 166 is CDR H3.

In one embodiment SEQ ID NO: 161 is CDR L1, SEQ ID NO: 162 is CDR L2, SEQ ID NO: 163 is CDR L3, SEQ ID NO: 164 is CDR H1, SEQ ID NO: 165 is CDR H2 and SEQ ID NO: 166 is CDR H3.

In one embodiment there is provided a variable region, such as a light chain variable region with the following sequence (Antibody 1153 anti-toxin B antibody; Light chain Variable region sequence) SEQ ID NO: 167:

DIQMTQSPSSLSASVGDRVTITCKASQNINKYLDWYQQKPGKVPKLLIYNIQSLHTGIPSRFS
GSGSGTDFTLTISSLQPEDVATYYCFQHNSGWTFGQGTRLEIK

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 167 is shown in Figure 10 and SEQ ID NO: 168 therein.

In one embodiment there is provided a variable region, such as a heavy chain variable region with the following sequence (Antibody 1153 anti-toxin B antibody heavy chain variable region sequence) SEQ ID NO: 169:

EVQLVESGGGLVQPGGSLKLSCAASGFTFTQAAMFWVRQASGKGLEGIARISTKSNNFATYYP
DSVKGRFTISRDDSKNTVYLQMNSLKIEDTAVYYCTAPAYYYDGTVPFAYWGQGTLVTVS

wherein the CDRs are underlined.

The polynucleotide sequence encoding SEQ ID NO: 169 is shown in Figure 10 and SEQ ID NO: 170 therein.

In one embodiment an antibody according to the invention comprises variable regions shown in SEQ ID NO: 167 and SEQ ID NO: 169.

In one embodiment there is provided antibody comprising 6 CDRs independently selected from SEQ ID NOs 1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 15, 16, 21, 22, 23, 24, 25, 26, 31, 32, 33, 34, 35, 36, 41, 42, 43, 44, 45, 46, 51, 52, 53, 54, 55, 56, 61, 62, 63, 64, 65, 66, 71, 72, 73, 74, 75, 76, 81, 82, 83, 84, 85, 86, 91, 92, 93, 94, 95, 96, 101, 102, 103, 104, 105, 106, 111, 112, 113, 114, 115, 116, 121,122, 123, 124, 125, 126, 131, 132, 133, 134, 135, 136, 141, 142, 143, 144, 145, 146, 151, 152, 153, 154, 155, 156, 161, 162, 163, 164, 165 and 166.

In one embodiment there is provided an anti-TcdA antibody comprising 6 CDRs independently selected from SEQ ID NOs 1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 15, 16, 21, 22, 23, 24, 25, 26, 31, 32, 33, 34, 35, 36, 41, 42, 43, 44, 45, 46, 51, 52, 53, 54, 55 and 56.

In one embodiment there is provided an anti-TcdB antibody comprising 6 CDRs independently selected from SEQ ID NOs 61, 62, 63, 64, 65, 66, 71, 72, 73, 74, 75, 76, 81, 82, 83, 84, 85, 86, 91, 92, 93, 94, 95, 96, 101, 102, 103, 104, 105, 106, 111, 112, 113, 114, 115, 116, 121,122, 123, 124, 125, 126, 131, 132, 133, 134, 135, 136, 141, 142, 143, 144, 145, 146, 151, 152, 153, 154, 155, 156, 161, 162, 163, 164, 165 and 166.

In one embodiment there is provided an antibody which comprises two variable regions independently selected from SEQ ID NOs: 7, 9, 17, 19, 27, 29, 37, 39, 47, 49, 57, 59, 67, 69, 77, 79, 87, 89, 97, 99, 107, 109, 117, 119, 127, 129, 137, 139, 147, 149, 157 and 159.

In one embodiment there is provided an antibody which comprises two variable regions independently selected from SEQ ID NOs: 7, 9, 17, 19, 27, 29, 37, 39, 47, 49, 57 and 59.

In one embodiment there is provided an antibody which comprises two variable regions independently selected from SEQ ID NOs: 67, 69, 77, 79, 87, 89, 97, 99, 107, 109, 117, 119, 127, 129, 137, 139, 147, 149, 157 and 159.

In one embodiment the antibodies according to the invention are humanized.

In one embodiment the antibody or antibodies are directed to the C terminal "cell binding" portion of the TcdA and/or TcdB toxin.

In one embodiment an antibody according to the invention is suitable for neutralising toxin A or toxin B.

Neutralising as employed herein is intended to refer to the elimination or reduction of harmful/deleterious effects of the target toxin, for example at least a 50% reduction in the relevant harmful effect.

The inventors have established by using internal comparisons between antibodies discovered in this application and by comparison against antibodies well described in the art (Babcock et al. 2006; Lowy et al., 2010) that some antibodies have the desirable characteristic of maintaining effective neutralization (for example low EC_{50} and high % protection) even at high toxin concentrations. Other antibodies including those described in the art do not maintain effective toxin neutralization at high toxin concentrations.

Effective toxin concentrations can be defined as a 'lethal dose' (LD) in titration studies in the absence of neutralizing antibodies. Neutralisation assays are typically conducted at an LD of 50% of complete cell killing (*i.e.* an LD_{50}) but may be more rigorously conducted at an LD_{80}.

Assays may also be performed under considerably more challenging conditions such as LD_{90}, LD_{95} and/or LD_{max} (LD_{max} is the maximal toxin quantity which can be included in an assay as constrained by assay volume and maximum toxin concentration / solubility). Such assays aim to mimic the early stages of infection of humans when *C. difficile* growth in the

bowel is rampant and diarrhea and other symptoms lead one to hypothesise that toxin concentrations are at their highest. Antibodies which effectively neutralize damaging toxin activities under high toxin concentration conditions are thought by the present inventors to have special clinical value for the control of symptoms in human infections. In one embodiment the antibody or antibodies of the present disclosure have useful, for example low EC_{50} values and/or high % protection from cell death for one or more the LD_{80}, LD_{90}, LD_{95} and/or LD_{max}. In one embodiment the EC_{50} in the one or more of the latter situations is 15ng/ml or less, for example 10ng/ml or less, such as 5ng/ml or less, in particular 1ng/ml or less. In one embodiment the % protection from cell death is >90%, or >75% or >50%.

Thus in one embodiment the present disclosure provides an antibody or a combination of antibodies which maintain toxin neutralization even in the presence of high levels of toxin, for example as measured in an assay provided herein.

The harmful effect of toxin may, for example be measured in a suitable in vitro assay. In one embodiment the neutralization is measured in an assay given in Example 1 below. Also provided is an antibody or antibodies identified in a neutralization assay, for example wherein the potency of the antibody is maintained in the presence of high levels of toxin.

Toxin A is used interchangeably with TcdA.

Toxin B is used interchangeably with TcdB.

In one embodiment an antibody according to the invention is a monoclonal antibody or binding fragment thereof.

In one embodiment a monoclonal antibody according to the invention is capable of neutralising TcdA with very high potency and affinity.

In one embodiment a monoclonal antibody according to the invention is capable of neutralising TcdA with very high potency and affinity and high avidity.

Avidity as employed herein refers to the combined strength of multiple binding affinities.

In one embodiment a monoclonal antibody according to the invention is capable of neutralising TcdA with very high potency and affinity and high avidity and high valency of binding.

Valency of binding as employed herein refers to the ability for a monoclonal antibody to bind to an antigen multiple times. High valency of binding hence results in high levels of decoration of antigen with antibodies and / or high levels of cross-linking of toxin molecules, which is thought to be advantageous.

Anti-TcdA Mabs according to the present disclosure may be suitable for neutralising the early effects of TcdA, for example on cells such as loss of tight junctions.

Tight junction as employed herein is intended to refer to impermeable zone of connection between cells within a monolayer or anatomical tissue structure. Fluid loss does not occur when tight junctions retain their structural and functional integrity. Loss of tight junctions is an indication that the cell has been compromised by toxin and is well documented as being an early step in the toxic effects of TcdA and TcdB (25) and results in loss of fluid containing serum, immunoglobulin and ions (26, 3). Loss of tight junctions is thought to be a first step on the onset of diarrhoea in humans.

The TEER assay system, can be used to measure the loss of tight junction *in vitro*. TEER is an acronym for trans epithelial electric resistance assay and it is generally employed to measure the permeability of a differentiated cell layer representative of a gut endothelial lining. However, in the context of screening for antibodies TEER loss can be employed to identify antibodies that slow or prevent damage to the tight junctions and hence is a surrogate for protection against tissue damage leading to diarrhoea.

Often Caco-2 cells are employed since they are derived from human colon cells and are known to form differentiated monolayers with cells connected by tight junctions. A kit is commercially available from Becton-Dickinson named the Caco-2 BioCoat HTS plate system (BD Biosciences/ 354802). The instructions in the kit are suitable for testing in the present context. The resistance of the membrane changes when the membrane has been compromised.

Generally the antibody will be pre-incubated with the toxin before addition to the TEER system to establish if the antibody can prevent or slow the damage to the membrane caused by the toxin. The assay may be performed over a suitable period, for example 24 hours taking measurements at certain time-points. The present inventors have established that the 4 hour time point is particularly discriminating for therapeutically useful antibodies.
The concentration of toxin employed in the TEER assay is generally in the range 100-200ng/ml, most preferably 125ng/ml

The concentration of antibody (for example IgG1) employed in the TEER assay is generally in the range of 4 to 2000ng/ml, for example 50 to 1000ng/ml, such as 100 to 500ng/ml.

In one embodiment the EC_{50} of the antibody in the TEER assay employed in said condition is at least 200ng/ml, for example less than 100ng/ml, such as about 60-80ng/ml.

In one embodiment there is provided an anti-TcdA antibody or an anti-TcdB antibody suitable for use as a therapeutic agent in the treatment or prevention of *C. difficile* infection, wherein said antibody was screened and selected employing a TEER assay.

In one aspect there is provided a method of screening an antibody in a TEER assay for the ability to slow or prevent loss of tight junctions. In one embodiment the antibody or

antibodies screened are anti-TcdA antibodies. In one embodiment the antibody or antibodies screened are anti-TcdB antibodies. In one embodiment the antibody or antibodies screened are a combination of anti-TcdA and anti-TcdB antibodies. In one embodiment the method comprises the step of identifying an appropriate antibody or antibodies and expressing suitable quantities of same. In one embodiment the method comprises the further step of formulating said antibody or antibodies in a pharmaceutical formulation. In one embodiment the method comprises the further step of administering said antibody or antibodies or said formulation to a patient in need thereof.

In one embodiment multiple antibodies of the disclosure may be capable of binding to the target toxin (TcdA or TcdB), which may aid immune clearance of the toxin.

Multiple antibodies as employed herein is intended to refer to multiple copies of an antibody with the same sequence or an antibody with the same amino acid sequence or an antibody specific to the same target antigen but with a different amino acid sequence.

In one embodiment the antibodies according to the invention are specific to the target antigen, for example specific to an epitope in the target antigen.

In one embodiment the antibodies of the invention are able to bind to the target antigen in two or more locations, for example two or three locations, such as four, five, six, seven, eight, nine, ten or more locations, for example the toxin may comprise repeating domains and thus an antibody may be specific to an epitope and in fact that epitope may be present in the antigen several times i.e. in more than one location. Thus given antibodies may bind the same epitope multiple times in different locations in the antigen.

In one embodiment the antibody binds to the given antigen multiple times, for example 2 to 20 times such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 times. In one embodiment the antibody binds the given antigen at least 3 times. This multiple binding is thought to be important in neutralisation and/or clearance of the toxin. Whilst not wishing to be bound by theory it is thought that multiple binding, for example 3 more times, i.e. by decoration with 3 or more Fc fragments is important in triggering rapid clearance of the toxin (24) primarily via the liver and spleen (27, 28).

In one embodiment the anti-TcdA antibody binds 3 or more times, for example 3 to 16 times.

In one embodiment the anti-TcdA antibody binds 12 times.

In one embodiment the anti-TcdA antibody binds 2 times.

In one embodiment an anti-TcdA antibody binds in the catalytic-terminal cell binding domain of TcdA.

In one embodiment the anti-Tcd B antibody binds 2 or more times, for example 2 times.

In one embodiment an anti-TcdB antibody binds in the catalytic-terminal cell binding domain of TcdB.

In one embodiment the antibody or antibodies according to disclosure are capable of cross-linking toxin molecules, for example one arm of the antibody molecule binds one toxin molecule and another of the antibody binds a epitope in a different toxin molecule, thereby forming a sort of immune complex. The formation of the latter may also facilitate activation of the immune system to clear the relate toxin and thereby minimise the deleterious *in vivo* effects of the same.

In one embodiment an innate immune response, such as complement is activated.

In one embodiment the antibody or antibodies of the disclosure have high potency against toxins derived from strains of different ribotypes, for example 003, 027, 078.

In one embodiment antibodies against TcdA may have an EC_{50} in the range of 0.1 – 100ng/ml, such as 1 to 10ng/ml and a maximal inhibition in the range of 50-100% at toxin concentrations of LD_{80-95}, for example against toxins from strains of ribotypes 003, 027 and 078.

In one embodiment antibodies against TcdA may have an EC_{50} in the range of 0.1 – 100ng/ml, such as 1 to 10ng/ml and a maximal inhibition in the range of 60-100%, 70-100%, 80-100% or 90-100% at toxin concentrations of LD_{80-95}, for example against toxins from strains of ribotypes 003, 027 and 078.

In one embodiment antibodies against TcdB may have EC_{50} in the range of 0.1 – 100ng/ml, such as 1 to 10ng/ml and a maximal inhibition in the range of 50-100% at toxin concentrations of LD_{80-95}, for example against toxins from strains of ribotype 003.

In one embodiment antibodies against TcdB may have EC_{50} in the range of 0.1 – 100ng/ml, such as 1 to 10ng/ml and a maximal inhibition in the range of 60-100%, 70-100%, 80-100% or 90-100% at toxin concentrations of LD_{80-95}, for example against toxins from strains of ribotype 003.

In one embodiment there are provided combinations of antibodies according to the invention, for example combinations of antibodies specific to TcdA, combinations of antibodies specific to TcdB or combinations of antibodies to specific to TcdA and antibodies specific to TcdB.

Combinations of antibodies specific to TcdA will generally refer to combinations of antibodies directed to different epitopes on the target antigen TcdA, or at least with different binding properties.

Combinations of antibodies specific to TcdB will generally refer to combinations of antibodies directed to different epitopes on the target antigen TcdB, or at least with different binding properties.

The combinations may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 distinct antibodies, for example 2, 3, 4 or 5 antibodies.

In one embodiment there is provided a combination of one anti-TcdA antibody and two anti-TcdB, for example wherein the anti-TcdA antibody is 997 and where the anti-TcdB antibodies are 1125 and 1151

In particular there is provided a combination of one anti-TcdA antibody comprising a heavy variable region with a sequence as shown in SEQ ID NO:49 and a light variable region with a sequence shown in SEQ ID NO: 47 and two anti-TcdB antibodies the first with a heavy variable region shown in SEQ ID NO: 129 and a light variable region shown in SEQ ID NO: 127, and the second with a heavy variable region shown in SEQ ID NO: 159 and light variable region shown in SEQ ID NO: 157.

Distinct antibodies as employed herein is intended to refer to antibodies with different amino acid sequences, which may bind the same epitope or different epitopes on the target antigen.

Also provided by the present invention is a specific region or epitope of TcdA which is bound by an antibody provided by the present invention, in particular an antibody comprising the heavy chain sequence given in SEQ ID NO:49 and the light chain sequence given in SEQ ID NO:47.

Also provided by the present invention is a specific region or epitope of TcdB which is bound by an antibody provided by the present invention, in particular an antibody comprising the heavy chain sequence given in SEQ ID NO:129 and the light chain sequence given in SEQ ID NO:127 or an antibody comprising the heavy chain sequence given in SEQ ID NO:159 and the light chain sequence given in SEQ ID NO:157.

This specific region or epitope of the TcdA or TcdB toxins can be identified by any suitable epitope mapping method known in the art in combination with any one of the antibodies provided by the present invention. Examples of such methods include screening peptides of varying lengths derived from the toxins for binding to the antibody of the present invention with the smallest fragment that can specifically bind to the antibody containing the sequence of the epitope recognised by the antibody. The peptides may be produced synthetically or by proteolytic digestion of the toxin polypeptide. Peptides that bind the antibody can be identified by, for example, mass spectrometric analysis. In another example, NMR spectroscopy or X-ray crystallography can be used to identify the epitope bound by an antibody of the present invention.

Once identified, the epitopic fragment which binds an antibody of the present invention can be used, if required, as an immunogen to obtain additional antagonistic antibodies which bind the same epitope.

Antibodies which cross-block the binding of an antibody according to the present invention may be similarly useful in neutralizing toxin activity. Accordingly, the present invention also provides a neutralizing antibody having specificity for TcdA or TcdB, which cross-blocks the binding of any one of the antibodies described above to TcdA or TcdB and/or is cross-blocked from binding these toxins by any one of those antibodies. In one embodiment, such an antibody binds to the same epitope as an antibody described herein above. In another embodiment the cross-blocking neutralising antibody binds to an epitope which borders and/or overlaps with the epitope bound by an antibody described herein above. In another embodiment the cross-blocking neutralising antibody of this aspect of the invention does not bind to the same epitope as an antibody of the present invention or an epitope that borders and/or overlaps with said epitope.

Cross-blocking antibodies can be identified using any suitable method in the art, for example by using competition ELISA or BIAcore assays where binding of the cross blocking antibody to TcdA or TcdB prevents the binding of an antibody of the present invention or *vice versa*.

In one embodiment there is provided a method of generating an anti-TcdA or anti-TcdB antibody, in particular a neutralizing antibody and/or an antibody which cross-blocks the binding of an antibody described herein, said method comprising the steps of immunizing a host with a suitable antigen, for example an antigen shown in any one of SEQ ID Nos 173 to 194 or a combination thereof. The said method may also comprise one or more the following steps, for example identifying an antibody of interest (in particular using a functional assay such as TEER assay), expressing the antibody of interest, and optionally formulating the antibody as a pharmaceutically acceptable composition.

Thus in one aspect the present disclosure provides a method of immunizing a host with an amino acid sequence shown in SEQ ID Nos 173 to 194 or a combination thereof.

In one embodiment the antibodies according to the invention have an affinity to the target antigen of 10nM or less, for example 1nM or less such as 900pM, in particular 800pM, 700pM, 600pM or500pM, such as 60pM.

In one embodiment the affinity is for TcdA or TcdB or a fragment thereof. In one example the fragment is TcdA123 corresponding to residues S1827-D2249 of TcdA. In one

example the fragment is TcdA456 corresponding to residues G2205-R2608. In one embodiment the fragment is TcdB1234 corresponding to residues S1833-E2366 of TcdB.

In one embodiment antibodies according to the invention or a combination thereof have an EC_{50} of 200ng/ml or less, for example 150ng/ml or less such as 100ng/ml or less, such as in the range 0.1 to 10ng/ml.

The individual component antibodies of mixtures are not required to have an EC_{50} in said range provided that when they are used in combination with one or more antibodies the combination has an EC_{50} in said range.

Advantageously, the antibodies of the invention are stable, for example are thermally stable at temperatures above 50°C such as 60 or 70°C.

The antibodies and combinations according to the present invention also have one or more of the following advantageous properties: slow off rate, high affinity, high potency, the ability to bind multiple times to the target antigen, to neutralise the toxin by a mechanism which reduces the loss of measurable TEER activity, to stimulate or assist the hosts natural immune response, to catalyse or assist in immune clearance of the pathogen (or toxin) and/or to educate the immune system to respond appropriately to the pathogen (or toxin).

The residues in antibody variable domains are conventionally numbered according to a system devised by Kabat *et al.* This system is set forth in Kabat et al., 1987, in Sequences of Proteins of Immunological Interest, US Department of Health and Human Services, NIH, USA (hereafter "Kabat et al. (supra)"). This numbering system is used in the present specification except where otherwise indicated.

The Kabat residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or complementarity determining region (CDR), of the basic variable domain structure. The correct Kabat numbering of residues may be determined for a given antibody by alignment of residues of homology in the sequence of the antibody with a "standard" Kabat numbered sequence.

The CDRs of the heavy chain variable domain are located at residues 31-35 (CDR-H1), residues 50-65 (CDR-H2) and residues 95-102 (CDR-H3) according to the Kabat numbering system. However, according to Chothia (Chothia, C. and Lesk, A.M. J. Mol. Biol., 196, 901-917 (1987)), the loop equivalent to CDR-H1 extends from residue 26 to residue 32. Thus unless indicated otherwise 'CDR-H1' as employed herein is intended to refer to residues 26 to

35, as described by a combination of the Kabat numbering system and Chothia's topological loop definition.

The CDRs of the light chain variable domain are located at residues 24-34 (CDR-L1), residues 50-56 (CDR-L2) and residues 89-97 (CDR-L3) according to the Kabat numbering system.

Antibodies for use in the present invention may be obtained using any suitable method known in the art. The toxin A and/or toxin B polypeptide/protein including fusion proteins, for example toxin-Fc fusions proteins or cells (recombinantly or naturally) expressing the polypeptide (such as activated T cells) can be used to produce antibodies which specifically recognise the target toxins. The toxin polypeptide may be the full length polypeptide or a biologically active fragment or derivative thereof.

Polypeptides may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems or they may be recovered from natural biological sources. In the present application, the term "polypeptides" includes peptides, polypeptides and proteins. These are used interchangeably unless otherwise specified. The sequence for TcdA from ribotype 027 is given in SEQ ID NO: 171 (Uniprot accession number C9YJ37) and the sequence for TcdB from ribotype 027 is given is SEQ ID NO: 172 (Uniprot accession number C9YJ35).

The antigen polypeptide may in some instances be part of a larger protein such as a fusion protein for example fused to an affinity tag.

Antibodies generated against the antigen polypeptide may be obtained, where immunisation of an animal is necessary, by administering the polypeptides to an animal, preferably a non-human animal, using well-known and routine protocols, see for example Handbook of Experimental Immunology, D. M. Weir (ed.), Vol 4, Blackwell Scientific Publishers, Oxford, England, 1986). Many warm-blooded animals, such as rabbits, mice, rats, sheep, cows, camels or pigs may be immunized. However, mice, rabbits, pigs and rats are generally most suitable.

Monoclonal antibodies may be prepared by any method known in the art such as the hybridoma technique (Kohler & Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today, 4:72) and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, pp77-96, Alan R Liss, Inc., 1985).

Antibodies for use in the invention may also be generated using single lymphocyte antibody methods by cloning and expressing immunoglobulin variable region cDNAs generated from single lymphocytes selected for the production of specific antibodies by, for

example, the methods described by Babcook, J. et al., 1996, Proc. Natl. Acad. Sci. USA 93(15):7843-7848l; WO92/02551; WO2004/051268 and International Patent Application number WO2004/106377.

Humanised antibodies (which include CDR-grafted antibodies) are antibody molecules having one or more complementarity determining regions (CDRs) from a non-human species and a framework region from a human immunoglobulin molecule (see, e.g. US 5,585,089; WO91/09967). It will be appreciated that it may only be necessary to transfer the specificity determining residues of the CDRs rather than the entire CDR (see for example, Kashmiri et al., 2005, Methods, 36, 25-34). Humanised antibodies may optionally further comprise one or more framework residues derived from the non-human species from which the CDRs were derived.

As used herein, the term 'humanised antibody molecule' refers to an antibody molecule wherein the heavy and/or light chain contains one or more CDRs (including, if desired, one or more modified CDRs) from a donor antibody (e.g. a murine monoclonal antibody) grafted into a heavy and/or light chain variable region framework of an acceptor antibody (e.g. a human antibody). For a review, see Vaughan et al, Nature Biotechnology, 16, 535-539, 1998. In one embodiment rather than the entire CDR being transferred, only one or more of the specificity determining residues from any one of the CDRs described herein above are transferred to the human antibody framework (see for example, Kashmiri et al., 2005, Methods, 36, 25-34). In one embodiment only the specificity determining residues from one or more of the CDRs described herein above are transferred to the human antibody framework. In another embodiment only the specificity determining residues from each of the CDRs described herein above are transferred to the human antibody framework.

When the CDRs or specificity determining residues are grafted, any appropriate acceptor variable region framework sequence may be used having regard to the class/type of the donor antibody from which the CDRs are derived, including mouse, primate and human framework regions. Suitably, the humanised antibody according to the present invention has a variable domain comprising human acceptor framework regions as well as one or more of the CDRs provided herein.

Thus, provided in one embodiment is a humanised antibody which binds toxin A or toxin B wherein the variable domain comprises human acceptor framework regions and non-human donor CDRs.

Examples of human frameworks which can be used in the present invention are KOL, NEWM, REI, EU, TUR, TEI, LAY and POM (Kabat et al., supra). For example, KOL and NEWM can be used for the heavy chain, REI can be used for the light chain and EU, LAY and

POM can be used for both the heavy chain and the light chain. Alternatively, human germline sequences may be used; these are available at: http://vbase.mrc-cpe.cam.ac.uk/

In a humanised antibody of the present invention, the acceptor heavy and light chains do not necessarily need to be derived from the same antibody and may, if desired, comprise composite chains having framework regions derived from different chains.

Also, in a humanised antibody of the present invention, the framework regions need not have exactly the same sequence as those of the acceptor antibody. For instance, unusual residues may be changed to more frequently-occurring residues for that acceptor chain class or type. Alternatively, selected residues in the acceptor framework regions may be changed so that they correspond to the residue found at the same position in the donor antibody (see Reichmann et al., 1998, Nature, 332, 323-324). Such changes should be kept to the minimum necessary to recover the affinity of the donor antibody. A protocol for selecting residues in the acceptor framework regions which may need to be changed is set forth in WO 91/09967.

Generally the antibody sequences disclosed in the present specification are humanised.

The invention also provides sequences which are 80%, 90%, 91%, 92%, 93% 94%, 95% 96%, 97%, 98% or 99% similar to a sequence or antibody disclosed herein.

"Identity", as used herein, indicates that at any particular position in the aligned sequences, the amino acid residue is identical between the sequences. "Similarity", as used herein, indicates that, at any particular position in the aligned sequences, the amino acid residue is of a similar type between the sequences. For example, leucine may be substituted for isoleucine or valine. Other amino acids which can often be substituted for one another include but are not limited to:

- phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains);
- lysine, arginine and histidine (amino acids having basic side chains);
- aspartate and glutamate (amino acids having acidic side chains);
- asparagine and glutamine (amino acids having amide side chains); and
- cysteine and methionine (amino acids having sulphur-containing side chains).

Degrees of identity and similarity can be readily calculated (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing. Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987, Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991, the BLAST™ software available from NCBI (Altschul, S.F. et al., 1990, J. Mol. Biol. 215:403-410; Gish, W. & States, D.J. 1993, Nature Genet. 3:266-272. Madden, T.L. et al.,

1996, Meth. Enzymol. 266:131-141; Altschul, S.F. et al., 1997, Nucleic Acids Res. 25:3389-3402; Zhang, J. & Madden, T.L. 1997, Genome Res. 7:649-656,).

The antibody molecules of the present invention include a complete antibody molecule having full length heavy and light chains or a fragment thereof and may be, but are not limited to Fab, modified Fab, Fab', modified Fab', F(ab')2, Fv, Fab-Fv, Fab-dsFv, single domain antibodies (e.g. VH or VL or VHH), scFv, bi, tri or tetra-valent antibodies, Bis-scFv, diabodies, triabodies, tetrabodies and epitope-binding fragments of any of the above (see for example Holliger and Hudson, 2005, Nature Biotech. 23(9):1126-1136; Adair and Lawson, 2005, Drug Design Reviews - Online 2(3), 209-217). The methods for creating and manufacturing these antibody fragments are well known in the art (see for example Verma et al., 1998, Journal of Immunological Methods, 216, 165-181). Other antibody fragments for use in the present invention include the Fab and Fab' fragments described in International patent applications WO2005/003169, WO2005/003170 and WO2005/003171. Multi-valent antibodies may comprise multiple specificities e.g bispecific or may be monospecific (see for example WO 92/22853 and WO05/113605). Bispecific and multispecific antibody variants are especially considered in this example since the aim is to neutralise two independent target proteins: TcdA and TcdB. Variable regions from antibodies disclosed herein may be configured in such a way as to produce a single antibody variant which is capable of binding to and neutralising TcdA and TcdB.

In one embodiment the antibody according to the present disclosure is provided as TcdA or TcdB binding antibody fusion protein which comprises an immunoglobulin moiety, for example a Fab or Fab' fragment, and one or two single domain antibodies (dAb) linked directly or indirectly thereto, for example as described in WO2009/040562.

In one embodiment the fusion protein comprises two domain antibodies, for example as a variable heavy (VH) and variable light (VL) pairing, optionally linked by a disulphide bond, for example as described in WO2010/035012.

In one embodiment the Fab or Fab' element of the fusion protein has the same or similar specificity to the single domain antibody or antibodies. In one embodiment the Fab or Fab' has a different specificity to the single domain antibody or antibodies, that is to say the fusion protein is multivalent. In one embodiment a multivalent fusion protein according to the present invention has an albumin binding site, for example a VH/VL pair therein provides an albumin binding site.

In one embodiment the multivalent fusion protein according to the invention binds TcdA and TcdB.

In one embodiment the multivalent fusion protein according to the invention binds TcdB in multiple positions, for example has distinct binding regions specific for two different epitopes.

The constant region domains of the antibody molecule of the present invention, if present, may be selected having regard to the proposed function of the antibody molecule, and in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgD, IgE, IgG or IgM domains. In particular, human IgG constant region domains may be used, especially of the IgG1 and IgG3 isotypes when the antibody molecule is intended for therapeutic uses and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simply neutralising or agonising an antigen. It will be appreciated that sequence variants of these constant region domains may also be used. For example IgG4 molecules in which the serine at position 241 has been changed to proline as described in Angal et al., Molecular Immunology, 1993, 30 (1), 105-108 may be used. It will also be understood by one skilled in the art that antibodies may undergo a variety of posttranslational modifications. The type and extent of these modifications often depends on the host cell line used to express the antibody as well as the culture conditions. Such modifications may include variations in glycosylation, methionine oxidation, diketopiperazine formation, aspartate isomerization and asparagine deamidation. A frequent modification is the loss of a carboxy-terminal basic residue (such as lysine or arginine) due to the action of carboxypeptidases (as described in Harris, RJ. Journal of Chromatography 705:129-134, 1995).

In one embodiment the antibody heavy chain comprises a CH1 domain and the antibody light chain comprises a CL domain, either kappa or lambda.

Biological molecules, such as antibodies or fragments, contain acidic and/or basic functional groups, thereby giving the molecule a net positive or negative charge. The amount of overall "observed" charge will depend on the absolute amino acid sequence of the entity, the local environment of the charged groups in the 3D structure and the environmental conditions of the molecule. The isoelectric point (pI) is the pH at which a particular molecule or solvent accessible surface thereof carries no net electrical charge. In one example, the antibody and fragments of the invention may be engineered to have an appropriate isoelectric point. This may lead to antibodies and/or fragments with more robust properties, in particular suitable solubility and/or stability profiles and/or improved purification characteristics.

Thus in one aspect the invention provides a humanised antibody engineered to have an isoelectric point different to that of the originally identified antibody from which it is derived.

The antibody may, for example be engineered by replacing an amino acid residue such as replacing an acidic amino acid residue with one or more basic amino acid residues. Alternatively, basic amino acid residues may be introduced or acidic amino acid residues can be removed. Alternatively, if the molecule has an unacceptably high pI value acidic residues may be introduced to lower the pI, as required. It is important that when manipulating the pI care must be taken to retain the desirable activity of the antibody or fragment. Thus in one embodiment the engineered antibody or fragment has the same or substantially the same activity as the "unmodified" antibody or fragment.

Programs such as ** ExPASY http://www.expasy.ch/tools/pi_tool.html, and http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html, may be used to predict the isoelectric point of the antibody or fragment.

It will be appreciated that the affinity of antibodies provided by the present invention may be altered using any suitable method known in the art. The affinity of the antibodies or variants thereof may be measured using any suitable method known in the art, including BIAcore, using an appropriate isolated natural or recombinant protein or a suitable fusion protein/polypeptide.

The present invention therefore also relates to variants of the antibody molecules of the present invention, which have an improved affinity for TcdA or TcdB, as appropriate. Such variants can be obtained by a number of affinity maturation protocols including mutating the CDRs (Yang et al., J. Mol. Biol., 254, 392-403, 1995), chain shuffling (Marks et al., Bio/Technology, 10, 779-783, 1992), use of mutator strains of E. coli (Low et al., J. Mol. Biol., 250, 359-368, 1996), DNA shuffling (Patten et al., Curr. Opin. Biotechnol., 8, 724-733, 1997), phage display (Thompson et al., J. Mol. Biol., 256, 77-88, 1996) and sexual PCR (Crameri et al., Nature, 391, 288-291, 1998). Vaughan et al. (supra) discusses these methods of affinity maturation.

Improved affinity as employed herein in this context refers to an improvement refers to an improvement over the starting molecule.

If desired an antibody for use in the present invention may be conjugated to one or more effector molecule(s). It will be appreciated that the effector molecule may comprise a single effector molecule or two or more such molecules so linked as to form a single moiety that can be attached to the antibodies of the present invention. Where it is desired to obtain an antibody fragment linked to an effector molecule, this may be prepared by standard chemical or recombinant DNA procedures in which the antibody fragment is linked either directly or via a coupling agent to the effector molecule. Techniques for conjugating such effector molecules to antibodies are well known in the art (see, Hellstrom et al., Controlled Drug Delivery, 2nd

Ed., Robinson et al., eds., 1987, pp. 623-53; Thorpe et al., 1982 , Immunol. Rev., 62:119-58 and Dubowchik et al., 1999, Pharmacology and Therapeutics, 83, 67-123). Particular chemical procedures include, for example, those described in WO 93/06231, WO 92/22583, WO 89/00195, WO 89/01476 and WO03031581. Alternatively, where the effector molecule is a protein or polypeptide the linkage may be achieved using recombinant DNA procedures, for example as described in WO 86/01533 and EP0392745.

The term effector molecule as used herein includes, for example, biologically active proteins, for example enzymes, other antibody or antibody fragments, synthetic or naturally occurring polymers, nucleic acids and fragments thereof e.g. DNA, RNA and fragments thereof, radionuclides, particularly radioiodide, radioisotopes, chelated metals, nanoparticles and reporter groups such as fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy.

Other effector molecules may include chelated radionuclides such as 111In and 90Y, Lu177, Bismuth213, Californium252, Iridium192 and Tungsten188/Rhenium188; or drugs such as but not limited to, alkylphosphocholines, topoisomerase I inhibitors, taxoids and suramin.

Other effector molecules include proteins, peptides and enzymes. Enzymes of interest include, but are not limited to, proteolytic enzymes, hydrolases, lyases, isomerases, transferases. Proteins, polypeptides and peptides of interest include, but are not limited to, immunoglobulins, toxins such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin, a protein such as insulin, tumour necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor or tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, e.g. angiostatin or endostatin, or, a biological response modifier such as a lymphokine, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), nerve growth factor (NGF) or other growth factor and immunoglobulins.

Other effector molecules may include detectable substances useful for example in diagnosis. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive nuclides, positron emitting metals (for use in positron emission tomography), and nonradioactive paramagnetic metal ions. See generally U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics. Suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; suitable prosthetic groups include streptavidin, avidin and biotin; suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine

fluorescein, dansyl chloride and phycoerythrin; suitable luminescent materials include luminol; suitable bioluminescent materials include luciferase, luciferin, and aequorin; and suitable radioactive nuclides include 125I, 131I, 111In and 99Tc.

In another example the effector molecule may increase the half-life of the antibody in vivo, and/or reduce immunogenicity of the antibody and/or enhance the delivery of an antibody across an epithelial barrier to the immune system. Examples of suitable effector molecules of this type include polymers, albumin, albumin binding proteins or albumin binding compounds such as those described in WO05/117984.

Where the effector molecule is a polymer it may, in general, be a synthetic or a naturally occurring polymer, for example an optionally substituted straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymer or a branched or unbranched polysaccharide, e.g. a homo- or hetero- polysaccharide.

Specific optional substituents which may be present on the above-mentioned synthetic polymers include one or more hydroxy, methyl or methoxy groups.

Specific examples of synthetic polymers include optionally substituted straight or branched chain poly(ethyleneglycol), poly(propyleneglycol) poly(vinylalcohol) or derivatives thereof, especially optionally substituted poly(ethyleneglycol) such as methoxypoly(ethyleneglycol) or derivatives thereof.

Specific naturally occurring polymers include lactose, amylose, dextran, glycogen or derivatives thereof.

"Derivatives" as used herein is intended to include reactive derivatives, for example thiol-selective reactive groups such as maleimides and the like. The reactive group may be linked directly or through a linker segment to the polymer. It will be appreciated that the residue of such a group will in some instances form part of the product as the linking group between the antibody fragment and the polymer.

The size of the polymer may be varied as desired, but will generally be in an average molecular weight range from 500Da to 50000Da, for example from 5000 to 40000Da such as from 20000 to 40000Da. The polymer size may in particular be selected on the basis of the intended use of the product for example ability to localize to certain tissues such as tumors or extend circulating half-life (for review see Chapman, 2002, Advanced Drug Delivery Reviews, 54, 531-545). Thus, for example, where the product is intended to leave the circulation and penetrate tissue, for example for use in the treatment of a tumour, it may be advantageous to use a small molecular weight polymer, for example with a molecular weight of around 5000Da. For applications where the product remains in the circulation, it may be advantageous to use a

higher molecular weight polymer, for example having a molecular weight in the range from 20000Da to 40000Da.

Suitable polymers include a polyalkylene polymer, such as a poly(ethyleneglycol) or, especially, a methoxypoly(ethyleneglycol) or a derivative thereof, and especially with a molecular weight in the range from about 15000Da to about 40000Da.

In one example antibodies for use in the present invention are attached to poly(ethyleneglycol) (PEG) moieties. In one particular example the antibody is an antibody fragment and the PEG molecules may be attached through any available amino acid side-chain or terminal amino acid functional group located in the antibody fragment, for example any free amino, imino, thiol, hydroxyl or carboxyl group. Such amino acids may occur naturally in the antibody fragment or may be engineered into the fragment using recombinant DNA methods (see for example US 5,219,996; US 5,667,425; WO98/25971, WO2008/038024). In one example the antibody molecule of the present invention is a modified Fab fragment wherein the modification is the addition to the C-terminal end of its heavy chain one or more amino acids to allow the attachment of an effector molecule. Suitably, the additional amino acids form a modified hinge region containing one or more cysteine residues to which the effector molecule may be attached. Multiple sites can be used to attach two or more PEG molecules.

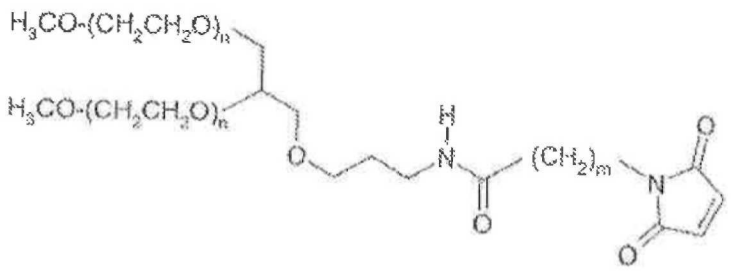
Suitably PEG molecules are covalently linked through a thiol group of at least one cysteine residue located in the antibody fragment. Each polymer molecule attached to the modified antibody fragment may be covalently linked to the sulphur atom of a cysteine residue located in the fragment. The covalent linkage will generally be a disulphide bond or, in particular, a sulphur-carbon bond. Where a thiol group is used as the point of attachment appropriately activated effector molecules, for example thiol selective derivatives such as maleimides and cysteine derivatives may be used. An activated polymer may be used as the starting material in the preparation of polymer-modified antibody fragments as described above. The activated polymer may be any polymer containing a thiol reactive group such as an α-halocarboxylic acid or ester, e.g. iodoacetamide, an imide, e.g. maleimide, a vinyl sulphone or a disulphide. Such starting materials may be obtained commercially (for example from Nektar, formerly Shearwater Polymers Inc., Huntsville, AL, USA) or may be prepared from commercially available starting materials using conventional chemical procedures. Particular PEG molecules include 20K methoxy-PEG-amine (obtainable from Nektar, formerly Shearwater; Rapp Polymere; and SunBio) and M-PEG-SPA (obtainable from Nektar, formerly Shearwater).

In one embodiment, the antibody is a modified Fab fragment or diFab which is PEGylated, i.e. has PEG (poly(ethyleneglycol)) covalently attached thereto, e.g. according to

the method disclosed in EP 0948544 or EP1090037 [see also "Poly(ethyleneglycol) Chemistry, Biotechnical and Biomedical Applications", 1992, J. Milton Harris (ed), Plenum Press, New York, "Poly(ethyleneglycol) Chemistry and Biological Applications", 1997, J. Milton Harris and S. Zalipsky (eds), American Chemical Society, Washington DC and "Bioconjugation Protein Coupling Techniques for the Biomedical Sciences", 1998, M. Aslam and A. Dent, Grove Publishers, New York; Chapman, A. 2002, Advanced Drug Delivery Reviews 2002, 54:531-545]. In one example PEG is attached to a cysteine in the hinge region. In one example, a PEG modified Fab fragment has a maleimide group covalently linked to a single thiol group in a modified hinge region. A lysine residue may be covalently linked to the maleimide group and to each of the amine groups on the lysine residue may be attached a methoxypoly(ethyleneglycol) polymer having a molecular weight of approximately 20,000Da. The total molecular weight of the PEG attached to the Fab fragment may therefore be approximately 40,000Da.

Particular PEG molecules include 2-[3-(N-maleimido)propionamido]ethyl amide of N,N'-bis(methoxypoly(ethylene glycol) MW 20,000) modified lysine, also known as PEG2MAL40K (obtainable from Nektar, formerly Shearwater).

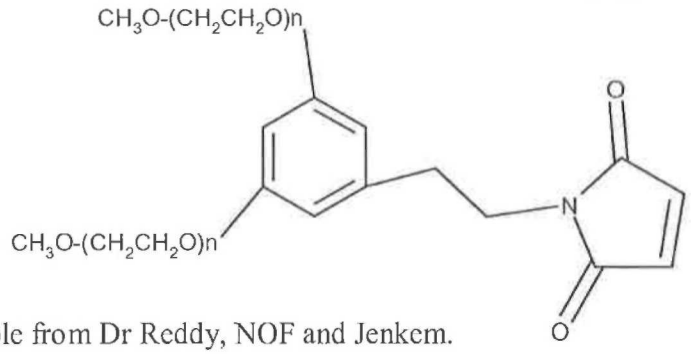
Alternative sources of PEG linkers include NOF who supply GL2-400MA2 (wherein m in the structure below is 5) and GL2-400MA (where m is 2) and n is approximately 450:



m is 2 or 5

That is to say each PEG is about 20,000Da.

Further alternative PEG effector molecules of the following type:



are available from Dr Reddy, NOF and Jenkem.

In one embodiment there is provided an antibody which is PEGylated (for example with a PEG described herein), attached through a cysteine amino acid residue at or about amino acid 226 in the chain, for example amino acid 226 of the heavy chain (by sequential numbering).

In one embodiment one certain antibodies according to the present disclosure have the following properties:

Antibody	Affinity (pM)		Valency of binding	EC_{50} (ng/ml)
	$TcdA_{123}$	$TcdA_{456}$	TcdA, est.	
CA922	4.06	2.59	16	1.21
CA923	64.7	312	12	160.42
CA995	nil	119	1	37.64
CA997	132	66.8	12	6.25
CA1000	73.3	84.1	2	19.73

The present invention also provides compositions such as a pharmaceutical composition of antibody or combination of antibodies defined herein.

The present invention also provides a composition that comprises at least two antibodies according to the invention, for example wherein at least one antibody therein is specific to TcdA and at least one antibody therein is specific to TcdB or alternatively at least two antibodies specific to TcdA or at least two antibodies specific to TcdB.

In one embodiment there is provided a composition that comprises multiple antibodies specific to TcdA and optionally one or more antibodies specific to TcdB.

In one embodiment there is provided a composition that comprises multiple antibodies specific to TcdB and optionally one or more antibodies specific to TcdA.

Thus in one embodiment there is provided a composition comprising 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 antibodies according to the invention i.e. distinct antibodies.

The invention describes one particular mixture comprising 3 Mabs, one Mab of which is specific for TcdA and two Mabs of which are specific for TcdB. This mixture demonstrated very high levels of protection from death and gut inflammation from a lethal infective oral dose of *Clostridium difficile* in hamsters.

In particular there is provided a composition comprising a combination of one anti-TcdA antibody comprising a heavy variable region with a sequence as shown in SEQ ID NO:49 and a light variable region with a sequence shown in SEQ ID NO: 47 and two anti-

TcdB the first with a heavy variable region shown in SEQ ID NO: 129 and a light variable region shown in SEQ ID NO: 127, and the second with a heavy variable region shown in SEQ ID NO: 159 and light variable region shown in SEQ ID NO: 157.

In one embodiment wherein the composition comprises 3 antibodies, such as one anti-TcdA and two anti-TcdB antibodies the antibodies are in the ratio of 50%, 25% and 25% respectively of the total antibody content thereof.

In one embodiment there is provided a composition comprising 2, 3, 4 or 5 antibodies specific to TcdA and optionally 1, 2, 3, 4 or 5 antibodies specific to TcdB.

In one embodiment the compositions provided according to the invention are well defined, for example are mixtures of monoclonal antibodies rather than simply polyclonal compositions derived from an immunised or immune competent host.

In one embodiment the composition of antibodies has an EC_{50} of 200ng/ml or less, for example 150ng/ml or less, such as 100ng/ml or less, such as 0.1 to 10ng/ml.

Advantageously the antibodies described herein have very high levels of biophysical stability and so are suitable for inclusion in mixtures of antibodies.

In one aspect a pharmaceutical formulation or composition according to the invention further comprises a pharmaceutically acceptable excipient.

Pharmaceutically acceptable carriers in therapeutic compositions may additionally contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents or pH buffering substances, may be present in such compositions. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries and suspensions, for ingestion by the patient.

Suitable forms for administration include forms suitable for parenteral administration, e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the product is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents, such as suspending, preservative, stabilising and/or dispersing agents. Alternatively, the antibody molecule may be in dry form, for reconstitution before use with an appropriate sterile liquid.

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals. However, in one or more embodiments the compositions are adapted for administration to human subjects.

Suitably in formulations according to the present disclosure, the pH of the final formulation is not similar to the value of the isoelectric point of the antibody or fragment, for example if the pH of the formulation is 7 then a pI of from 8-9 or above may be appropriate.

Whilst not wishing to be bound by theory it is thought that this may ultimately provide a final formulation with improved stability, for example the antibody or fragment remains in solution.

In one embodiment the composition or formulation of the present disclosure comprises 1-200mg/mL of antibodies, that this to say the combined antibody content, for example 150mg/mL or less, such as 100mg/mL or less, in particular 90, 80, 70 , 60, 50, 40, 30, 20, 10mg/mL or less.

In one embodiment a composition or formulation according to the present disclosure comprises 20mg/mL of each antibody therein.

In one embodiment there is provided a formulation comprising:

33mg/mL or less of one anti-TcdA antibody comprising a heavy variable region with a sequence as shown in SEQ ID NO: 49 and a light variable region with a sequence shown in SEQ ID NO: 47, and

28mg/mL or less of a first anti-TcdB with a heavy variable region shown in SEQ ID NO: 129 and a light variable region shown in SEQ ID NO: 127, and

25mg/mL of a second anti-TcdB with a heavy variable region shown in SEQ ID NO: 159 and light variable region shown in SEQ ID NO: 157.

In one embodiment the pharmaceutical formulation at a pH in the range of 4.0 to 7.0 comprises: 1 to 200mg/mL of an antibody according to the present disclosure, 1 to 100mM of a buffer, 0.001 to 1% of a surfactant,

a) 10 to 500mM of a stabiliser,

b) 5 to 500 mM of a tonicity agent , or

c) 10 to 500mM of a stabiliser and 5 to 500 mM of a tonicity agent.

In one embodiment the composition or formulation according to the present disclosure comprises the buffer phosphate buffered saline.

For example the formulation at approximately pH6 may comprise 1 to 50mg/mL of antibody, 20mM L-histidine HCl, 240 mM trehalose and 0.02% polysorbate 20. Alternatively a formulation at approximately pH 5.5 may comprise 1 to 50mg/mL of antibody, 20mM citrate buffer, 240mM sucrose, 20mM arginine, and 0.02% polysorbate 20.

The pharmaceutical compositions of this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, transcutaneous (for example, see WO98/20734), subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, intravaginal or rectal routes. Hyposprays may also be used to administer the pharmaceutical compositions of the invention. Typically, the therapeutic compositions may be prepared as

injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared.

Direct delivery of the compositions will generally be accomplished by injection, subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue.

The compositions can also be administered into a lesion or directly into the gastrointestinal tract by for examples, encapsulated oral dosage for swallowing, through a nasogastric tube to the stomach or ileum, through a rectal tube or enema solutions or by rectal capsule. Dosage treatment may be a single dose schedule or a multiple dose schedule.

It will be appreciated that the active ingredient in the composition will be an antibody molecule. As such, it will be susceptible to degradation in the gastrointestinal tract. Thus, if the composition is to be administered by a route using the gastrointestinal tract, the composition will need to contain agents which protect the antibody from degradation but which release the antibody once it has been absorbed from the gastrointestinal tract.

A thorough discussion of pharmaceutically acceptable carriers is available in Remington's Pharmaceutical Sciences (Mack Publishing Company, N.J. 1991).

The present invention also provides an antibody or antibody combination or a composition comprising the same, as described herein, for treatment, for example for the treatment or prophylaxis of *C. difficile* infection or complications associated with the same such as diarrhoea, colitis in particular pseudomembranous colitis, bloating, abdominal pain and toxic megacolon.

Prophylaxis can also be achieved by the administration of pre-formed complexes of inactivated toxin antigen (toxoid) and antibody in order to create a vaccine.

In one embodiment the antibodies, combinations thereof and compositions comprising the same according to the invention are suitable for treating infection with so-called super strains of *C. difficile*, i.e. hypervirulent strains such as ribotype 027.

The antibodies and compositions according to the present invention are suitable for use in the treatment or prophylaxis of acute and/or chronic effects of the relevant *C. difficile* toxins during primary infection.

The antibodies and compositions according to the present invention are suitable for use in the treatment or prophylaxis of effects of the relevant *C. difficile* toxins during secondary infection or re-infection. International guidelines enshrine time intervals after a primary infection which hence defines a secondary (or recurrent) infection as being distinct from a continuation of existing symptoms sometimes described as a relapse (29). Research has shown that secondary infections can be the result of the same strain or ribotype as the primary

infection. In such cases recurrence rather than relapse relies on agreed temporal constraints. However, research also clearly shows that secondary infection can also be the result of infection of a strain or ribotype distinct from that of the primary infection. In one study, 48% of disease recurrences were the result of a second strain distinct from that having caused the first infection (30). In another study, more than 56% of disease recurrences were the result of a second strain distinct from that having caused the first infection (31).

In one embodiment the antibodies, combinations thereof and compositions comprising the same according to the invention are suitable for use in the prevention of damage, for example long term structural damage to the epithelium of the colon.

In one embodiment the antibodies, combinations and composition are suitable for preventing *C. difficile* infection including recurrence of infection, in particular nosocomial infection.

In one embodiment the antibodies, combinations thereof and compositions comprising the same according to the invention are suitable for reducing the risk of recurrence of *C. difficile* infection.

Advantageously, the antibodies of the present disclosure can be administered prophylactically to prevent infection or re-infection because in the absence of toxin to which the antibody is specific the antibody is simply to be cleared from the body without causing adverse interactions with the subjects body tissues.

Advantageously the antibodies of the present disclosure seem to elicit a rapid response after administration, for example within one or two days of administration rapid clearance of the target toxin is invoked, this may prevent vital organs such as the lungs, heart and kidneys being damaged. This is the first time that agents have been made available with can be employed to prevent damage or injury to a patient by toxins A and/or B in the acute *C. difficile* infection stage.

Thus in one embodiment the antibodies, combinations thereof and compositions comprising the same according to the invention are suitable for preventing damage to vital organs.

In one embodiment the antibody, combinations or formulations described herein are suitable for preventing death of an infected patient, if administered within an appropriate time frame before irreparable damage has been done by the toxins.

The antibodies of the present disclosure have fast on-rates, which facilitates the rapid *effect in vivo*.

In one embodiment the patient population is over 60, such as over 65 years of age.

In one embodiment the patient population is 5 years old or less.

The antibodies according the invention may be employed in combination with antibiotic treatment for example metronidazole, vancomycin or fidaxomicin.

A range of *in vitro* data exemplify the properties of the Mabs and Mab mixtures. We show that one mixture of 3 Mabs (50% molar quantities of anti-TcdA and 50% molar quantities of anti-TcdB components) was able to protect hamsters from a lethal CDI.

In one embodiment there is provided a method of treating a patient in need thereof by administering a therapeutically effective amount of an antibody as described herein or antibody combination or a composition comprising the same, for example in the treatment or prophylaxis of *C. difficile* infection or complications associated with the same such as diarrhoea, colitis in particular pseudomembranous colitis, bloating, abdominal pain and toxic megacolon.

In one embodiment the antibody, combination or formulation is administered by a parenteral route, for example subcutaneously, intraperitoneally, intravenously or intramuscularly. The data in the Examples generated in hamsters indicates that the doses administered by this route reach the gut and thus are able to generate a therapeutic effect.

In one embodiment the antibody, combination or formulation is administered orally, for example an enterically coated formulation.

In one embodiment there is provided use of an antibody, combination or formulation as described herein for the manufacture of a medicament for the treatment or prophylaxis of *C. difficile* infection.

In one embodiment the dose administered is in the range 1 to 1000mg/Kg, for example 10 to 75mg/Kg, such 20 to 50mg/Kg.

In one embodiment the half-life of the antibody or antibodies in mice and hamsters *in vivo* is in the range 6 to 8 days in healthy (uninfected) animals and hence are expected to have half-lives in humans in the range of 14-28 days.

In one embodiment the antibody or antibodies are given as one dose only.

In one embodiment the antibody or antibodies are given as a weekly or biweekly dose.

In one embodiment the antibody or antibodies are given as once daily doses.

In one embodiment there is provided complex comprising TcdA or an immunogenic fragment thereof, complexed with one or more anti-TcdA antibodies defined herein. The complex may be employed as the antigen in a vaccine formulation, for example suitable for generating protective antibodies to toxin A in vivo after administration to a human.

In one embodiment there is provided complex comprising TcdB or an immunogenic fragment thereof, complexed with one or more anti-TcdB antibodies defined herein. The

complex may be employed as the antigen in a vaccine formulation, for example suitable for generating protective antibodies to toxin B in vivo after administration to a human.

Th1-type immunostimulants which may be formulated to produce adjuvants suitable for use in the present invention include and are not restricted to the following.

In one embodiment there is provided a complex comprising TcdA or an immunogenic fragment thereof and TcdB or an immunogenic fragment thereof, wherein each toxin or fragment is complexed with one or more antibodies specific thereto, wherein the complex is suitable for administration as a vaccine formulation.

Antibody:antigen complexes are known to be taken up by the immune system in an Fc receptor mediated process (27, 28) and pre-formed complexes of antibody:antigen complexes have been successfully use as vaccines in human clinical trials (22).

In one or more embodiments the vaccine formulation further comprises an adjuvant as an immunostimulant.

Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL), is a preferred Th1-type immunostimulant for use in the invention. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers *et al.*, 1986, *Int.Arch.Allergy.Immunol.*, 79(4):392-6; Hilgers *et al.*, 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1). A preferred form of 3D-MPL is in the form of a particulate formulation having a small particle size less than 0.2mm in diameter, and its method of manufacture is disclosed in EP 0 689 454.

Saponins are also preferred Th1 immunostimulants in accordance with the invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. J.

Immunology vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711. One such system is known as an Iscorn and may contain one or more saponins.

Another preferred immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al., J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon g and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have also now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ; Brazolot-Millan *et al., Proc.Natl.Acad.Sci.*, USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and or metallic salts, including

aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287); CpG may be formulated with alum (Davis *et al. supra* ; Brazolot-Millan *supra*) or with other cationic carriers.

Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention. Alternatively the saponin may be formulated in a liposome or in an Iscorn and combined with an immunostimulatory oligonucleotide.

Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt.

Thus is one embodiment the adjuvant is a combination of QS21 and 3D-MPL in an oil in water or liposomal formulation.

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739. This combination may additionally comprise an immunostimulatory oligonucleotide.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred formulation for use in the invention.

Another preferred formulation comprises a CpG oligonucleotide alone or together with an aluminium salt.

In a further aspect of the present invention there is provided a method of manufacture of a vaccine formulation as herein described, wherein the method comprises admixing a polypeptide according to the invention with a suitable adjuvant.

Particularly suitable adjuvant combinations for use in the formulations according to the invention are as follows:

i) 3D-MPL + QS21 in a liposome

ii) Alum + 3D-MPL

iii) Alum + QS21 in a liposome + 3D-MPL

iv) Alum + CpG

v) 3D-MPL + QS21 + oil in water emulsion

vi) CpG

As used herein, the term "comprising" in context of the present specification should be interpreted as "including".

Embodiments and preferences may be combined as technically appropriate.

The disclosure herein describes embodiments comprising certain integers. The disclosure also extends to the same embodiments consisting or consisting essentially of said integers.

FIGURES

Fig 1-10 shows various antibody and fragment sequences

Fig 11 shows sera titres for TcdA and TcdB

Fig 12 shows anti TcdA (Ribotype 003) in-vitro neutralization data for single Mabs

Fig 13 shows anti TcdA (Ribotype 003) in-vitro neutralization data for single and paired Mabs

Fig 14-15 shows anti TcdA (Ribotype 003) in-vitro neutralization data for paired Mabs

Fig 16-18 shows anti TcdA (Ribotype 003) in-vitro neutralization data for three Mab mixtures

Fig 19-20 shows anti TcdA (Ribotype 003) in-vitro neutralization data for four and five Mab mixtures

Fig 21-22 shows anti TcdA (Ribotype 003) in-vitro neutralization data for single and paired Mabs at different TcdA concentrations

Fig 23-24 shows anti TcdA (Ribotype 003) in-vitro neutralization data for single and to five Mab mixtures at different TcdA concentrations

Fig 25-26 shows anti TcdB (Ribotype 003) in-vitro neutralization data for single Mabs

Fig 27-30 shows anti TcdB (Ribotype 003) in-vitro neutralization data for paired Mabs

Fig 31-33 shows anti TcdB (Ribotype 003) in-vitro neutralization data for three Mab mixtures

Fig 34-40 shows anti TcdB (Ribotype 003) in-vitro neutralization data for two Mab mixtures at different toxin concentrations

Fig 41-45 shows anti TcdB (Ribotype 003) in-vitro neutralization data for two Mab mixtures at different relative Mab ratios and different toxin concentrations

Fig 46-59 shows TcdB neutralisation data for single antibodies and pairs of antibodies

Fig 60 shows the amino acid sequence for TcdA

Fig 61 shows the amino acid sequence for TcdB

Fig 62 shows TEER assay data for TcdA in a histogram format

Fig 62A shows TEER assay data for TcdA in line graph format

Fig 63 shows a meier-kaplan curve for the combination of antibodies 997, 1125 and 1151, high concentration is 50mg/Kg and low concentration is 5mg/Kg 50mg/kg' dose gave 100% protection to day 11, ~82% protection to day 28. 5mg/kg' dose resulted in non-durable and incomplete protection.

Fig 64 shows bodyweight changes for vancomycin and vehicle treated hamsters

Fig 65 shows the bodyweight for low dose antibodies 5mg/Kg and high dose antibodies 50mg/Kg

Fig 66 shows photographs of a colon where the animal received treatment with antibodies according to the present disclosure vs a control

Fig 67-68 show effects of vortexing on antibody stability

Fig 69 shows a comparison of aggregation stability for various antibodies

Fig 70-73 show neutralisation of TcdA for various ribotypes

EXAMPLES

Antibody Generation

A range of different immunogens and screening reagents were either purchased or produced by conventional *E. coli* expression techniques in order to provide a diverse and broad immune response and to facilitate identification and characterisation of monoclonal antibodies (listed in Table 1). In cases where recombinant proteins or peptides were generated, sequences were based on ribotype 027. The sequence for TcdA from ribotype 027 is given in SEQ ID NO: 171 (Uniprot accession number C9YJ37) and the sequence for TcdB from ribotype 027 is given is SEQ ID NO: 172 (Uniprot accession number C9YJ35).

Sprague Dawley rats and half-lop rabbits were immunised with either synthetic peptides mapping to regions common to both TcdA and TdcdB full-length toxin, formaldehyde-inactivated toxoid A, binding domain fragments of Toxin A (CROPs1,2,3 or CROPs4,5,6) or binding domain fragment of Toxin B (CROPs1,2,3,4), or in some cases, a combination of the above. Following 2 to 6 immunisations, animals were sacrificed and PBMC, spleen and bone marrow harvested. Sera were monitored for binding to Toxin A domains, toxin B domains, toxin or toxoid by ELISA. Sera titres of 2 such immunisations are shown in figure 11.

UCB SLAM was used as a means to generate monoclonal antibodies. B cells were cultured directly from immunised animals (Zubler et al., 1985). This step enabled sampling of a large percentage of the B cell repertoire. By incorporating the selected lymphocyte antibody method (SLAM) (Babcook et al., 1996) it was possible to deconvolute positive culture wells and

identify antigen-specific antibody-secreting cells. Here we used a modified version of SLAM (UCB SLAM (Tickle et al. 2009)) that utilises a fluorescence-based method to identify antigen-specific B cells from culture wells. B cell cultures were set up and supernatants were first screened for their ability to bind a relevant purified toxin domain (binding, translocation or catalytic) in a bead-based assay using an Applied Biosystem 8200 cellular detection system. This was a homogeneous assay using B cell culture supernatant containing IgG, biotinylated toxin domains coated onto streptavidin beads and a goat anti-rat/rabbit Fc-Cy5 conjugate. Cell cultures positive for binding to TcdA or TcdB components from this assay were selected for use in cell-based functional assays to identify neutralisers of toxin-induced cytotoxicity. Approximately 12,000 toxin-specific positives were identified in the primary binding screen from a total of 40 x 50-plate experiments. This equated to the screening of approximately 0.5 billion B cells. Heavy and light variable region gene pairs were isolated from single cells harvested by micromanipulation from approximately 100 toxin-neutralising wells following reverse transcription (RT)-PCR. These V-region genes were then cloned as mouse IgG1/kappa full-length antibodies for rat variable regions and rabbit IgG/kappa full-length antibodies for rabbit variable regions. Antibodies were re-expressed in a HEK-293 transient expression system. These recombinant antibodies were then retested for their ability to neutralise toxin in cell based assays. Recombinant antibodies were also screened by BIAcore to determine affinity for a given toxin domain and to also determine the specificity and approximate the number of binding events of antibody to toxin. Based on in vitro activity in cell based assays and affinity measurements, lead candidates were selected for humanisation. Unless otherwise stated, all the data herein was generated using the humanised antibodies.

A panel of recombinant, *E. coli*-produced toxin fragments (TcdA), *C. difficile*-derived toxin or toxoid (A) and synthetic peptides (B) were generated or purchased from commercial sources.

Table 1. Toxin A (TcdA) sequence related reagents for screening and immunizations.

Fragment	Residue number	Source
TcdA catalytic	M1-E659	UCB E. coli expression
TcdA translocation	K577-D1350	UCB E. coli expression
TcdA $CROPS_{123}$ (TcdA123)	S1827-D2249	UCB E. coli expression
TcdA $CROPS_{456}$ (TcdA456)	G2205-R2608	UCB E. coli expression
TcdA $CROP_1$	S1827-N1978	UCB E. coli expression
TcdA $CROP_2$	G1966-N2133	UCB E. coli expression
TcdA $CROP_3$	G2100-D2249	UCB E. coli expression
TcdA $CROP_4$	G2213-N2381	UCB E. coli expression

TcdA $CROP_5$	G2328-N2494	UCB E. coli expression
TcdA $CROP_6$	G2462-N2609	UCB E. coli expression
TcdA $CROP_7$	R2554-D2701	UCB E. coli expression
TcdB catalytic	M1-A593	UCB E. coli expression
TcdB translocation	R576-D1349	UCB E. coli expression
TcdB binding (TcdB1234)	S1833-E2366	UCB E. coli expression
TcdB $CROP_1$	S1833-S1981	UCB E. coli expression
TcdB $CROP_2$	G1968-D2113	UCB E. coli expression
TcdB $CROP_3$	G2100-E2247	UCB E. coli expression
TcdB $CROP_4$	G2234-E2366	UCB E. coli expression
Toxin A	Full length	purchased
Toxin B	Full length	purchased
Toxoid A	Full length	purchased

Table 2. Toxin B (TcdB) sequence related reagents for screening and immunizations.

Toxin Domain	**Amino acid Sequence**	
Catalytic	SPVEKNLHFVWIGGEVSD	SEQ ID NO: 173
Catalytic	NLAAASDIVRL	SEQ ID NO: 174
Catalytic	CGGVYLDVDMLPGIH	SEQ ID NO: 175
Catalytic	CGGVYLDVDMLPGIHSDLFK	SEQ ID NO: 176
Catalytic	CWEMIKLEAIMKYK	SEQ ID NO: 177
Catalytic	CTNLVIEQVKNR	SEQ ID NO: 178
Catalytic	PEARSTISLSGP	SEQ ID NO: 179
Catalytic	CSNLIVKQIENR	SEQ ID NO: 180
Catalytic	TEQEINSLWSFDQA	SEQ ID NO: 181
Catalytic	TEQEINSLWSFDPEARSTISLSGPC	SEQ ID NO: 182
Translocation	NVEETYPGKLLLC	SEQ ID NO: 183
Translocation	Acetyl-CANQYEVRINSEGR	SEQ ID NO: 184
Translocation	VNTLNAAFFIQSLIC	SEQ ID NO: 185
Translocation	YAQLFSTGLNTIC	SEQ ID NO: 186
Translocation	CAGISAGIPSLVNNEL	SEQ ID NO: 187
Translocation	DDLVISEIDFNNNSIC	SEQ ID NO: 188
Translocation	MEGGSGHTVT	SEQ ID NO: 189
Translocation	AVNDTINVLPTITEGIPIVSTILDGINLGAAIKEL	

	SEQ ID NO: 190	
Binding	CGFEYFAPANTDANNIEGQA	SEQ ID NO: 191
Binding	CGYKYFAPANTVNDNIYGQA	SEQ ID NO: 192
Binding	CKYYFNTNTAEA	SEQ ID NO: 193
Binding	CKYYFDEDTAEA	SEQ ID NO: 194

Expression and purification of *C. difficile* anti-toxin Mabs

Separate light chain and heavy chain mammalian expression plasmids were combined in equimolar ratios and used to transfect HEK-293 or CHO-S cells. For small scale expression studies lipofectamine and HEK-293 cells were used whereas for production of larger batches of IgG electroporation into CHO-S was preferred.

Culture supernatants were loaded onto a MabSelect SuRe column (in PBS pH 7.4). Antibody was eluted with 100% 0.1M Sodium Citrate pH 3.4 buffer. Samples were neutralized to pH7.4 with Tris.Cl pH8.0. Aggregate was removed by Superdex 200 Gel Filtration column in PBS pH 7.4.

TABLE 3

Antibody	Cell type	Volume of SN (L)	Expression type	Amount purified (mg)
CA164_00997.g1_P3	CHO	10	Transient	755.93
CA164_00922.g1_P3	CHO	0.5	Transient	129.36
CA164_01125.g2_P3	CHO	10	Transient	498.96
CA164_01151.g4_P3	CHO	5	Transient	262.43

Example 1 In-vitro neutralization of TcdA activity by purified Mabs

All neutralisation screening assays were run in 96 well polystyrene plates. The assay uses CACO-2 cells grown, and screened in MEM + 20% FCS, 2mM Q, and NEAA. Any antibody combinations are at equal molar ratios unless stated otherwise. **Day 1:** Cells were plated @ 3000 per well in 50 µl media, and incubated for 24 hrs; **Day 2:** Purified samples of humanised Mab were added to 96 well round bottomed polypropylene sterile plates; Spike PP plates with toxin A at a concentration sufficient to generated the appropriate lethal dose i.e. LD_{80} or above and incubate for 1 hr, at 37oC; Add 50 µl of this mixture to cell plates and incubate for 96 hrs; **Day 5:** Add Methylene blue (0.5% Methylene Blue 50% ethanol); Incubate for 1 hr at room temperature; Lyse the cells with 1% N-Lauryl Sarcosine, and Read on the BIOTEK Synergy2 plate reader at 405nm. The results are shown in Fig 12 to 24. EC_{50} and % maximum neutralization of TcdA activity shown confirm that the selected antibodies have very high potencies as single agents. Combinations of 2 to 5 of these did not improve upon the best EC_{50}

or % maximum neutralization. Lack of any synergy when combining Mabs CA922, 923, 995, 997 and 1000 is an important observation and may be due to the fact the each antibody alone has exceptionally high levels of affinity and potency. Supporting data in Example 5 also show that some of the Mabs (e.g. CA997) are capable of binding to TcdA subdomains many times. Hence it seems probable that these 5 Mabs represent that the maximum affinity, potency and valency that is achievable when targeting the C-terminal cell binding domain of TcdA. The antibodies were also effective at neutralising very high toxin concentrations ranging from LD80 to greater than LD_{95} (LD_{max}) but some modest increases in EC_{50} (i.e. decreases in potency) were observed with very high levels of [TcdA]. These data are also surprising since others have shown substantial reductions in potency when testing elevated TcdA concentrations (20).

The high potency and affinity of the Mabs described here, e.g. for CA997; is not due solely to their high valency of binding. Others (20 and WO06/071877) describe anti-TcdA Mabs capable of binding up to 14 times. These Mabs only had affinities in the range 0.3 to 100nM and showed incomplete protection against TcdA mediated cell killing, alone (26-63% protection) or in pairs (31-73% protection). Hence it has been demon-strated that high valency of binding to TcdA does not necessarily invoke either high affinity of binding to or neutralisation of TcdA. Neither the affinities nor valency of binding to TcdA were described for Mab CDA-1 (18 and US7625559). Thus Mabs described herein to have surprising affinity, potency and valency.

TABLE 4 Anti TcdA 1, 2 & 3 Mab combinations at a single TcdA conc. (LD_{80})

Antibody	Final (highest) Mab conc.ng/ml	EC_{50}(ng/ml)
922	500	1.21
923	500	160.42
995	500	37.64
997	500	6.25
1000	500	19.73
922+923	500	3.58
922+925	500	3.326
922+997	500	2.88
922+1000	500	2.64
923+995	500	60.23
923+997	500	7.54
923+1000	500	9.24
995+997	500	7.29
995+1000	500	19.63
997+1000	500	4.46
922+923+995	500	4.72
922+923+997	500	3.23
922+923+1000	500	3.21
922+995+997	500	2.22
922+995+1000	500	2.85
922+997+1000	500	2.22
923+995+997	500	5.04
923+995+1000	500	9.49
995+997+1000	500	5.84
922+923+995+997	500	2.75
922+923+995+1000	500	3.75
922+995+997+1000	500	3.46
923+995+997+1000	500	4.81
922+923+997+1000	500	3.06
922+923+995+997+1000	500	4.72

TABLE 5 Anti TcdA single, paired, and triplet Mab combinations at various TcdA concentrations, where TcdA is at its LD_{80}, LD_{90}, LD_{95} and LD_{max}.

Toxin TcdA	Sample	Final Mab conc.ng/ml	EC_{50} (ng/ml)
@ 3000 pg/ml (LD_{MAX})	922	500	4.89
	997	500	10.99
	1000	500	50.17
	922+997	500	7.18
	922+1000	500	6.99
	997+1000	500	9.437
	922+997+1000	500	10.80
	922+997+1000+995	500	15.03
	922+997+1000+995+923	500	7.16
@ 1000 pg/ml (LD_{95})	922	500	1.24
	997	500	3.42
	1000	500	9.60
	922+997	500	1.85
	922+1000	500	2.51
	997+1000	500	3.61
	922+997+1000	500	2.40
	922+997+1000+995	500	2.74
	922+997+1000+995+923	500	2.38
@ 700 pg/ml (LD_{90})	922	500	0.84
	997	500	2.40
	1000	500	6.23
	922+997	500	1.19
	922+1000	500	1.33
	997+1000	500	2.68
	922+997+1000	500	1.84
	922+997+1000+995	500	2.17
	922+997+1000+995+923	500	2.06
@ 350 pg/ml (LD_{80})	922	500	0.39
	997	500	1.18
	1000	500	2.76
	922+997	500	0.67
	922+1000	500	0.85
	997+1000	500	2.06
	922+997+1000	500	0.83
	922+997+1000+995	500	0.97
	922+997+1000+995+923	500	0.98

Example 2 Anti TcdB *in-vitro* neutralization by purified Mab.

Assay methods description:

All neutralisation screening assays were run in 96 well polystyrene plates.

The assay uses CACO-2 cells grown, and screened in MEM + 20% FCS, 2mM Q, and NEAA. Unless stated all Ab combinations are in equal ratios.

- Day 1: Cells are plated @ 3000 per well in 50 µl media, and incubated for 24 hrs
- Day 2: Purified samples of humanised Mab were added to 96 well round bottomed polypropylene sterile plates
- Spike PP plates with toxin B lot # 031 and incubate for 1 hr, at 37oC
- Add 50 µl of this mixture to cell plates
- Incubate for 96 hrs
- Day 5: Add Methylene blue (0.5%Methylene Blue 50% ethanol)
- Incubate for 1 hr at room temperature
- Lyse the cells with 1% N-Lauryl Sarcosine
- Read on the BIOTEK Synergy2 plate reader at 405nm

The data in Figures 25 to 33 show that single Mabs alone were relatively ineffective at neutralizing TcdB, both in terms of % maximum neutralization and activity (EC_{50}). However, when the antibodies were combined in two's and three's considerable improvements in both % maximum neutralization and activity (EC_{50}) were observed. 1125 and 1151 were selected as a best pairing, although other good pairings were observed: 1125+1153, 1125+1134.

The most effective pairs of Mabs were selected empirically and were found retrospectively to make unexpected and surprising combinations when regarding the individual potencies of each Mab. For example, in Table 6 only CA927 had a TcdB neutralisation potential which could result in a defined EC_{50} whilst the TcdB neutralisation potential of both CA1125 and CA1151 were insufficient under these assay conditions to result in a defined EC_{50}. However, CA927 was not found to be the most effective Mab to use within a combination. The best CA927 containing combination had an EC_{50} of 13.5ng/ml whereas other two Mab combinations had EC_{50}'s as low as 2.59 and 4.71ng/ml. In another example, in Table 8 CA1099 had the lowest TcdB neutralisation EC_{50} under the assay conditions used. However, CA1099 was not found to be the most effective Mab to use within a combination. The best CA1099 containing combination had an EC_{50} of 6ng/ml whereas other two Mab combinations had EC_{50}'s as low as 2 and 1ng/ml. We might speculate that the most effective pairings of Mabs are defined by their cooperative binding modalities especially as defined by having non-overlapping epitopes.

TABLE 6 Anti-TcdB Mab combinations and relative Mab ratios at constant toxin concentration.

Sample	Final Mab conc.ng/ml	EC_{50}(ng/ml)
1125.g2	1000	>1000
1134.g5	1000	>1000
927.g2	1000	12.89
1153.g8	1000	>1000
1102.g4	1000	>1000
927+1099	1000	>1000
927+1102	1000	>1000
927+1114	1000	>111.111
927+1125	1000	13.55
927+1134	1000	51.58
1099+1114	1000	>1000
1102+1114	1000	>333.333
1102+1125	1000	15.51
1114+1134	1000	19.70
1114+1151	1000	25.69
1114+1153	1000	27.48
1125+1134	1000	2.59
1125+1151	1000	4.71
1125+1153	1000	21.23
1125+1134+1114	1000	3.77
1125+1134+927	1000	2.63
1125+1151+1114	1000	4.90
1125+1151+927	1000	5.69
1125.g2+1134.g5+927.g2	1000	5.83
1125.g2+1134.g5+1153.g8	1000	9.89
1125.g2+1134.g5+1102.g4	1000	2.72

Example 3 Neutralisation of TcdB by combinations of purified Mab.

All neutralisation screening assays were run in 96 well polystyrene plates.

The assay uses CACO-2 cells grown, and screened in MEM + 20% FCS, 2mM Q, and NEAA.

- Day 1: Cells are plated @ 3000 per well in 50 µl media, and incubated for 24 hrs
- Day 2: Purified samples of humanised Mab were added to 96 well round bottomed polypropylene sterile plates
- Spike PP plates with toxin B (VPI 10463) and incubate for 1 hr, at 37oC
- Add 50 µl of this mixture to cell plates
- Incubate for 72 hrs
- Day 5: Add Methylene blue (0.5%Methylene Blue 50% ETOH)
- Incubate for 1 hr at room temperature
- Lyse the cells with 1% N-Lauryl Sarcosine
- Read on the BIOTEK Synergy2 plate reader at 405nm

The results are shown in Figures 34 to 45.

These data show that the best pair of Mabs for neutralizing TcdB at a range of toxin concentrations was CA1125 and CA1151. Moreover, the 1125+1151 combination was largely unaffected by changes in the relative molar ratios which is in contrast to 1125+1153.

TABLE 7 Anti-TcdB Mab combinations and relative Mab ratios at 3 different toxin concs.

	EC50 values (ng/ml)		
Antibody combination	**TcdB LD60**	**TcdB LD77**	**TcdB LD85**
1125.g2 + 927.g2 (50:50)	2.8	6	11.3
1125.g2 + 1102.g4 (50:50)	4	13	44
1125.g2 + 1114.g8 (50:50)	3.5	7.1	25.4
1125.g2 + 1134.g5 (50:50)	0.48	1.4	4
1125.g2 + 1151.g4 (50:50)	0.85	0.85	1.5
1125.g2 + 1153.g8 (50:50)	2.7	5.2	25.2
1125.g2 + 1134.g5 (25:75)	<0.15	0.84	7.2
1125.g2 + 1151.g4 (25:75)	0.73	1	2.1
1125.g2 + 1153.g8 (25:75)	7	10	27
1125.g2 + 1134.g5 (75:25)	0.66	1.2	2.5
1125.g2 + 1151.g4 (75:25)	1.4	1.2	8.3
1125.g2 + 1153.g8 (75:25)	2.9	7.5	30

The data show that even the most active specific paired combinations have surprisingly and non-predictably different properties relative to each other. The EC_{50} of the preferred combination of CA1125 and CA1151 in equimolar ratios is largely unaffected by an increasing [TcdB]. The three

relative molar ratios of Mabs tested (i.e. 25:75 vs 50:50 vs 75:25) have very similar EC_{50}'s to each other, suggesting that CA1125 and CA1151 have especially complementary modes of action. This is in contrast to the combination of CA1125 with CA1134 where the increase in EC_{50} (i.e. reduction of potency) with higher [TcdB] is more substantial and where the three Mab molar ratios are not equally effective: The CA1125:CA1134 ratio of 25:75 is notably less potent than 50:50 and 75:25. This suggests that the combined potency of CA1125+CA1134 is more dependent upon the CA1125 component. The EC_{50} of all three molar combinations of CA1125 and CA1153 is substantially affected by increasing [TcdB] suggesting that CA1153 is a less suitable partner for combination with CA1125. *In toto*, these data show that CA1125 and CA1151 are a particularly favourable combination since the highest potency is maintained across a range of Mab and TcdB molar ratios.

TABLE 8 TcdB neutralisation – 1 or 2 anti-TcdB Mabs at constant toxin dose (LD_{80}).

Antibody	IC50 (ng/ml)
1099	2
1102	N/A
1114	103
1125	N/A
1134	8
1151	182
1153	260
926	N/A
927	N/A
1099 + 1125	6
1114 + 1125	7
1151 + 1125	2
1134 + 1125	1
1102 + 1125	6
1125 + 1153	12
926 + 1125	42
927 + 1125	4

TABLE 9 TcdB neutralisation – 1 or 2 anti-TcdB Mabs at various TcdB doses.

	EC50 values (ng/ml)			Maximum neutralisation		
Antibody combination	TcdB LD75	TcdB LD86	TcdB LD90	TcdB LD75	TcdB LD86	TcdB LD90
1125.g2	n/a	n/a	n/a	40%	25%	15%
1114.g8	n/a	n/a	n/a	45%	25%	15%
1134.g5	n/a	n/a	n/a	45%	25%	15%
1151.g4	n/a	n/a	n/a	45%	25%	20%
1153.g8	28.3	n/a	n/a	65%	35%	28%
1125.g2 + 1114.g8 (50:50)	10.1	243.8	n/a	85%	65%	40%
1125.g2 + 1134.g5 (50:50)	1.7	22.6	n/a	87%	60%	40%
1125.g2 + 1153.g8 (50:50)	6.1	32.2	n/a	95%	75%	48%
1125.g2 + 1151.g4 (50:50)	0.8	2.8	19.1	85%	80%	55%
1125.g2 + 1151.g4 (25:75)	1.2	2.8	47.2	85%	75%	60%
1125.g2 + 1151.g4 (75:25)	2.9	3.8	2.6	75%	70%	60%

These data show that combination of Mabs, especially CA1125 and CA1151 improve both the potency as measured by EC_{50} but also as measured by % maximum protection. The % maximum protection is of particular relevance in this assay method since the Mab:TcdB mixture is incubated with cells for a long time (72h). Since TcdB is toxic to Caco-2 cells in the range of pg/ml in 2-4h this measure may be considered to be a very difficult test of Mab neutralisation ability and may reflect the ability of Mab mixture with regard to their binding kinetics or modalities. In turn this may reflect the ability of Mab mixtures to protect against the effects of TcdB during an established infection when there may be substantial quantities of TcdB within tissues for many hours. Selected data from Tables 6-9 are further illustrated in Figures 46-59.

Example 4 Valency of binding of Mabs to TcdB sub-domains.

The number of moles of binding events of anti-*C. difficile* TcdB antibodies to $TcdB_{1234}$ was determined by Surface Plasmon Resonance (SPR) on a Biacore 3000 (GE Healthcare). Streptavidin was immobilized on a CM5 sensor chip (GE Healthcare) to a level of ~4000RU via amine coupling and biotinylated $TcdB_{1234}$ was bound at 500-600RU. Two 20µl injections of the same anti-TcdB antibody mixtures (final concentration of each antibody was 500nM) were injected over this surface at 10µl/min and the saturating binding response recorded. The surface was regenerated after every cycle using HCl. All the data was corrected for background binding using the response to the streptavidin only reference flowcell.

Table 10: Surface plasmon resonance analysis of the number of IgG binding sites on $TcdB_{1234}$

Antibody combination	No. of binding cycle repeats	Binding Response (RU)	Binding relative to CA927 average response
CA1125.g2	10	750	0.9
CA1151.g4	10	1232	1.6
CA1125_CA1151	4	1941	2.5
CA1125_CA927	3	1570	2.0
CA1151_CA927	3	1959	2.5
CA927	8	791	1.0

All responses have been expressed relative to a multiple of CA927 average response (final column table 10) since CA927 appears to be representative of a Mab which binds to $TcdB_{1234}$ once only.

Immobilized CA1125, when bound to $TcdB_{1234}$, does not allow CA1125 to bind further supporting the idea that CA1125 has one binding site on $TcdB_{1234}$ and that after this has been saturated that no other binding site for CA1125 can be found. However, when $TcdB_{1234}$ has been saturated by CA1125, CA1151 can still bind. This demonstrates that CA1151 binds at alternative sites to that occupied by CA1125. Together these data show that CA1125 is a single binder of $TcdB_{1234}$ whereas 1151 IgG binds to $TcdB_{1234}$ more than once, most likely twice. Hence a mixture of CA1125 and CA1151 can bind to $TcdB_{1234}$ approximately 3 times.
All antibodies combinations have an additive binding response showing that there are 2 or more non-competitive sites on $TcdB_{1234}$ bound by these combinations.

Example 5 Valency of binding of Mabs to TcdA sub-domains.

The number of moles of binding events of anti-*C.difficile* TcdA antibodies to $TcdA_{123}$ and A_{456} were determined by Surface Plasmon Resonance (SPR) on a Biacore 3000 (GE Healthcare). Streptavidin was immobilized on a CM5 sensor chip (GE Healthcare) via amine coupling to a level of ~4000RU and biotinylated $TcdA_{123}$ was bound to one flowcell and $TcdA_{456}$ was bound to a different flowcell to a response of ~500RU. Two 30µl injections of the same anti-TcdA antibody at 1µM were injected over both flowcells at 10µl/min and the saturating binding response recorded. The surface was regenerated after every cycle using HCl. All the data was corrected for background binding using the response to the streptavidin only reference flowcell.

Table 11: SPR analysis of the binding responses of IgGs to immobilised $TcdA_{123}$ and $TcdA_{456}$

	CA997	CA1000	CA997/CA1000 ratio
TcdA123	1069	166	6
TcdA456	1285	407	3

Antibodies CA997 and CA1000 bind to $TcdA_{123}$ in a ratio of six CA997's to one CA1000 whereas they bind to $TcdA_{456}$ in a ratio of three CA997's to one CA1000 (Table 2).

The maximum antibody response for CA997, corrected for molecular weight and immobilized toxin level is similar for $TcdA_{123}$ and $TcdA_{456}$. This suggests that CA997 binds $TcdA_{456}$ six times and CA1000 binds twice to $TcdA_{456}$. Hence antibody CA997 likely binds to TcdA whole toxin (TcdA) approximately 12 times.

Overall CA997 binds six times or more to A_{123} and six times or more to A_{456}, whereas CA1000 binds at least once to A_{123} and twice to A_{456}.

Increased valency of binding to TcdA and TcdB may have two important effects *in vivo*. The first is that any Mab or Mab mixture which is capable of binding TcdB more than once will have increased potential to form inter-toxin binding events and hence immunoprecipitation. Immunoprecipitation can contribute to potency by reducing the solubility of toxin and forming very large macromolecular complexes which hence reduce the effective working concentration of toxin. Such large protein complexes may be taken up by macrophages and monocytes resident in the tissue and may contribute to an augmented host immune respone. Antigen:antibody complexes bearing Fc fragments have been specifically shown to be capable of priming a host immune respone against a gut pathogen (21). Also, soluble antigen:antibody complexes have been successfully used as a vaccine directed against the antigen in human clinical trials (22). In addition, immune decoration of toxin with Fc bearing IgG may contribute to immune clearance using normal mechanisms through the liver and spleen. In general, higher levels of Fc decoration of antigen lead to faster and more complete levels of clearance (23) Critically, it may be that presence of 2 or more Mab Fc domains per toxin, especially 3 Fc domains per toxin may represent a critical number of Fcs required for very rapid and substantial clearance of toxin (24). The anti-TcdA Mab CA997 is likely capable of binding to TcdA up to 12 times and the combination of CA1125 and CA1151 is likely capable of binding to TcdB 3 times. Hence the 3 Mab mixture is very likely to be capable of providing for these kinds of additional potency mechanisms *in vivo*.

Example 6 Mab neutralisation of loss of TEER caused by TcdA.

C.difficile monolayer integrity assay is performed using the Becton-Dickinson (BD) Caco-2 BioCoat HTS plate system.

Day 1 – Caco-2 cells seeded @ $2x10^5$/ml per well of the plate insert in 500µl Basal seeding medium (provided by BD). 35ml of Basal seeding medium added to the feeder tray. Cells incubated for 24 hours at 37°C. **Day 2** – Basal seeding medium removed from inserts and feeder tray, and replaced with Entero-STIM differentiation medium (supplied by BD). 500µl added per well insert and 35ml to the feeder tray. Cells incubate for a further 72hrs at 37°C. **Day 5** – Antibodies prepared at 2x concentration relative to that to be used in the assay well in a polypropylene plate and toxin A. Toxin A added to antibodies at a concentration of 125ng/ml and plate incubated for 1hr at 37°C. 1ml of Caco-2 growth medium (MEM + 20% FCS, 2mM Q, NEAA) added to each well of a standard 24-well TC plate. BioCoat insert plate transferred to the 24-well TC plate. Entero-STIM medium removed from inserts and replaced with 400µl of toxin:Ab mixture.

Loss of tight junctions between gut cells is the key early effect of TcdA on cell monolayers and gut tissue sections and is the primary cause of diarrhoea. Albumin and other serum proteins are lost into the gut lumen along with accompanying serum fluid. The loss of trans-epithelial electrical resistance in differentiated cultured cells which have formed a monolayer is a useful surrogate for the protection against the acute effects of TcdA. Three antibodies shown have good levels of protection against TEER loss, Figure 62. It is notable and surprising that the abilities of these Mabs in TEER assays do not reflect those seen in toxin neutralization as measured in a cell proliferation assay. CA922 has the best performance in a cell proliferation assay (EC_{50} = 1.21ng/ml) and yet this is considerably out-performed in the TEER assay by an antibody (CA1000) which has >10x lower potency in a cell proliferation assay (EC_{50} = 19.73ng/ml). CA997 had the best performance in the TEER assay since it had both high levels of protection and maintained this at the lower Mab concs. CA997 had a substantial potential to neutralize TEER loss with maximal inhibition approaching 80% and an EC_{50} of approximately 80ng/ml at 4h. These observations are unexpected since the Mabs in question all had high affinities for TcdA domains (CA922 ~4pM, CA997 ~132pM, CA1000 ~73pM). These data suggest that CA997 and CA1000 recognise epitopes important in TEER loss or neutralize TcdA by different mechanism to other Mabs. Furthermore, since CA1000 is estimated to bind to holotoxin twice (once in $TcdA_{123}$ and once in $TcdA_{456}$) CA1000 may define 'TEER critical' epitopes within the TcdA cell binding regions which might have particular value for defining vaccine immunogens. Results are shown in Figures 62.

Example 7 Affinity of anti-*C. difficile* toxin antibodies for sub-domains of TcdA and TcdB: $TcdA_{123}$, $TcdA_{456}$ and $TcdB_{1234}$.

Kinetic constants for the interactions of anti-*C. difficile* TcdA and TcdB antibodies were determined by surface plasmon resonance conducted on a BIAcore 3000 using CM5 sensor chips. All experiments were performed at 25°C. Affinipure $F(ab')_2$ fragment goat anti-human IgG, Fc fragment specific (Jackson ImmunoResearch) was immobilised on a CM5 Sensor Chip (GE) via amine coupling chemistry to a capture level of ≈7000 response units (RUs). HBS-EP buffer (10mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % Surfactant P20, Biacore AB) was used as the running buffer with a flow rate of 10 µL/min. A 10 µL injection of each antibody at 1ug/ml or lower was used for capture by the immobilised anti-human IgG, Fc. TcdA123, TcdA456 or TcdB1234 were titrated over captured purified antibodies at doubling dilutions from 12.5nM at a flow rate of 30 µL/min. For antibodies present in culture supernatants, a single concentration of 12.5nM of TcdA123 or TcdA456 and 50nM of TcdB1234 was passed over the antibodies at 30ul/min. Kinetics were calculated on n=2 The surface was regenerated at a flowrate of 10uL/min by two 10 µL injections of 40 mM HCl, and a 5 µL injection of 5 mM NaOH.

Double referenced background subtracted binding curves were analysed using the BIAevaluation software (version 3.2) following standard procedures. Kinetic parameters were determined from the fitting algorithm.

TABLE 12 Anti-TcdA Mab affinities and binding kinetics

	Antibody ID	ka (1/Ms)	kd (1/s)	KD (M)	KD(pM)	Material/Assay
TcdA123	CA164_00922.g1	1.09E+06	4.43E-06	4.06E-12	4.06	Purified Mab 5 point titration
	CA164_00923.g1	5.36E+05	3.47E-05	6.47E-11	64.7	
	CA164_00995.g1	No binding			No binding	
	CA164_00997.g1	7.84E+05	1.03E-04	1.32E-10	132	
	CA164_01000.g1	1.33E+05	9.78E-06	7.33E-11	73.3	
	CA164_00993.g1	9.00E+05	5.00E-06	5.56E-12	5.56	Supernatant 2x 1point titration
TcdA456	CA164_00922.g1	1.29E+06	3.33E-06	2.59E-12	2.59	Purified Mab 5 point titration
	CA164_00923.g1	6.16E+05	1.92E-04	3.12E-10	312	
	CA164_00995.g1	2.87E+05	3.42E-05	1.19E-10	119	
	CA164_00997.g1	9.21E+05	6.15E-05	6.68E-11	66.8	

	CA164_01000.g1	3.55E+05	2.98E-05	8.41E-11	84.1	
	CA164_00993.g1	1.25E+06	5.00E-06	4.00E-12	4.00	Supernatant 2x 1point titration

TABLE 13 Anti-TcdB Mab affinities and binding kinetics

	Antibody ID	ka (1/Ms)	kd (1/s)	KD(M)	KD (pM)	Material/Assay
TcdB1234	CA164_1125.g2	2.64E+05	3.23E-05	1.22E-10	122	Purified Mab 3 point titration
	CA164_1151.g4	7.49E+05	4.13E-04	5.51E-10	551	Purified Mab 3 point titration
	CA164_926.g1	1.38E+05	7.12E-05	5.16E-10	516	Supernatant 2x 1point titration
	CA164_927.g2	3.97E+05	3.61E-05	9.11E-11	91	Purified Mab 3 point titration
	CA164_1099.g2	5.24E+05	1.63E-05	3.10E-11	31	Purified Mab 3 point titration
	CA164_1102.g4	1.17E+05	3.78E-04	3.25E-09	3250	Supernatant 2x 1point titration
	CA164_1114.g2	2.87E+05	1.97E-03	6.87E-09	6870	Supernatant 2x 1point titration
	CA164_1114.g8	2.55E+05	1.85E-03	7.25E-09	7250	Supernatant 2x 1point titration
	CA164_1129.g1	1.89E+05	2.30E-04	1.22E-09	1220	Supernatant 2x 1point titration
	CA164_1134.g5	5.09E+05	2.45E-05	4.81E-11	48	Purified Mab 3 point titration
	CA164_1153.g8	1.43E+05	4.48E-05	3.14E-10	314	Purified Mab 3 point titration

The anti-TcdA affinities are particularly high compared to the published affinities of other Mabs. We demonstrate that affinities as low as 4pM are achievable. The preferred CA997 has an affinity of 132pM, CA1125 122pM and CA115 551pM. CA995 clearly shows that it does not bind to

CROPs A_{123} and hence that demonstrates that the Mab shown here have properties which are different from each other in surprising and unexpected ways. CA922, 923, 997 and 1000 do bind at least once to CROPs A123 and A456. Hence these 4 Mabs confirming that each must bind to holotoxin at least twice. We have been unable to derive affinities for the binding of these Mabs to holotoxin due to technical constraints. However, given the high affinities and valencies demonstrated for the anti-TcdA Mabs it is possible to speculate that the functional affinities against holotoxin may be even stronger than those illustrated for binding to toxin sub-domains.
The anti-TcdB Mabs also demonstrated strong affinities reaching as low as 31pM. In particular CA1125, 1151, 927, 1099, 1134 and 1153 show affinities which surpass those demonstrated by others.

Example 8 Biophysical characteristion of *C. difficile* anti-toxin humanised IgG1 Molecules.

Molecules analysed

Anti-TcdA IgG1:

CA164_00922.g1

CA164_0923.g1

CA164_0995.g1

CA164_0997.g1

CA164_01000.g1

Anti-TcdB IgG1

CA164_01125.g1

CA164_01125.g2

CA164_01134.g4

CA164_01134.g5

CA164_01134.g6

CA164_01102.g1

CA164_01102.g4

CA164_01151.g4

Antibody combinations need to be made up of Mabs having high levels of stability in order to mitigate potential risks of aggregation during long term storage. Thermal stability (Tm) is used as one measure. Of special value to Mab mixtures is measuring their propensity to aggregate due to physical stress such as agitation or shaking. Aggregates are undesirable components of drug

compositions since they may reduce storage life time and may pose a safety risk to patients at certain levels. The Tm data show that all 5 anti-TcdA Mabs have high Tm stability, whilst three (CA922, 923 and 997) have very high Tm's in the range of 79-81°C. Of the anti-TcdB Mabs tested all but two have very high Tm's. Of note is that CA997, CA1125 and CA1151 which were tested in the hamster infection study (Example 9) had very high Tm's (79.2°C, 79.3°C and 80.8°C respectively) which makes them suitable for use in a Mab mixture.

In the shaking aggregation assay, CA997 and 922 had the lowest propensity to aggregate of the 5 anti-TcdA Mabs. Similarly, CA115 and 1151 had the lowest aggregation propensities of the anti-TcdB Mabs. Hence the use of CA997, 1125 and 1151 as a Mab mixture may have special value since they are more likely to survive co-formulation and storage at high protein concentrations.

Estimation of isoelectric point (pI) by capillary IEF

Samples were prepared by mixing the following: 30ul Protein sample at 2mg/ml, 0.35% Methylcellulose, 4% pH3-10 ampholytes (Pharmalyte), synthetic pI markers (4.65 and 9.77), 1ul of each stock solution, and HPLC grade water to make up the final volume to 200ul. The mixture was then analysed using iCE280 IEF analyzer(pre-focusing at 1500V for 1 min followed by focusing at 3000V for 6mins). The calibrated electropherograms were then integrated using Empower software (from Waters)

Thermal stability (Tm) measured *via* Thermofluor assay.

This method uses Sypro orange fluorescent dye to monitor the unfolding process of protein domains. The dye binds to exposed hydrophobic regions that become exposed as a consequence of unfolding which results in a change to the emission spectrum.

The sample (5ul at 1mg/ml) is mixed with a 5ul of a stock solution of Sypro orange (30x) and the volume made up to 50ul with PBS,pH 7.40.

10ul aliquots of this solution is applied towells in a 384 well plate (n=4).

The plate is placed in a 7900HT fast real-time PCR system containing a heating device for accurate temperature control. The temperature is increased from 20°C to 99° C (Ramp rate of 1.1°C/min).A CCD device simultaneously monitors the fluorescence changes in the wells. An algorithm is used to process intensity data and take into account multiple transitions.

Stressing of samples by agitation.

During manufacture antibody samples are subjected to mechanical stress generated by processes such as pumping and filtration. This may cause denaturation and consequently aggregation due to exposure of the protein to air-liquid interfaces and shear forces resulting in the ultimate loss of

bioactivity. Stress by vortexing is a method to screen the robustness of the antibody samples for prediction of aggregation stability.

Both anti-TcdA and anti-TcdB IgG1 molecules were subjected to stress by agitation, by vortexing using an Eppendorf Thermomixer Comfort at 25 °C, 1400rpm. Sample size was 250uL, (x3 per sample) in a 1.5 mL conical Eppendorf-style capped tube (plastic), in PBS pH 7.4. Each sample was brought to a concentration of 1mg/ml (using extinction coefficient calculated from sequence) and aggregation was monitored by absorbance at 340nm and/or 595nm, by use of a Varian Cary 50-Bio spectrophotometer, measured at intervals for up to 24 hours.

Results Table 14 provides a summary of the measured pI and Tm data for both anti-TcdA and anti-TcdB IgG1 molecules.

Table 14 : Compilation of pI and Tm Data

Anti-TcdA IgG1	**measured pI**	**Tm(Fab) in PBS**	**Tm(CH2)**
CA164_00922.g1	8.8	81	69.2
CA164_0923.g1	9.2	79	69.3
CA164_0995.g1	8.5	71	no data*
CA164_0997.g1	8.3	79.2	68.4
CA164_01000.g1	7.74	70.5	no data*
Anti-TcdB IgG1			
CA164_01125.g1	9.2	79.3	69.4
CA164_01125.g2	9.2	79.5	69.3
CA164_01134.g4	9.3	78.4	69.4
CA164_01134.g5	9.2	76.4	69.2
CA164_01134.g6	9.2	76.6	69.6
CA164_01102.g1	9.1	69	no data*
CA164_01102.g4	9.1	69.1	no data*
CA164_01151.g4	9.2	80.8	69.8

*denotes that it was not possible to discern the Fab and CH2 domains.

Measured pI

The measured pI of the molecules were high (except for CA164_01000.g1_P3) and away from the pH of formulation buffers such as PBS, pH 7.4 and 50m sodium acetate/125mM sodium chloride,

pH 5. This may mean that buffers with pH's suitable for co-formulation of two or more Mabs can be selected.

Thermal Stability (Tm) Measured *via* Thermofluor assay

Since all of the molecules are IgG1, the Tm of the Fc domain (Tm(CH2)) are the same. The difference in thermal stability between the molecules can be determined by the Tm of the Fab' domain (Tm(Fab)).

For the anti-TcdA molecules, the rank order (most stable first) was CA922≥997>923>995>1000 and for the anti-TcdB molecules (most stable first) was CA1151.g4>1125.g1,g4>1134.g4>1134.g5≥1134.g6>1102.g1=1102.g4.

Stressing of samples by agitation

It was possible to determine different aggregation stability between the different antibodies, Figure 67 shows the effect of agitation *via* vortexing on different anti-TcdA IgG1 molecules in PBS,pH 7.4.

It was possible to determine a ranking order (most aggregation stable first) : CA922≥997>923≥995>1000

Figure 68 shows the effect of agitation *via* vortexing on different anti-TcdB molecules.

It was possible to rank the order of aggregation stability, such that the CA1125 grafts appeared more stable than the CA1134 molecules which were more stable than the CA1102 molecules.

A further study was performed to compare directly the aggregation stability of the anti-TcdB molecule (CA1151.g4) with the more stable molecule CA1125.g2 (see Figure 2) and more aggregation stable anti-TcdA molecules (CA922.g1 and CA997.g1). The results can be seen in Figure 69.

Further results for these 4 Mabs are also shown in Figures 67 and 68.

For the anti-TcdA molecules, CA922.g1 and CA977.g1, CA922 were preferable based on the analyses above, although apart from CA1000) all molecules could be considered suitable candidates for use as therapeutic IgG1.

For the anti- TcdB molecules, the biophysical characteristics could be grouped within the family of grafts based on the aggregation stability and Tm, such that the CA1125 grafts potentially proved more stable. The CA1102 grafts showed poorest Tm data and also showed the greatest tendancy to aggregate *via* stress by agitation.

A study using CA1151.g4 showed that this molecule exhibited slightly increased aggregation stability relative to CA11125.g2 and seemed equivalent to the TcdA molecules (CA922.g1 and

CA997.g1.All four molecules showed equivalent Tm values. CA997, CA1125 and CA1151 show very high levels of thermostability and very low levels of aggregate formation after agitation.

Example 9 Anti-*C. difficile* toxin Mab hamster infection study.

The hamster infection study was performed by Ricerca Biosciences LLC, Cleveland, Ohio, USA. The study protocol was approved by the Ricerca IACUC committee. Active and control components (composition and dose) were blinded to Ricerca staff until after completion of the planned 28 day study period.

Golden Syrian male hamsters (weight 82-103g, 54 days old) were individually housed in HEPA filtered disposable cages and fed Teklad Global Diet 2016 and water *ad libitum*. After acclimatisation, hamsters were pre-dosed (i.p.) with Mab mixtures or PBS (vehicle control) once a day for each of 4 days: days -3, -2, -1 and 0. Two doses of Mab were investigated: high dose = 50mg/kg each of anti-TcdA and anti-TcdB components and low dose 5mg/kg each of anti-TcdA and anti-TcdB components.

The drug combination tested was composed of one anti-TcdA antibody (CA997.g1) which constituted 50% of the injected protein and two anti-TcdB antibodies (CA1125.g2 and CA1151.g4) which together constituted 50% of the injected protein but which alone constituted 25% of the injected protein. Hamsters were sensitised (day -1) with 50mg/kg of Clindamycin phosphate in PBS (s.c.) before being challenged 1 day later (day 0) with 3.4 x 106 c.f.u. of vegetative cells from strain ATCC43596. Vancomycin was dosed at 5mg/kg twice a day for 5 days (p.o.) on days 1, 2, 3, 4, 5.

Viability checks were performed on animals twice a day, animals found to be *in extremis* were euthanised and counted as dead. Body weights were determined on each day of dosing, then twice weekly and before euthanising survivors. Gross necropsy was performed on all animals. Survival curves were created by the method of Kaplan and Meier. Survival curves were analysed using the P value from the log rank test compared to the Bonferroni corrected threshold of P = 0.005. The Vancomycin treated group were not included in the analysis. All statistical tests were done with Prism v5.04. All groups contained 11 animals, except the Vancomycin control group which contained 5 animals.

Survival curves can be seen in Figure 63. Hamsters receiving PBS (control) all died on days +2 and +3, whilst those receiving vancomycin treatment for 5 days all died on days +10 and +11. Hamsters receiving the high dose of UCB Mab mixture all survived until day +11, thereafter only two animals died until the end of the 28 day study. Hamsters receiving the low dose of UCB Mab

mixture all survived until day +3, thereafter animals were lost fairly steadily until day +16 when all had died. The data show exceptional levels and duration of protection when compared to published data for use of anti-toxin Mabs in hamsters (18). These in vivo data support the in vitro observations of very high level performance for neutralization and stability.

There is no apparent link between death and body weight during the acute phase (days 1-5) of the infection, Figures 64-65. Hence it may be supposed that hamsters die of overwhelming direct and indirect effects of TcdA and TcdB. Hamsters which survive the acute period due to partial protection (UCB low dose) of neutralizing Mabs lose weight, presumably due to gut damage and altered nutritional status. It was notable that many of the hamsters which went on to survive the 28 period of the study due to the protective effects of the UCB high dose Mabs recovered from weight loss and indeed even gained weight. This may be taken as evidence of the superior protective effects of the UCB Mabs enabling the gut to function as normal.

Table 15. Gross pathology scores

Group	Black caecum	Dark red caecum	Red caecum	Pink caecum	Normal caecum	Anogenital staining 'wet-tail'	Red small intestine
PBS control	1	9	1	0	0	1	1
UCB low	0	4	5	2	0	4	1
UCB high	0	0	1	1	9	3	0

It is clear that UCB Mabs were able to protect the large and small intestines from the bloody effusions caused by TcdA and TcdB.

The results are shown in Figures 63 to 66

The photographs in Figure 66 show typical gross pathologies for the swelling and bloody effusions of caeca caused by TcdA and TcdB (left image, PBS control, animal death on day 2) and a normal stool filled caeca after protection by UCB high dose Mabs (right image, UCB high dose, animal surviving to day 28). These data show that after protection with a high dose of UCB Mabs the large intestine can return to normal morphology and function.

Example 10 Neutralisation of TcdA from different ribotyped strains by purified Mab.

Clinical infections are caused by a variety of different strains. Strain differences are characterized using a number of different methods of which ribotyping is a key one. Different ribotype strains are observed to have different pathogenicity, infection and sporulation properties. All of the TcdA

neutralization shown above used TcdA purified from strain known as VPI10463. However, the predominant aggressively pathogenic strain associated with out-breaks is called ribotype 027. Other key ribotypes include 078, 001, 106. Amino acid sequence difference have been observed between toxins produced by different ribotypes and hence it is important that Mabs are capable of neutralizing toxin from a diverse set of clinical isolates. CA922, 997 and 1000 were tested for their ability to neutralize TcdA from strains 027 and 078 and compared to their abilities against TcdA from VPI10463. Mabs were tested at 4 [TcdA] and found to be capable of neutralizing all toxins without significant difference at LD_{80}, LD_{90} and LD_{95}

Table 16

	EC50 values (ng/ml) - TcdA strain VPI 10463			
Antibody	**LD80**	**LD90**	**LD95**	**LDmax**
CA164_922	0.27	0.9	1.2	>500
CA164_997	1	2.5	3.5	25.4
CA164_1000	3.6	13.5	19.3	>500

Table 17

	EC50 values (ng/ml) - TcdA ribotype 027			
Antibody	**LD80**	**LD90**	**LD95**	**LDmax**
CA164_922	0.19	0.25	0.41	1.46
CA164_997	0.92	1.27	1.75	7.19
CA164_1000	2.25	2.49	3.52	16.32

Table 18

	EC50 values (ng/ml) - TcdA ribotype 078			
Antibody	**LD80**	**LD90**	**LD95**	**LDmax**
CA164_922	0.11	0.12	0.25	0.68
CA164_997	0.33	0.64	1.11	2.57
CA164_1000	2.04	2.41	5.03	14.16

Example 11 PK data

A PK study of a human IgG1 (20mg/kg) in healthy hamsters. The hamster PK was found a half-life similar to Mabs in mice or rats. (t½ 6-8 days). i.p. and s.c. dosing were essentially the same.
The pharmacokinetics and distribution to the gut of a hIgG1 Mab was studied in 'normal' (non-infected) golden Syrian hamsters. Purified Mab was administered to male hamsters (120-135g) by CARE Research LLC, Fort Collins, Colorado, USA and samples were assayed by UCB Pharma. The study was approved by the CARE IACUC committee. Eight animals each received a single dose of 20 mg/kg of IgG1, four were dosed *i.p.*, four were dosed *s.c.* Blood was collected at 1, 3, 8, 24, 48, 72, 103 and 168 hours post-dose, serum was separated before storage at -80°C. Blood was also taken from two untreated hamsters in order to provide assay controls. Following euthanasia, a

2cm length of colon was cut from the caeca junction onwards from each hamster. The colon section was flushed with wash buffer (50% (v/v) PBS containing 50% (v/v) Sigma protease inhibitor cocktail (P2714) before being opened and separation and removal of the mucosa from the underlying muscle. Mucosal samples were placed in 0.5ml of wash buffer homogenized until visually uniform and stored at 4°C before immediate shipping on wet ice. For the anti-human IgG1 ELISA Nunc maxisorp 96 well plates were coated overnight in 0.1M $NaHCO_3$ pH 8.3 with Goat $F(ab')_2$ anti-human IgG-Fcγ fragment (Jackson 109-006-098), plates were washed with PBS-Tween (PBS/0.1% (v/v) Tween 20) and then blocked with 1.0% (w/v) BSA & 0.1% (v/v) Tween in PBS. Serum samples were diluted in sample-conjugate buffer (1% (w/v) BSA, 0.2% Tween in PBS) and after washing were revealed with goat anti-human kappa-HRP (Cambridge Bioscience 2060-05) in sample-conjugate buffer and TMB with a 2.5M H_2SO_4 stop solution.

Gut, Mucosa and Serum Levels:

Serum samples collected from blood taken at 168 hour time point and colon samples were removed after this.

20mg/kg IP at 168 hour

Sample	ng/mL per cm mucosa	serum µg/mL
1001	23.2	75.0
1002	13.7	90.8
1003	21.8	70.5
1004	53.8	119.4

20mg/kg SC at 168 hour

Sample	ng/mL per cm mucosa	serum µg/mL
2001	41.4	108.7
2002	62.1	76.6
2003	35.6	163.7
2004	37.3	153.3

Serum Data

		Hamster *i.p.*		Hamster *s.c.*	
		Mean	SE of mean	Mean	SE of mean
C_{max}:	µg/mL	202	12	186	21
T_{max}:	hr	36	7	76	16
$AUC_{(last)}$:	hr·µg/mL	22626	1378	22371	2258
$AUC_{(inf)}$:	hr·µg/mL	43287	7169	61290	17637
% Extrapolation:		43.7	9.2	54	11.7
CL/F	mL/hr/kg	0.50	0.07	0.43	0.13
MRT_{inf}	h	223	53	310	88
$t_{½,z}$:	h	149.2	36.9	188.5	61.9

The data is also shown in Figure 70 and 71

Hamster ID		Mean	SE
IP serum kinetics			
C_{max}:	µg/mL	202	12
T_{max}:	hr	36	7
$AUC_{(last)}$:	hr·µg/mL	22626	1378
$AUC_{(inf)}$:	hr·µg/mL	43287	7169
% Extrapolation:		43.7	9.2
CL/F	mL/hr/kg	0.50	0.07
MRT_{inf}	h	223	53
$t_{½,z}$:	h	149.2	36.9
SC serum kinetics			
Hamster ID		Mean	SE
C_{max}:	µg/mL	186	21
T_{max}:	hr	76	16

$AUC_{(last)}$:	hr·µg/mL	22371	2258
$AUC_{(inf)}$:	hr·µg/mL	61290	17637
% Extrapolation:		54	11.7
CL/F	mL/hr/kg	0.43	0.13
MRT_{inf}	h	310	88
$t_{½}$:	h	188.5	61.9

It was also shown that hIgG1 could be found in 'scrapings' of the gut i.e that hIgG1 gets into the vasculature of healthy gut – and so could be protective in 'prophylactic dosing'. This effect would be even more profound in humans since they have a cognate hFcRn.

Example 12 Serum Levels in Hamsters with C. difficile Infection

This study was to determine the serum concentration of CA725.0, CA726.0, CA997.g1 CA1125.g2, and CA01151.g4 following i.p. administration (various doses detailed below) in the Golden Syrian Hamster.

Humanised Mabs were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis following tryptic digestion. Quantitation was achieved by comparison to authentic standard material spiked at known concentrations into blank matrix, with spiked horse myoglobin used as the internal standard.

A unique ("proteotypic") peptide common to all of the humanised Mabs investigated was selected (DTLMISR, a CH2 region peptide) and both samples and calibration samples were tryptically digested as outlined. Tryptic digest of 5 µl serum samples was performed overnight using sequencing grade modified Trypsin (Promega, Southampton, UK) following denaturation / reduction with acetonitrile / Tris (2-carboxyethyl) phosphine and carbamido-methylation with iodoacetamide (Sigma-Aldrich, Poole, UK).

The LC-MS/MS system consisted of a CTC HTS-x Autosampler (CTC Analytics, Zwingen, Switzerland), a Agilent 1290 LC system (Agilent Technologies, Stockport, UK) and a Sciex 5500 QTrap MS system (AB Sciex, Warrington, UK), equipped with a Turbo V ion source operated in electrospray mode. Analytes were separated using an Onyx Monolithic C18 column (100x4.6 mm, Phenomenex, Macclesfield, UK) with a gradient of 2 to 95 % (v/v) water/acetonitrile (0.1 %

formic acid) delivered at 1.5 mL/min over 6 minutes. The injection volume was 10 µL; all of the eluent was introduced into the mass spectrometer source. The source temperature of the mass spectrometer was maintained at 600 °C and other source parameters (e.g. collision energy, declustering potential, curtain gas pressure etc.) were optimized to achieve maximum sensitivity for the peptide of interest. Selective transitions for each proteotypic peptide of interest were monitored.

Unique ("proteotypic) peptides were selected for all of the analytes of interest; samples were analysed following tryptic digestion.

Plasma concentrations calculated based on the peptides monitored are outlined below.

For CA164_00997 and CA164_01151, interfering peaks were observed in the MRM traces. For this reason, these two analytes could not be quantified in the samples.

Total h-IgG was quantified in all samples using a peptide common to all analytes of interest. This was done using a combined standard curve of all five analytes. The validity of this approach is demonstrated by the fact that the sum of the concentrations observed for CA164_00725 and CA164_00726 are in good agreement (within experimental error) of the concentration observed for total h-IgG.

Using this approach, the total concentration of h-IgG in the samples of animals dosed with CA164_00997, CA164_01125 and CA164_01151 was determined.

Overall the data obtained indicate that the exposure of all five analytes of interest was similar for a given dose.

Study groups

Blinded labels		Actual Treatments	Dose days	Treatment components		
Grp	Treatment			Anti-toxin A	Anti-toxin B	
4	Treatment 3	Vehicle PBS 5mL/kg i.p.	3, 2, 1, 0			
2	Vancomycin	Vancomycin 5mg/kg b.i.d. p.o.	1, 2, 3, 4, 5			
1	Treatment 1	UCB LD* 5mg/kg A 5mg/kg i.p.	3, 2, 1, 0	CA997.g1_P3 5mg/kg	CA1125.g2_P3 2.5mg/kg	CA1151.g4_P3 2.5mg/kg
5	Treatment 4	UCB HD* 50mg/kg A 50mg/kg i.p.	3, 2, 1, 0	CA997.g1_P3 50mg/kg	CA1125.g2_P3 25mg/kg	CA1151.g4_P3 25mg/kg
6	Treatment 5	Competitor LD* 5mg/kg A 5mg/kg i.p.	3, 2, 1, 0	CA726_P3 5mg/kg	CA725_P3 5mg/kg	
3	Treatment 2	Competitor HD* 50mg/kg A 50mg/kg i.p.	3, -2, -1, 0	CA726_P3 50mg/kg	CA725_P3 50mg/kg	

Table 19

Group/time	Day	Animal No	Dose	Serum conc µg/mLtotal h-IgG
1	1	44	5 mg/kg 997, 2.5 mg/kg 1125, 2.5 mg/kg 1151	280
	1	45		302
	1	46		182
	6	45		61
	6	47		71
	6	49		45
3	1	60	50 mg/kg 725, 50 mg/kg 726	3040
	1	61		3330
	1	62		2990
	6	62		583
	6	63		913
	6	64		1240
	28	64		199
	28	65		36
4	1	71	Vehicle	nd
	1	72		nd
	1	73		nd
5	1	82	50 mg/kg 997, 25 mg/kg 1125, 25 mg/kg 1151	3050
	1	83		2790
	1	84		2370
	6	82		838
	6	83		645
	6	84		855
	28	82		116
	28	83		65
	28	84		66
	28	85		44
	28	86		101
	28	87		89

	28	88		27
	28	89		31
	28	90		66
6	1	93	5 mg/kg 725, 5 mg/kg 726	335
	1	94		322
	1	95		260
	6	200		103
	6	202		62
	6	203		79
	28	203		nd

nd - not detected (LOQ = 2.5 µg/mL for all analytes

na - not analysed: interference in the sample was observed for 997 and 1151

Table 20 Antibody CA725 is prior art antibody MDX1388. Antibody CA726 is prior art antibody CDA1 as described (15) A summary of this data is presented in Figure 72.

Group	**Caecal pathology**					**Small intestine pathology**	
	Black	Dark Red	Red	Pink	Normal	Dark Red	Red
PBS control	1	9	1	0	0	0	1
MDX high 50mg/Kg x4	0	1	4	4	2	1	0
UCB high 50mg/Kg x4	0	0	1	1	9	0	0

References

1. Kuehne, S et al., "The role of toxin A and toxin B in *Clostridium difficile* Infection" Nature (2010) 467: 711-713.
2. Davies AH et al., "Super toxins from a super bug: structure and function of *Clostridium difficile* toxins" Biochem. J (2011) 436: 517-526.
3. Rothman, S et al., "Differential Cytotoxic Effects of Toxins A and B Isolated from *Clostridium difficile*" Infect. Imm. (1984) 46: 324-331.
4. Du, T and Alfa, MJ "Translocation of *Clostridium difficile* toxin B across polarized Caco-2 cell monolayers is enhanced by toxin A" Can J Infect Dis. (2004) 15: 83-88.

5. Kim, Iaconis and Rolfe. "Immunization of Adult Hamsters against *Clostridium diffcile*-Associated Ileocecitis and Transfer of Protection to Infant Hamsters" Infect. Imm. (1987) 55:2984-2992
6. Rupnik JCM (2003) 41:1118-1125
7. Chaves-Olarte JBC (1999) 274:11046-11052.
8. Lylerly, DM et al., "Passive Immunization of Hamsters against Disease Caused by *Clostridium difficile* by Use of Bovine Immunoglobulin G Concentrate" Infection and Immunity (1991) 59:2215-2218.
9. Lylerly, DM et al., "Vaccination against Lethal *Clostridium difficile* Enterocolitis with a Nontoxic Recombinant Peptide of Toxin A" Current Microbiology (1990) 21:29-32.
10. Lylerly, DM et al., "Characterization of Toxins A and B of *Clostridium difficile* with Monoclonal Antibodies" Infect. Imm. (1986) 54:70-76.
11. Corthier et al., "Protection against Experimental Pseudomembranous Colitis in Gnotobiotic Mice by Use of Monoclonal Antibodies against *Clostridium difficile* Toxin A" Infect. Imm. (1991) 59: 1192-1195.
12. Kink JA and Williams JA, "Antibodies to Recombinant *Clostridium difficile* Toxins A and B Are an Effective Treatment and Prevent Relapse of *C. difficile*-Associated Disease in a Hamster Model of Infection" Infect. Imm. (1998) 66:2018-2025.
13. Ma D, et al., Progenics inc. ASM Poster 27th May 2010
14. Hansen, G and Demarest, SJ. WO 2006/0718877 A2
15. Babcock GJ, et al., "Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamster" Infect. Imm.(2006) 74:6339-6347.
16. Lowy I et al., "Treatment with Monoclonal Antibodies against *Clostridium difficile* Toxins" NEJM (2010) 362: 197-205.
17. Zubler, R. H., Erard, F., Lees, R. K., Van, L. M., Mingari, C., Moretta, L. & MacDonald, H. R. (1985). Mutant EL-4 thymoma cells polyclonally activate murine and human B cells via direct cell interaction. J. Immunol. 134, 3662-3668
18. Babcook, J. S., Leslie, K. B., Olsen, O. A., Salmon, R. A. & Schrader, J. W. (1996). A novel strategy for generating monoclonal antibodies from single, isolated lymphocytes producing antibodies of defined specificities. Proc. Natl. Acad. Sci. U. S. A 93, 7843-7848
19. Tickle, S., Adams, R., Brown, D., Griffiths, M., Lightwood, D. & Lawson, A. (2010). High-Throughput Screening for High Affinity Antibodies ., pp. 303-307.
20. Demarest et al., mAbs (2010) 2:190-198

21. Yoshida et al., J. Clin. Invest. (2006) 116: 2142-2151
22. Xu et al., Vaccine (2005) 23:2658-2664.
23. Yousaf et al., Clin. Exp. Immunol. (1986) 66:654-660
24. Mannik et al., J. Exp. Med. (1971) 133: 713-739
25. Nusrat et al., Infection and Immunity (2001) 69:1329-1336
26. Lima et al., Infect Immun (1988) 56:582-588
27. Ravichandran et al J of Pharmacology and Experimental Therapeutics. (2006) 318: 1343-1351
28. Takahashi et al., (2009) Vaccine 27:2616-2619
29. Cohen et al., Infect. Cont. and Hosp. Epidem. (2010) 31: 431-455
30. Barbut et al., J. Clin. Microbiol. (2000) 38: 2386-2388
31. Wilcox et al., J. Hospital Infection (1998) 38: 93-100.

Claims

1. A monoclonal antibody specific to antigen TcdA wherein the antibody comprises a heavy chain wherein the variable domain of the heavy chain comprises 3 CDRs having the sequence given in SEQ ID NO:44 for CDR-H1, a CDR having the sequence given in SEQ ID NO:45 for CDR-H2 and a CDR having the sequence given in SEQ ID NO:46 for CDR-H3, and a light chain wherein the variable domain of the light chain comprises at least 3 CDRs having the sequence given in SEQ ID NO:41 for CDR-L1, a CDR having the sequence given in SEQ ID NO:42 for CDR-L2 and a CDR having the sequence given in SEQ ID NO:43 for CDR-L3.
2. A monoclonal antibody according to claim 1 having a heavy chain comprising the sequence given in SEQ ID NO:49 and a light chain comprising the sequence given in SEQ ID NO:47.
3. A pharmaceutical composition comprising a monoclonal antibody as defined in any one of claim 1 or 2.
4. A pharmaceutical composition according to claim 3 further comprising a monoclonal antibody which specifically binds TcdA comprising a heavy chain wherein the variable domain of the heavy chain comprises 3 CDRs having the sequence given in SEQ ID NO:54 for CDR-H1, a CDR having the sequence given in SEQ ID NO:55 for CDR-H2 and a CDR having the sequence given in SEQ ID NO:56 for CDR-H3 and a light chain wherein the variable domain of the light chain comprises 3 CDRs having the sequence given in SEQ ID NO:51 for CDR-L1, a CDR having the sequence given in SEQ ID NO:52 for CDR-L2 and a CDR having the sequence given in SEQ ID NO:53 for CDR-L3.
5. A pharmaceutical composition according to claim 4 comprising a monoclonal antibody having a heavy chain comprising the sequence given in SEQ ID NO:59 and a light chain comprising the sequence given in SEQ ID NO:57.
6. A pharmaceutical composition according to any one of claims 3 to 5 comprising a monoclonal antibody which specifically binds TcdB comprising a heavy chain wherein the variable domain of the heavy chain comprises at least one of a CDR having the sequence given in SEQ ID NO:124 for CDR-H1, a CDR having the sequence given in SEQ ID NO:125 for CDR-H2 and a CDR having the sequence given in SEQ ID NO:126 for CDR-H3 and a light chain wherein the variable domain of the light chain comprises at least one of a CDR having the sequence given in SEQ ID NO:121 for CDR-L1, a CDR having the sequence given in SEQ ID NO:122 for CDR-L2 and a CDR having the sequence given in SEQ ID NO:123 for CDR-L3.

7. A pharmaceutical composition according to claim 6 comprising a monoclonal antibody having a heavy chain comprising the sequence given in SEQ ID NO:129 and a light chain comprising the sequence given in SEQ ID NO:127.

8. A pharmaceutical composition according to any one of claims 3 to 7 comprising a monoclonal antibody which specifically binds TcdB comprising a heavy chain wherein the variable domain of the heavy chain comprises 3 CDRs having the sequence given in SEQ ID NO:154 for CDR-H1, a CDR having the sequence given in SEQ ID NO:155 for CDR-H2 and a CDR having the sequence given in SEQ ID NO:156 for CDR-H3 and a light chain wherein the variable domain of the light chain comprises 3 CDRs having the sequence given in SEQ ID NO:151 for CDR-L1, a CDR having the sequence given in SEQ ID NO:152 for CDR-L2 and a CDR having the sequence given in SEQ ID NO:153 for CDR-L3.

9. A pharmaceutical composition according to claim 8, comprising a monoclonal antibody having a heavy chain comprising the sequence given in SEQ ID NO:159 and a light chain comprising the sequence given in SEQ ID NO:157.

10. A pharmaceutical composition according to any one of claims 3 to 9 comprising a monoclonal antibody which specifically binds TcdA having a heavy chain and a light chain wherein the heavy chain variable region comprises a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:19, SEQ ID NO:29 and SEQ ID NO:39 and the light chain variable region comprises a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:17, SEQ ID NO:27 and SEQ ID NO:37.

11. A pharmaceutical composition according to any one of claims 3 to 10 comprising a monoclonal antibody which specifically binds TcdB having a heavy chain and a light chain wherein the heavy chain variable region comprises a sequence selected from the group consisting of SEQ ID NO:69, SEQ ID NO:79, SEQ ID NO:89, SEQ ID NO:99, SEQ ID NO:109, SEQ ID NO:119, SEQ ID NO:139, SEQ ID NO:149 and SEQ ID NO:159 and the light chain variable region comprises a sequence selected from the group consisting of SEQ ID NO:67, SEQ ID NO:77, SEQ ID NO:87, SEQ ID NO:97, SEQ ID NO:107, SEQ ID NO:117, SEQ ID NO:137, SEQ ID NO:147 and SEQ ID NO:157.

12. A pharmaceutical composition according to any one of claims 3 to 11, wherein at least one antibody in the composition is specific to TcdA and at least one antibody in the composition is specific to TcdB.

13. A pharmaceutical composition according to claim 12, wherein the composition further comprises at least a second antibody specific to TcdB.

14. Use of a monoclonal antibody as defined in any one of claim 1 or 2, in the manufacture of a medicament for the treatment of *C. difficile* infection.
15. Use of a monoclonal antibody according to claim 14, wherein the *C. difficile* infection is a hypervirulent strain.
16. Use of a monoclonal antibody according to claim 15, wherein the hypervirulent strain is ribotype 027.