Production and Assessment of Ovine Polyclonal Antibodies
to Treat Clostridium difficile Infections

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“Perfer et obdura, dolor hic tibi proderit olim”
ABSTRACT

The clinical manifestations of Clostridium difficile infections (CDI) are due to the release of two powerful exotoxins, TcdA and TcdB. The aim of this study was to raise polyclonal antibodies (PcAb) in sheep that bind to and neutralise these toxins with a view to developing potent therapeutic agents for use in humans.

Dose response studies were performed with recombinant fragments of each of the toxins, TxA4 and TxB4, in separate flocks of sheep. The PcAb produced showed high binding titres in an immunoassay and protected Vero cells against the two natural toxins in-vitro. TcdB was considerably more cytotoxic than TcdA with LC50 of 16 pg/mL and 1000 pg/mL respectively. The TxA4 immunogen stimulated a greater toxin neutralising immune response than TxB4. Thus, 1 mL of anti-TxA4 sera neutralised 1,800 µg of natural TcdA, whereas 1 mL of anti-TxB4 neutralised only 15 µg of TcdB.

There was no correlation between binding titre and neutralising potency, suggesting that toxin inactivation was dependant on PcAb binding to specific regions on the natural toxins. Affinity purified PcAb, directed against distinct regions of TcdB, were fractionated and those directed to the central region possessed neutralising potencies of up to 250% greater than those directed elsewhere. These investigations identified the quantity and potency of specific PcAb directed to each of three distinct TcdB regions.

A novel cell based assay, simulating the colonic epithelial barrier, was developed to investigate the translocation of TcdA and TcdB from simulated colonic lumen to the systemic circulation and to determine the effects of various toxin neutralising strategies. TcdA was shown to cause colonic epithelial damage allowing TcdB to translocate to the systemic circulation. Addition of neutralising PcAb either directly to simulated colonic lumen or systemic circulation provided protection to the intestinal epithelial layer and reduced or prevented toxin translocation.
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>%CV</td>
<td>Percentage Coefficient of Variation</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>aa</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-PCR</td>
<td>Arbitrarily Primed Polymerase Chain Reaction</td>
</tr>
<tr>
<td>CDI</td>
<td><em>Clostridium difficile</em> Infection</td>
</tr>
<tr>
<td>CD</td>
<td>Challenge Dose</td>
</tr>
<tr>
<td>CDT</td>
<td><em>Clostridium difficile</em> Binary Toxin</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>cm²</td>
<td>Centimetres Squared</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CROP</td>
<td>Combined Repetitive Oligopeptide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DOH</td>
<td>Department of Health</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>Concentration to Protect 50% Cell Monolayer</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immunoassay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Fv</td>
<td>Final Volume</td>
</tr>
<tr>
<td>g/L</td>
<td>Grams per Litre</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HPA</td>
<td>Health Protection Agency</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IgY</td>
<td>Immunoglobulin Y</td>
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IQR     Inter-Quartile Range
kDa     Kilo Dalton
Kg     Kilogram
K-W     Kruskal-Wallis Statistical Test
LC₅₀     Toxin Concentration at 50% Cell Rounding
M     Moles
mAb     Monoclonal Antibody
mau     Milli Absorbance Units
mg     Milligram
MIC     Minimum Inhibitory Concentration
mL/min     Millilitres per Minute
mM     Millimoles
mmol/L     Millimoles per Litre
MPh     MicroPharm
MRSA     Methicillin Resistant Staphylococcus Aureus
MW     Molecular Weight
MWCO     Molecular Weight Cut-off
M-W     Mann-Whitney Statistical Test
NaCl     Sodium Chloride
nm     Nanometer
NRA     Neutral Red Assay
NSS     Normal Sheep Serum
OD     Optical Density
OD₂₈₀     Optical Density at a Wavelength of 280 nm
PBS     Phosphate Buffered Saline
PBSa     Dulbecco’s Phosphate Buffered Saline
PBST     Phosphate Buffered Saline with Tween
PBW     Phosphate Buffer with 0.5M Sodium Chloride
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PcAb</td>
<td>Polyclonal Antibodies</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed Field Gel Electrophoresis</td>
</tr>
<tr>
<td>PHE</td>
<td>Public Health England</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per Million</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose Nucleic Acid</td>
</tr>
<tr>
<td>SCS</td>
<td>Sodium Citrate Buffered Saline</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
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<tr>
<td>SSAC</td>
<td>Small Scale Affinity Chromatography</td>
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<td>TcdA</td>
<td><em>Clostridium difficile</em> Toxin A</td>
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<tr>
<td>TcdB</td>
<td><em>Clostridium difficile</em> Toxin B</td>
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<tr>
<td>TcsH</td>
<td><em>Clostridium sordellii</em> Haemorrhagic Toxin</td>
</tr>
<tr>
<td>TcsL</td>
<td><em>Clostridium sordellii</em> Lethal Toxin</td>
</tr>
<tr>
<td>TFF</td>
<td>Tangential Flow Filtration</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TxA4</td>
<td>Recombinant Fragment of TcdA</td>
</tr>
<tr>
<td>TxB4</td>
<td>Recombinant Fragment of TcdB</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine Diphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>Vab</td>
<td>Volume of Antibody</td>
</tr>
<tr>
<td>Vcd</td>
<td>Volume of Challenge Dose</td>
</tr>
<tr>
<td>VPI</td>
<td>Virginia Polytechnic Institute</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin Resistant Enterococci</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per Volume</td>
</tr>
<tr>
<td>WFI</td>
<td>Water for Injection</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per Volume</td>
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<td>w/w</td>
<td>Weight per Weight</td>
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CHAPTER ONE

INTRODUCTION
1.1 Intestinal Environment and Flora

The human gastrointestinal tract is colonised by an abundant and diverse community of hundreds of different species of bacteria (Robles Alonso and Guarner, 2013). Whilst much is known about some of these species, many remain to be characterised. Thus, it has been found that 40% to 80% of the bacteria that can be seen by microscopic examination of diluted faecal samples cannot be cultured in the laboratory (Suau et al., 1999; Guarner and Malagelada, 2003). Recently, the application of molecular biological techniques has enabled researchers to identify many more of these previously unknown microbes, with the current known strains numbering in excess of 1,000 (Sekirov et al., 2010; Robles Alonso and Guarner, 2013).

The low numbers (approximately $1 \times 10^1$ to $1 \times 10^3$ colony forming units [CFU] per gram of luminal content) of microbes in the stomach and duodenum reflect the acidic and proteolytic nature of the luminal contents, which effectively inactivates most microbes. Continuous peristaltic activity along the small intestine (jejunum and ileum) also impedes bacterial adherence and colonisation (Guarner and Malagelada, 2003). Higher numbers of colonising microbes are found in the ileum with CFU densities of $1 \times 10^4$ to $1 \times 10^7$ per gram of luminal contents (Guarner and Malagelada, 2003). In contrast to the upper gastrointestinal tract, the large intestine (colon) is colonised by a vast number of bacteria at approximately $1 \times 10^{11}$ to $1 \times 10^{12}$ CFU per gram of luminal content (Guarner and Malagelada, 2003; O'Hara and Shanahan, 2006). In fact, bacteria make up approximately 60% of the dry weight of human faeces (Stephen and Cummings, 1980). These numbers are analogous to those of bacteria cultured under optimum conditions on laboratory plates. Moreover, the number of bacterial cells present in the gut lumen is approximately 10 times greater than the total number of eukaryotic cells that comprise the human body (O'Hara and Shanahan, 2006; Suau et al., 1999; Guarner and Malagelada, 2003).
The foetal gut is sterile, but colonisation of the gastrointestinal tract of newborns begins immediately after birth. The type of delivery (vaginal versus caesarean section) and diet (breast fed versus bottle fed) affects the colonisation pattern, but enterobacteria and bifidobacteria represent the early colonisers (Long and Swenson, 1977). It has been suggested by Hooper et al, (2001), that these pioneering bacteria are able to modulate gene expression in the host to create a more suitable environment for themselves whilst inhibiting colonisation by other species of bacteria.

Bacteriological analysis of faecal flora has shown that anaerobic bacteria outnumber their aerobic counterparts by a factor of 100 to 1000 (Guarner and Malagelada, 2003; Xu and Gordon, 2003). The anaerobic genera Bacterioides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus and Ruminococcus are reported to be the predominant bacterial species found in the human colon with the facultative anaerobic genera Escherichia, Enterobacter, Enterococcus, Klebsiella and Lactobacillus found amongst the subdominant species (Xu and Gordon, 2003; Suau et al., 1999; Salminen et al., 1998). The ratios of the predominant microbes colonising the colon vary greatly between individuals and some of these organisms have the potential to cause infection and sepsis under certain conditions, for example when the integrity of the bowel barrier is physically or functionally breached (Guarner and Malagelada, 2003). However, the continuous interaction between host and colonising microbes can infer important health benefits to the human host (Salminen et al., 1998). Thus, studies carried out using gnotobiotic animals (those bred under germ free conditions or colonised by one or more known species of microbe) suggest that gut flora have several important metabolic, trophic and protective functions (Xu and Gordon, 2003; Suau et al., 1999).
1.1.1 Metabolic functions of the gut flora

The fermentation of otherwise non-digestible dietary and endogenous residues, such as large polysaccharides and mucus, is the most important metabolic function of the intestinal microbiota. The microbial community provides a variety of enzymes and biochemical pathways that are not naturally available to the host and result in a far greater recovery of metabolic energy (in the form of short-chain fatty acids) and absorbable substrates for the host whilst also furnishing the microbiota with a supply of energy and nutrients for growth and proliferation. Colonic microorganisms also have an important role in vitamin synthesis and in absorption of calcium, magnesium and iron (Guarner and Malagelada, 2003).

1.1.2 Trophic functions of the gut flora

Short-chain fatty acids, produced by the activities of the gut microbiota, have been shown to have a trophic effect on intestinal epithelial cells (Frankel et al., 1994). In studies with gnotobiotic rats, non-colonised animals had fewer Crypts of Lieberkuhn (tubular invaginations around villi), stem cells and a lower cell production rate than animals colonised by the normal diversity of microbiota. This indicates that bacteria found in the intestinal lumen play a role in cell proliferation and differentiation in the colon (Alam et al., 1994).

1.1.3 Protective functions of the gut flora

The intestinal mucosa is the site of primary interaction between host and colonising microbiota, where the bacterial community is separated from the host’s systemic
circulation by just a single layer of epithelial cells (Shanahan, 2002). Adherent non-pathogenic (commensal) bacteria influence the development and function of the mucosal immune system with many diverse interactions between microbes, epithelial and gut associated lymphoid tissue being involved in modelling the memory mechanisms of systemic immunity. For example, studies using gnotobiotic animals have shown that non-colonised intestinal tracts contain low densities of lymphoid tissue compared to those populated with the normal diversity of microbiota (Alam et al., 1994; Frankel et al., 1994). In addition to their immunogenic role, communities of adherent commensal bacteria tend to out-compete any opportunistic and potentially pathogenic species for both binding sites and nutrients, thereby restricting their growth. The resident microbiota functions as a barrier, which can prevent attachment and subsequent entry of invasive pathogens into the epithelial cells (Shanahan, 2002). This equilibrium between species of resident bacteria forms a stable microbial population within the same individual under normal conditions. However, use of antibiotics can disrupt the ecological balance and allow overgrowth of pathogenic species such as toxigenic Clostridium difficile (O'Hara and Shanahan, 2006).

1.2 Clostridia

First described in 1880 by Prazmowski, the clostridia are anaerobic, Gram positive, endospore forming bacteria that make up one of the largest of all the bacterial genera with 120 currently described species (Rood et al., 1997). The natural habitat of this genera is the soil, where obligate anaerobes exist in areas made anoxic (oxygen free) by facultative organisms metabolising organic compounds. Some members of this group produce exo-toxins which include some of the most lethal molecules currently known (Stephen and Pietrowski, 1986; Proft, 2013). The role that the clostridial toxins play in the natural habitat of the organism is not currently understood. However, these organisms are also found naturally in the anoxic colon of the mammalian intestinal tract where they are
capable of causing severe disease in humans and animals under certain conditions (Rood et al., 1997; Keto-Timonen et al., 2006; Madigan et al., 2009).

A number of the clostridia species are of great biotechnological importance owing to unique metabolic pathways which enable the microbes to ferment various substrates, including sugars and amino acids, to produce acetone and butanol (Bahl and Dürre, 2001). At one time acetone-butanol fermentation by clostridia was the main commercial source of these products. Clostridia are also utilised to produce commercial enzymes such as amylase, cellulase and pectinase, with *Clostridium histolyticum* used to produce collagenase for use as a medical treatment (Bahl and Dürre, 2001).

Some clostridial species are of great medical importance, including *Clostridium botulinum*, *Clostridium tetani*, *Clostridium perfringenes* and *Clostridium difficile* (Table 1.1). These clostridia are metabolically similar to other species of their genera, however they are distinct in that they are capable of producing one or more powerful exotoxins (Durre, 2005; Madigan et al., 2009; Rood et al., 1997).

### 1.3 *Clostridium difficile*

First described by Hall and O’Toole (1935), this Gram positive, endospore forming, obligate anaerobe was originally named *Bacillus difficilis* due to the problems encountered in its culture (Wilcox, 2006). *B. difficilis* was first isolated from the meconium and stools of neonates and in a follow up study by Snyder (1937), it was detected in 10% of infant faecal specimens (Brazier, 1998). These early investigators also demonstrated that filtrates taken from the broth culture of *B. difficilis* were lethal to mice and appeared to contain a component only 10 to 100 times less potent than *C. botulinum* toxin (Bartlett, 2008). Later, *B. difficilis* was correctly assigned to the genus *Clostridium* and in the early 1970’s *C. difficile* began to be recognised as a pathogen of medical importance (Brazier, 1998).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Disease</th>
<th>Toxin or Factor</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Botulism</td>
<td>Neurotoxins</td>
<td>Flaccid paralysis</td>
</tr>
<tr>
<td><em>Clostridium difficile</em></td>
<td>Pseudomembranous colitis</td>
<td>Toxin A</td>
<td>Enterotoxin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toxin B</td>
<td>Cytotoxin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Binary Toxin</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>Gas gangrene, food poisoning</td>
<td>α-Toxin</td>
<td>Haemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-Toxin</td>
<td>Haemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ-Toxin</td>
<td>Haemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>δ-Toxin</td>
<td>Haemolysis (cardiotoxin)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>κ-Toxin</td>
<td>Collagenase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>λ-Toxin</td>
<td>Protease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterotoxin</td>
<td>Alters permeability of intestinal epithelium</td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td>Tetanus</td>
<td>Neurotoxin</td>
<td>Spastic paralysis</td>
</tr>
</tbody>
</table>

Table 1.1 Clostridia of Medical Importance
Compiled from Madigan et al. (2009) and Voth and Ballard (2005)
The microbiota of the human gastrointestinal tract functions as a barrier which can help prevent attachment and subsequent entry of invasive pathogens into epithelial cells. An equilibrium between species of resident bacteria and the host allows a stable microbial population to form within the individual under normal conditions. However, use of antibiotics can alter the microbial balance resulting in a loss of microbial diversity (dysbiosis) and allowing colonisation by *C. difficile* (Guarner and Malagelada, 2003; O'Hara and Shanahan, 2006). Thus, *C. difficile* infection (CDI) has become the most common hospital acquired (nosocomial) disease in patients treated with broad spectrum antibiotics, such as clindamycin and cephalosporins. Symptoms of CDI range from mild, self-limiting diarrhoea to life threatening pseudomembranous colitis and toxic megacolon (Brazier, 1998; Mitchell and Gardner, 2012). Additional pre-disposing risk factors for CDI include advanced age, severe underlying illness and use of proton pump inhibitors (Loo et al., 2011).

*C. difficile* occurs in both toxigenic and non-toxigenic strains. The former produce at least one of two large exotoxins which are referred to as toxin A (TcdA) and toxin B (TcdB). TcdA has a molecular weight of 308 kDa and is known as an enterotoxin due to it’s activity in a rabbit ileal loop assay (Wilcox, 2006). TcdB has a molecular weight of 270 kDa and is a potent cytotoxin. Both toxins are glucosyltransferases that inactivate Rho, Rac and Cdc42 proteins within target cells (Wilcox, 2006; Voth and Ballard, 2005). Diarrhoea associated isolates normally produce both TcdA and TcdB, although variants have been identified which produce only TcdB. These variants are known as toxinotype A-B+ (Voth and Ballard, 2005). Non-toxigenic strains produce no detectable toxin, as determined by antibody based enzyme immunoassay and cytotoxicity assay, and do not cause disease (Wilcox, 2006; Voth and Ballard, 2005). Thus, CDI is a toxin mediated disease.
1.4 *Clostridium difficile* Toxin Structure and Mechanism of Action

The main virulence factors of *C. difficile*, TcdA and TcdB, are encoded by genes *tcdA* and *tcdB* on the 19.6 kb pathogenicity locus (Figure 1.1). Regulatory genes (*tcdC* and *tcdD*) are also present along with a gene (*tcdE*) which encodes a protein thought to act as a porin involved in the permeabilisation of the *C. difficile* cell wall and release of TcdA and TcdB (Loo *et al.*, 2011). The *tcdC* gene is transcribed in the opposite direction to the main toxin genes and is expressed to a higher degree in the early exponential growth phase and decreases towards the stationary phase. The decrease in *tcdC* expression coincides with a greater expression of *tcdD* and subsequent toxin genes. This occurs during the late log and stationary phases of cell growth and is thought to be in response to a range of poorly understood environmental stimuli (Voth and Ballard, 2005). Thus, the production and release of toxin appears to be dependant on a lower expression of tcdC (negative regulator) and an increased expression of tcdD (positive regulator) and tcdE.

The TcdA and TcdB toxin proteins are remarkably similar in both structure and function (Figure 1.2). Both comprise an enzymatic domain located at the N-terminus, followed by a cysteine protease domain, a central “transmembrane” domain and a receptor binding domain at the C-terminus (Genth *et al.*, 2008). The structure may be considered as two distinct regions, the glucosyltransferase domain (located at the N-terminus) with the remaining structure termed the delivery domain (Genth *et al.*, 2008). The enzymatic region of TcdA and TcdB encompass amino acid (aa) residues 1 to 542 and 1 to 543 for each respectively and are reported to have a sequence homology of 74%, which explains the similarity in their substrate specificity (Voth and Ballard, 2005; Genth *et al.*, 2008; Giesemann *et al.*, 2008). The cysteine protease domain is located close to the auto-cleavage site of the N-terminus and is conserved in both toxins (Popoff and Geny, 2011).
Figure 1.1  The arrangement of genes along the *C. difficile* pathogenicity locus (Voth and Ballard, 2005)
Figure 1.2 Proposed protein domain structure for TcdB (Genth et al., 2008).
The central domain forms the majority of the toxin protein, encompassing aa 767 to 1850 (TcdA) and 767 to 1852 (TcdB), and includes a small hydrophobic region, which is believed to be involved with membrane insertion during translocation (Giesemann et al., 2008; Popoff and Geny, 2011). The receptor binding domain at the C-terminus, located at aa 1850 to 2710 (TcdA) and aa 1852 to 2366 (TcdB), is composed of many short, repeating amino acid sequences of between 21 to 50 residues in length (Voth and Ballard, 2005; Maynard-Smith et al., 2014). These repeating sequences are termed Combined Repetitive Oligopeptides (CROPs) and are present in five distinct groups on both toxins, with four CROP groups on TcdB showing a high degree of homology to those found on TcdA (Voth and Ballard, 2005).

The receptor binding domain of TcdA is believed to recognise the tri-saccharide Gal-α1-3Gal-β1-4GlcNac. However, as yet the binding specificity of TcdB remains unknown (Voth and Ballard, 2005; Popoff and Geny, 2011). Once bound to its target site, the central transmembrane region is believed to trigger endocytosis into the target cell (Figure 1.3). The acidic environment of the endosome then induces a structural rearrangement of the central region of the toxin allowing the transmembrane domain to form a pore in the endosome wall. The glucosyltransferase domain passes through this pore into the cytosol and is then cleaved off from the delivery domain of the protein by the cysteine protease (Genth et al., 2008). Co-factors present in the cytosol, such as inositol phosphate (InsP-6), are believed to play a role in the activation of the protease and facilitate cleavage of the active toxin fragment (Giesemann et al., 2008). The free glucosyltransferase domain contains an aspartate-any amino acid-aspartate (D-X-D) sequence, along with a conserved tryptophan, which is involved in the coordination of the sugar donor UDP-glucose. This enzymatic toxin fragment transfers the glucose to a threonine residue on low molecular mass GTP-binding proteins of the Rho sub-family (Thr-37 in RhoA). This results in irreversible functional inactivation as the signalling protein is no longer able to couple to effector and regulatory proteins. The Rho, Rac and Cdc42 proteins regulate actin cables,
Figure 1.3 Theoretical model of TcdA and TcdB toxin uptake, processing and release of the glucosylating enzymatic region into cellular cytosol. Modified from Giesemann et al. (2008)
formation of lamellipodia protrusions, membrane ruffles and trigger filopodial extensions at the cell periphery (Genth et al., 2008). Following TcdA or TcdB intoxication of a host cell, the integrity of the actin cytoskeleton is lost resulting in cell rounding and destruction of the barrier function of intestinal tight junctions (Genth et al., 2008; Voth and Ballard, 2005). Loss of the mucosal barrier function and increased permeability of the intoxicated host cell wall allows the influx of fluids into the intestine, resulting in secretory diarrhoea, and acts as a conduit for neutrophil recruitment to the infected site. Mast cells and macrophages produce and release inflammatory mediators at the infected site which adds further to the pathologies observed with pseudomembranous colitis (Voth and Ballard, 2005; Genth et al., 2008).

Up to 35% of all toxigenic strains of C. difficile produce a binary toxin (CDT) in addition to TcdA and TcdB (Popoff et al., 1988; Goldenberg and French, 2011). Unlike the two main toxins, this additional virulence factor is encoded on the CDT locus (cdtLoc) and is expressed throughout all phases of bacterial growth (Popoff et al., 1988; Carman et al., 2011). CDT is similar in structure and function to C. botulinum C2 toxin and comprises two independently expressed polypeptide components, cdtA (48 kDa) and cdtB (75 kDa), which are initially unlinked and non-functional (Popoff et al., 1988; Carman et al., 2011). When exposed to a serine protease (such as trypsin), a peptide is cleaved from cdtB enabling it to link with cdtA to form the active binary toxin which is enterotoxic and has been shown to cause cytopathic effects in-vitro (Popoff et al., 1988; Sundriyal et al., 2010; Carman et al., 2011). In its active form, the enzymatic cdtA component is a mono ADP-ribosyltransferase whilst the cdtB component functions as a binding and translocation protein. Once internalised into target cells, CDT functions in a similar manner to TcdA and TcdB, but is specific only to G-actin. Disruption to the actin cytoskeleton caused by CDT interrupts the metabolic processes of the cell causing cell rounding, death and nutrient release into the surrounding environment (Carman et al., 2011). It has been suggested that CDI caused by CDT producing strains may exhibit increased severity (Goldenberg and
French, 2011). There is also some evidence that TcdA and TcdB negative, binary toxin-positive strains (A’B’C’) of *C. difficile* are capable of causing disease (Elliott *et al.*, 2009). Although the specific role of CDT in the clinical manifestations of CDI is currently not well understood, it is believed to aid *C. difficile* colonisation of the colon by inducing microtubule based protrusions from the intestinal epithelium (Carman *et al.*, 2011). The release of nutrients from CDT intoxicated host cells may contribute to oxygen depletion from tissues around the site of infection, thereby resulting in a more anoxic environment conducive to colonisation and proliferation of *C. difficile* or other microorganisms of medical importance.

1.5 *Clostridium difficile* Ribotype and Toxinotype Classifications

Various methods exist to differentiate between strains of *C. difficile* including serotyping, immunoblotting, pulsed-field gel electrophoresis (PFGE), arbitrarily primed polymerase chain reaction (AP-PCR) and restriction endonuclease analysis (O'Neill *et al.*, 1996; Stubbs *et al.*, 1999). In 1993, a modified version of the AP-PCR technique was developed based on amplification of the spacer region between the 16S and 23S ribosomal RNA genes of *C. difficile* (Gürtler, 1993). Using this PCR ribotyping method, it was discovered that the absence or presence of specific variable length RNA spacer regions differed between *C. difficile* strains (Gürtler, 1993). In 1995, Gürtlers’ method was further refined to simplify and expedite the process, thereby allowing for the analysis of larger numbers of samples and underpinning its routine use across clinical laboratories (O'Neill *et al.*, 1996). The PCR ribotyping technique has now become the method of choice, due to its superior discriminatory power and reproducibility, and was used by the Anaerobic Reference Laboratory (Cardiff, UK) to compile the first *C. difficile* ribotype library in 1999 (O'Neill *et al.*, 1996; Stubbs *et al.*, 1999). This pioneering library initially identified 116 different ribotypes based on the analysis of over 2,000 *C. difficile* isolates obtained from
various sources including hospital patients, hospital environment, veterinary practice, general community and from official reference collections (Stubbs et al., 1999). It was discovered that the predominant strain causing CDI in hospital patients at that time was ribotype 001, which was detected in 55% of CDI patient samples (Stubbs et al., 1999).

In addition to the ribotyping technique, small variations in \textit{C. difficile} toxin genes can be used to differentiate between specific sub-types of the bacteria. The toxin gene variants are known as toxinotypes and 31 distinct toxinotypes have been identified thus far (Rupnik, 2010). The reference strain of \textit{C. difficile} (VPI 10463) is designated toxinotype 0, while the variants are designated from toxinotype I to XXXI (Rupnik et al., 2001; Rupnik, 2010). Based on their toxinotype (Table 1.2), sub-strains of \textit{C. difficile} may be capable of producing both TcdA and TcdB (A$^+$B$^+$), they may produce no TcdA but still express TcdB (A$^-$B$^+$) or they may be non-toxigenic (A$^-$B$^-$) (Rupnik et al., 2001; Rupnik, 2010). These toxinotypes may or may not also produce CDT, in fact some A$^-$B$^-$ strains are capable of expressing CDT (Elliott et al., 2009; Rupnik, 2010).

1.6 Clinical Manifestations

Typically, CDI affects hospitalised patients over the age of 65 who have received broad-spectrum antibiotics. The protective intestinal microbiota are disrupted by the antibiotics and, as the immune response of the elderly is often impaired (Simor et al., 2002), \textit{C. difficile} can colonise unchallenged (Adams and Mercer, 2007). The clinical symptoms of CDI range from mild, self-limiting episodes of diarrhoea to fulminant colitis, which may lead to bowel perforation, sepsis and death (Table 1.3) (Adams and Mercer, 2007; Baldoni et al., 2014). An increasing number of patients suffer from a refractory form of CDI characterised by prolonged diarrhoea that is resistant to conventional therapy (Adams and Mercer, 2007). The ‘in hospital’ mortality rate for patients infected with the
<table>
<thead>
<tr>
<th>PCR Ribotype</th>
<th>Toxin Production (A / B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>+ / +</td>
</tr>
<tr>
<td>002</td>
<td>+ / +</td>
</tr>
<tr>
<td>005</td>
<td>+ / +</td>
</tr>
<tr>
<td>014</td>
<td>+ / +</td>
</tr>
<tr>
<td>015</td>
<td>+ / +</td>
</tr>
<tr>
<td>016</td>
<td>+ / +</td>
</tr>
<tr>
<td>017</td>
<td>- / +</td>
</tr>
<tr>
<td>020</td>
<td>+ / +</td>
</tr>
<tr>
<td>023</td>
<td>+ / +</td>
</tr>
<tr>
<td>026</td>
<td>+ / +</td>
</tr>
<tr>
<td>027</td>
<td>+ / +</td>
</tr>
<tr>
<td>078</td>
<td>+ / +</td>
</tr>
<tr>
<td>106</td>
<td>+ / +</td>
</tr>
</tbody>
</table>

Table 1.2: Predominant *C. difficile* Ribotypes and Associated Toxin Production
Compiled from Rupnik *et al.* (2001) and Health Protection Agency (2014).
<table>
<thead>
<tr>
<th>Categories</th>
<th>Clinical Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>Three to five bowel movements daily for less than 5 days. Often self-limiting.</td>
</tr>
<tr>
<td>Moderate</td>
<td>Three to five bowel movements daily for 5 to 10 days.</td>
</tr>
<tr>
<td>Severe</td>
<td>Diarrhoea (often accompanied by abdominal pain, distension and tenderness) for more than 10 days.</td>
</tr>
<tr>
<td>Refractory</td>
<td>Diarrhoea persisting for more than 10 days despite adequate antibiotic therapy.</td>
</tr>
<tr>
<td>Recurrent</td>
<td>Two or more episodes of diarrhoea that initially respond to antibiotics, but not long term.</td>
</tr>
<tr>
<td>Complicated</td>
<td>Approximately 3% of patients develop one or more complications including, peritonitis, paralytic ileus, toxic megacolon and fulminant pseudomembranous colitis.</td>
</tr>
</tbody>
</table>

Table 1.3 Categories of *C. difficile* Infection and their Clinical Manifestations
Compiled from Adams and Mercer (2007)
more virulent, toxigenic strains of *C. difficile* ranges from 8% to 37% (Mitchell and Gardner, 2012), and the recurrence rate is also high, with between 15% to 25% of patients experiencing a relapse of symptoms after withdrawal of specific treatments (Kyne and Kelly, 2001). Of those patients who suffer a relapse, up to 65% are likely to suffer from multiple recurrences (Kyne and Kelly, 2001). It is unclear whether these relapses are due to incomplete initial treatment of the infection or are re-infections due to the persistence of *C. difficile* spores in the patient’s environment. It is likely that the dysbiosis in the patient’s colon, following antimicrobial treatments, will aid re-colonisation of *C. difficile* from the environment due to lack of competition for attachment sites.

1.7 The Burden of *Clostridium difficile* Infection on the Healthcare System

Over the past decade highly virulent, epidemic strains of *C. difficile* have emerged and have been implicated with CDI cases becoming more frequent, more severe and increasingly difficult to treat (Vardakas *et al.*, 2012; Wiegand *et al.*, 2012). Reported CDI cases in the UK rose sharply from approximately 20,000 in the year 2000 to 56,000 in 2007 (Health Protection Agency, 2013). Epidemic strains exhibit several enhanced virulence factors including multidrug resistance and increased sporulation capacity (Dubberke and Wertheimer, 2009). One particularly virulent strain (*C. difficile* NAP1/027, known as ribotype 027) has been reported to produce 16 times more TcdA and 23 times more TcdB than conventional strains, due to a deletion of the *tcdC* negative regulator gene. It also expresses CDT in addition to the two main toxins (Kuijper *et al.*, 2006; Dubberke and Wertheimer, 2009). These enhanced virulence factors explain the observed rise in numbers and severity of CDI cases over this period and, with increasing multidrug resistance, indicates why the disease has become more refractory to treatment.

Although the vegetative cells of *C. difficile* become non-viable within minutes after exposure to air, their spores are extremely robust and can persist for several months in the
hospital or healthcare facility environment (Dubberke and Wertheimer, 2009). Spores are shed in vast numbers in the stools of infected patients, resulting in contamination of patient’s skin, bedding and clothing (Guerrero et al., 2012). As the spores are resistant to alcohol hand rubs and many disinfectants, the healthcare environment becomes contaminated and effectively acts as a reservoir for *C. difficile*, with spores being recoverable from 20% to 70% of surfaces (Wilcox, 2006; Dubberke and Wertheimer, 2009; Wilcox et al., 2011). There is also some evidence that demonstrates the aerial dissemination of *C. difficile* spores within the healthcare environment (Roberts et al., 2008). It is the spores that are responsible for the transmission of *C. difficile* between patients and, although individuals may pick up spores directly from contaminated surfaces, it is believed that an important vector for transmission is on the gloved hands of healthcare workers (Dubberke and Wertheimer, 2009; Guerrero et al., 2012).

CDI, and any relapse, will inevitably increase a patient’s length of stay in hospital and contribute to healthcare costs. Little data is available on actual healthcare costs directly attributable to CDI. However, a recent study conducted in the USA estimated a cost of $4.8 billion to US acute care facilities in 2008 (Dubberke and Olsen, 2012). A European study estimated the comparative costs per patient to be between £4,577 (in Ireland) and £8,843 (in Germany) with the cost per patient in the UK estimated at £6,986 (Wiegand et al., 2012).

In the USA, CDI was listed as the primary cause of death for 793 patients in 1999, rising dramatically to 7483 in 2008 (Lessa et al., 2012). In the UK, there has been a steady reduction in reported CDI cases since 2007, falling from ~38,000 in 2008 to ~13,000 in 2012 (Health Protection Agency, 2013). This is likely due to the implementation of improved diagnostic assays, prompt CDI patient isolation strategies and more effective sporicidal cleaning regimes (Department of Health and Health Protection Agency, 2008; Shapey et al., 2008; Gouliouris et al., 2009; Wilcox et al., 2010). Following the peak in reported UK infections in 2007, the predominant strains associated with CDI have changed
Ribotype 027 was detected in more than 60% of CDI patients across healthcare institutions in England in 2007. However, this has steadily decreased to less than 10% in 2013 over the same region whilst the incidence of previously uncommon ribotypes has increased correspondingly (Health Protection Agency, 2014).

1.8 Prevention and Control of *Clostridium difficile* Infection

Since the emergence of the 027 strain of *C. difficile*, new guidance has been issued by the Health Protection Agency (HPA) and Department of Health (DOH) providing information on appropriate treatment algorithms, isolation of infected patients from the uninfected hospital population and decontamination of isolation rooms following discharge of CDI patients (Department of Health and Health Protection Agency, 2008). Good antimicrobial stewardship has become important in the prevention and management of CDI because any antibiotic that can disrupt the intestinal microbiota, but does not suppress *C. difficile*, has a great potential to cause or prolong a case of CDI (Owens et al., 2008). It was recommended in the 2008 guidelines that all healthcare trusts should establish antimicrobial management teams to ensure the prudent use of antibiotics and to restrict the use of clindamycin, cephalosporins, fluoroquinolones, carbapenems and prolonged courses of aminopenicillins (Department of Health and Health Protection Agency, 2008).

It has been recommended that any patient with suspected or laboratory diagnosed CDI is immediately moved to an isolation room, with a self contained toilet. Anyone entering the isolation room, staff or visitors, should wear disposable gloves and apron and use soap and water to wash their hands before and after contact with the patient or their environment (Department of Health and Health Protection Agency, 2008). As *C. difficile* spores are sensitive to chlorine, the isolation room and toilet should be cleaned a minimum of once daily with a chlorine based disinfectant of at least 1000 ppm (Department of Health and Health Protection Agency, 2008). Following discharge of the patient, the room
Figure 1.4  Distribution of predominant *C. difficile* ribotypes across healthcare institutions in England from 2007 to 2013 (Health Protection Agency, 2014).
should again be thoroughly cleaned with a chlorine based disinfectant to eliminate remaining spores; however, a study by Shapey et al (2008) demonstrated that a higher level of \textit{C. difficile} spore removal can be achieved by using a vaporised hydrogen peroxide system (Department of Health and Health Protection Agency, 2008; Shapey \textit{et al.}, 2008). Given that CDI is transmitted by spores, prompt isolation of those infected followed by a thorough room decontamination to eliminate spores is likely to have contributed considerably to the decline in reported CDI cases since 2007.

1.9 Current Treatment Strategy

The current protocol for CDI treatment usually involves discontinuing any inciting broad spectrum antibiotic, if appropriate, after which mild symptoms resolve within a few days in approximately 23\% of cases (Dubberke and Wertheimer, 2009). If symptoms persist, or if there is any evidence of colonic inflammation, patients are normally given oral metronidazole (400 mg to 500 mg three times per day) or oral vancomycin (125 mg four times per day) for 7 to 10 days (Wilcox, 2006). Metronidazole is the preferred first line treatment as it is less expensive than vancomycin. Patients with more severe CDI, where peristalsis through the intestinal tract has been adversely affected (paralytic ileus) or those who cannot tolerate these antibiotics orally, may be given both metronidazole and vancomycin simultaneously by intravenous infusion (Wilcox, 2006). Patients with severe or complicated CDI that is refractory to treatment are likely to need surgical intervention to remove the infected colon, usually before their serum lactate level exceeds 5 mmol/L or the white blood cell count reaches 50 x 10^9 cells/L (Lamontagne \textit{et al.}, 2007).

Although the current treatment of CDI with metronidazole or vancomycin is successful in the majority of cases, the rate of treatment failure can be up to 22\% (Vardakas \textit{et al.}, 2012). The selection for metronidazole-resistant sub-populations of \textit{C. difficile} has been demonstrated \textit{in-vitro} and there is also evidence of emerging
metronidazole-resistance in some clinical isolates (Peláez et al., 2008). The susceptibility of *C. difficile* to vancomycin treatment remains high. In-fact the only report of vancomycin-resistance originated from an *in-vitro* experiment conducted in Poland in 1991 (Huang et al., 2009). However, the frequent or inappropriate use of vancomycin within the healthcare environment is undesirable due to valid concerns regarding the selection for vancomycin-resistant enterococci (VRE) (Baldoni et al., 2014). The spectrum of antimicrobial activity of metronidazole and vancomycin is also very broad, resulting in the destruction and further imbalance of protective intestinal microbiota (Baldoni et al., 2014).

An efficacious antibiotic for *C. difficile* should exhibit a narrow range of antimicrobial activity and should remain un-absorbed in the intestine, thereby limiting systemic exposure following oral administration (Baldoni et al., 2014). The steroid drug, fusidic acid, fulfills these criteria and has shown similar efficacy to metronidazole and vancomycin in clinical use (Huang et al., 2009). However, the use of fusidic acid has been shown to rapidly select for resistant *C. difficile* strains (Huang et al., 2009) and is therefore not appropriate as a routine treatment for CDI.

Linezolid was approved for clinical use in 2000 and was the first of the oxazolidinone class of antibiotics to be marketed (Vinh and Rubinstein, 2009). This drug is effective against both susceptible and multidrug-resistant Gram-positive pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), and demonstrates a near 100% bioavailability when administered in its oral form (Vinh and Rubinstein, 2009). Although linezolid was welcomed as a treatment option for CDI, its use has been restricted to severe cases following experimental evidence that resistance is likely to emerge quickly (Diekema and Jones, 2001).

Following its approval by the US Food and Drug Administration (FDA) in May 2011, fidaxomicin has become a new treatment option for CDI (Lancaster and Matthews, 2012). It has been reported that the minimum inhibitory concentration (MIC) for metronidazole and vancomycin against *C. difficile* is ~0.5 µg/mL, but increases to ~2
µg/mL when used against the 027 strain. However, the MIC for fidaxomicin against both 027 and non-027 strains remains constant at ~0.125 µg/mL (Lancaster and Matthews, 2012). When compared to metronidazole and vancomycin, the therapeutic use of fidaxomicin in CDI tends to result in a lower relapse rate and higher overall cure rate (Baldoni et al., 2014). This increased efficacy may be due, in part, to reduced non-specific destruction of intestinal microbiota (Mullane, 2014). However, while fidaxomicin (marketed as Dificlir in the UK) appears to be a valuable alternative to metronidazole and vancomycin, it is very expensive at $135 for a 200 mg tablet compared to $0.72 for a 500 mg dose of metronidazole (Lancaster, 2012). The cost of fidaxomicin treatment must be balanced carefully against the potential cost savings gained by a reduced number of hospital bed days and fewer interventions required per CDI patient. A recent analysis determined that using fidaxomicin as a routine first line treatment for CDI would not be cost effective, however, it may be more economically viable if deployed appropriately (Bartsch et al., 2013).

Cadazolid is the latest antibiotic of the oxazolidinone class and is currently in phase III clinical trials (Baldoni et al., 2014). Its structure is different to that of linezolid, in that it contains chemical structures common to both the oxazolidinone and fluoroquinolone classes of antimicrobial drug (Locher et al., 2014). This novel structure enables the drug to interfere with the early stages of bacterial protein synthesis, similar to linezolid, and also inhibit bacterial DNA synthesis, as do the fluoroquinolones (Locher et al., 2014). Cadazolid has a narrow range of antimicrobial activity and exhibits an MIC against C. difficile of 0.06 to 0.5 µg/mL; it has also been shown to inhibit toxin production and spore formation in-vitro (Baldoni et al., 2014). Being an orally administered drug with a low aqueous solubility, cadazolid has demonstrated low systemic exposure in animal studies and human clinical trials (Baldoni et al., 2014). Experimental evidence also indicates that cadazolid is unlikely to induce microbial resistance quickly (Locher et al., 2014). These
properties make cadazolid an important new drug for the treatment of CDI and it may have
great potential as a first line treatment option if clinical trials are successful.

Whilst antibiotics are used to inhibit \textit{C. difficile} growth or otherwise destroy the
bacteria, they do not eradicate toxins. Moreover, lysis of the bacteria may initially
exacerbate CDI symptoms in some cases, due to rapid release of intracellular toxin, and
potentially create favourable conditions for \textit{C. difficile} re-colonisation and subsequent CDI
relapse.

1.10 Alternative Treatments

1.10.1 Probiotics

The management of CDI becomes increasingly challenging when relapses occur,
particularly when the bacteria are refractory to antibiotic treatment and the normal
diversity of microbiota in the colon have been severely disrupted (Guo \textit{et al.}, 2012).

The growth of the normal intestinal microbiota may be stimulated by the taking of
non-digestible, fermentable foods (known as prebiotics) (Quigley, 2010). Prebiotics alone
are unlikely to offer any direct therapeutic benefit to patients with CDI, but research into
the use of probiotics (single or mixed cultures of live organisms) indicates some potential
as an adjunctive therapy alongside the current first line treatments (McFarland, 2009). The
strategy to re-populate the disrupted colon with probiotic strains, as a prevention
(prophylactic) or treatment for CDI, seems appropriate. Their presence may increase
colonisation resistance and aid in modulation of the mucosal immune system, some strains
even produce proteases which are capable of denaturing the toxins produced by \textit{C difficile}
(McFarland, 2009). A limited number of clinical trials using probiotic strains, such as
\textit{Saccharomyces boulardii, Lactobacillus casei, Streptococcus thermophilus and
Lactobacillus bulgaricus}, appear to demonstrate significantly fewer cases of relapse in
treated groups of patients with CDI compared to those receiving placebo (McFarland, 2009). However, these trials have been criticised for their poor design, low number of participants and lack of consensus on effective dose (Miller, 2009). Routine use of probiotics in the healthcare environment may also pose a small but considerable risk. Adverse reactions in patients are rare, but translocation of probiotic organisms and subsequent systemic infection has been documented and the transfer of antibiotic resistance genes is also a great threat (McFarland, 2009). There is an obvious need for further, more adequately controlled clinical trials of sufficient size before any clear conclusions can be drawn on the prophylactic or therapeutic use of probiotics. Moreover, the specific strains responsible for promoting colonisation resistance are, as yet, unknown (McFarland, 2009).

1.10.2 Faecal Transplant

A promising alternative therapy has been developed whereby patients suffering from severe CDI receive an infusion of commensal intestinal microorganisms (from the stool of a healthy donor) administered, via either a colonoscopic or nasojejunal tube, directly into the intestine (Gough et al., 2011; Guo et al., 2012; Petrof et al., 2013). This approach aims to re-populate the diseased intestine with the full spectrum of microbiota and quickly re-establish the normal diversity that was likely to have been lost through the antimicrobial treatment that preceded C. difficile colonisation. In relation to the efficacy of this approach, there is currently a paucity of evidence from clinical studies. However, the effectiveness of this faecal transplant therapy appears to be high with resolution of CDI symptoms being reported in 83% to 92% of cases following the first infusion (Gough et al., 2011; Guo et al., 2012). Stool donations from a healthy individual, related to and from the same household as the CDI patient, have been the preferred source as the diversity of their intestinal microbiota is likely to be similar to that of the patient. A recent study, however,
has proven the efficacy of using frozen faecal samples from healthy donors unrelated to the patient, with a cure rate of 90% (Youngster et al., 2014). It is reasonable to assume that repopulating the diseased intestine, by faecal transplant, effectively displaces *C. difficile* as the dominant microbe and could be a valuable alternative or adjunctive therapy for complicated, refractory or recurrent CDI cases.

Although this approach, like that of taking probiotics, aims to displace *C. difficile* rather than destroy it with antibiotics, the same problems associated with the post treatment persistence of active toxins remain. Thus, residual TcdA and TcdB must be neutralised by the patient’s already compromised immune system.

1.10.3 Toxin Binding Polymers

Drug companies continue to research new and better antimicrobial therapies for CDI, but these are unlikely to be the final solution due to their effects on intestinal microbiota, inability to neutralise toxin and because the constantly evolving *C. difficile* strains will inevitably develop antimicrobial resistance. One explored alternative was to administer an oral product that irreversibly adsorbed TcdA and TcdB, effectively neutralising their activity. The bile acid sequestrant, cholestyramine, acts as a strong anion exchange resin and has been used in Crohn’s disease to reduce the concentration of highly osmotic bile salts in the colon, thereby alleviating watery diarrhoea (Weiss, 2009). Some reports have suggested the efficacy in using cholestyramines to treat CDI, however no clinical evidence currently exists (Weiss, 2009). Cholestyramine may be considered as a treatment option of last resort in refractory or complicated CDI, but its ability to bind vancomycin poses a major drawback to routine use in CDI management (Weiss, 2009).

The high molecular mass, non-antimicrobial polymer, tolevamer, was designed by the Genzyme Corporation to specifically bind TcdA and TcdB with high affinity (Braunlin et al., 2004). Early studies with the polymer demonstrated efficacy in binding and
neutralising toxins in cell-based, rat ileal loop and hamster model assays (Braunlin et al., 2004). Phase III clinical trials of tolevamer concluded that, although it significantly reduced relapse rates in CDI patients, its effectiveness was inferior to that of metronidazole or vancomycin and further development of the polymer was therefore abandoned (Weiss, 2009). Being non-antimicrobial and therefore having no detrimental effect on host intestinal microbiota, the significantly reduced relapse rate associated with tolevamer treatment is likely the result of the normal gut flora becoming re-established, facilitated by the inactivation of TcdA and TcdB, thus displacing and inhibiting re-colonisation by C. difficile.

1.10.4 Active Immunisations (Vaccines)

Work relating to the active immunisation of humans against the risk of contracting CDI has been led by Acambis Inc (Cambridge, Massachusetts). A vaccine, comprising a mixture of partially purified TcdA and TcdB, was prepared and administered to healthy volunteers by intramuscular injection (Kotloff et al., 2001). The vaccine was well tolerated and over 90% of the subjects showed a marked elevation in circulating antibodies directed against both toxins (Kotloff et al., 2001). In a later study, three patients with recurring CDI were given the vaccine with beneficial results (Sougioultzis et al., 2005). Whilst vaccination may provide long term protection, the response was too slow to consider using this vaccine as a therapy immediately following a diagnosis of CDI. Moreover, the elderly, who are most at risk of contracting CDI, may fail to mount a normal antibody response due to immune senescence (Owens Jr, 2007).
1.10.5 Passive Immunisations

Rifkin et al. (1977) demonstrated the presence of a toxin in the stools of patients with antibiotic associated pseudomembranous colitis, which was neutralised by Clostridium sordellii anti-toxin. It was deduced that the toxin was most likely produced by C. sordellii. However, C. sordellii has never been implicated in cases of antibiotic associated pseudomembranous colitis and it is more likely that this was an early case of toxigenic C. difficile infection and that the C. sordellii anti-toxin cross reacted with the C. difficile toxins. In fact, according to Voth and Ballard (2005), C. sordellii haemorrhagic toxin (TcsH – an enterotoxin) is homologous to TcdA and C. sordellii lethal toxin (TcsL – a cytotoxin) is homologous to TcdB. Thus antibodies raised against C. sordellii toxin show cross reactivity with their C. difficile equivalents and vice versa.

In 1991, Lyerly and his colleagues raised antibodies against C. difficile toxins by immunising gestating cows with formalin treated C. difficile culture filtrates. The colostrum from these cows was collected at parturition and processed to produce an immunoglobulin concentrate which was then used in the well established hamster model of CDI. The hamsters were given the concentrate orally prior to, and at eight hourly intervals following the oral administration of a toxigenic strain of C. difficile. Animals given the immunoglobulins were protected against CDI, whilst untreated (control) animals developed diarrhoea within 2 to 7 days following exposure and died within 24 hrs of the onset of symptoms (Lyerly et al., 1991). When the treatment was stopped, the previously protected animals died within 72 hours, suggesting that the concentrate provided protection against the toxin, but did not eliminate the organism. Further, when treatment was restarted, following the emergence of symptoms, it offered no protection to the hamsters. This showed that treatment offered protection from CDI if given prophylactically, but was not able to treat an established infection. The researchers also commented that once CDI...
was established in the hamster, there was significant damage to the intestinal mucosa which facilitated rapid systemic intoxication (Lyerly et al., 1991). It is probable that orally administered polyclonal antibodies (PcAb) would not be sufficient to neutralise systemic *C. difficile* toxins, as they are not absorbed into the circulation. Systemically administered PcAb may show greater efficacy in cases of systemic intoxication. This might explain why the approach used by Lyerly *et al* (1991) failed to treat hamsters with established CDI.

Using the same hamster model, Kink and Williams (1998) showed that IgY based PcAb, raised in hens against recombinant fragments (C-terminus / receptor binding domain) of TcdA and TcdB, could be successfully used to prevent CDI if anti-TcdA was given prophylactically (via a feeding tube), or to treat an established infection if both anti-TcdA and anti-TcdB were administered orally (Kink and Williams, 1998). The demonstration that only anti-TcdA was required to prevent infection, if given prophylactically, supports the findings of Lyerly *et al.* (1991), and suggests that TcdA may be a pre-requisite for toxigenic strains of *C. difficile* to successfully colonise the intestine. It also indicates that antibodies directed against the receptor binding domains of both TcdA and TcdB were required to effectively treat an established case of CDI. Furthermore, Kink and Williams (1998) found that infected hamsters, which had been successfully treated with anti-toxin (40 mg anti-TcdA and 40 mg anti-TcdB per 80 g to 90 g animal), were protected against subsequent exposure to *C. difficile* and did not suffer any relapse of CDI symptoms when re-infected even after several months. This is in contrast to vancomycin treatments used in the same hamster model, where the relapse rate is 100% (Kink and Williams, 1998). These results seem to contradict the findings of Lyerly *et al.* (1991), although the immunogens, methods used to raise the antibodies and the antibody type (IgA versus IgY) were markedly different, which may account for the differences in efficacy. It is possible that the crude culture filtrates used in the earlier study may have lacked sufficient concentrations of TcdB. Thus, the PcAb from the colostrum may have lacked
specificity for TcdB which, therefore, would not have successfully treated the infected hamsters, as shown by Kink and Williams (1998).

A study by Salcedo et al. (1997) noted that two elderly CDI patients, who did not respond to standard antimicrobial treatment, appeared to show a rapid resolution of symptoms following an intravenous infusion of normal pooled human immunoglobulins which had been shown to neutralise C. difficile toxins \textit{in vitro} (Salcedo et al., 1997). This demonstrated that human serum/plasma contains PcAb directed against TcdA and TcdB that can be used to neutralise C. difficile toxins in patients with established CDI and by, doing so, the severity of CDI can be reduced or eliminated. The reason for the presence of such antibodies is that the donors would have been colonised by toxigenic C. difficile, usually asymptptomatically, in earlier life.

Investigating the use of human monoclonal antibodies (mAbs) directed against TcdA and TcdB, Babcock et al. (2006) identified two promising mAb candidates, CDA1 (anti-TcdA) and MDX1388 (anti-TcdB) both with specificity to the C-terminal receptor binding domain of their respective toxin. These mAbs were further assessed using the hamster model where CDA1 alone, MDX1388 alone, or both concurrently were administered, by intraperitoneal injection, before the animals were experimentally infected with C. difficile. The study concluded that CDA1 alone conferred early protection against the onset of disease, but this protection faded over a few days. MDX1388 alone offered no protection, but the combination of both mabs simultaneously provided an enhanced and longer lasting protection compared to CDA1 alone (Babcock et al., 2006). These researchers noted that whilst the MDX1388 mAb did not protect against toxin lethality \textit{in-vivo}, the use of goat anti-TcdB PcAb demonstrated a potent toxin neutralising effect with a hamster survival rate of 98% following systemic administration (Babcock et al., 2006).
1.11 Immunotherapy for *Clostridium difficile* Infection

There is growing evidence that naturally elevated host serum levels of antibodies, directed against *C. difficile* toxins, correlate with improved patient outcomes and reduced relapse rates (Kyne *et al.*, 2001; Leav *et al.*, 2010; Kelly and Kyne, 2011; Solomon *et al.*, 2013). These elevated levels of toxin neutralising PcAb are likely the result of an appropriate host immune response to the infection. As CDI is toxin mediated, neutralising PcAb may protect against tissue damage and subsequent symptoms, whilst the normal intestinal microbiota re-establishes and displaces *C. difficile* colonisation.

Infusions of normal pooled human immunoglobulins, shown to neutralise TcdA and TcdB *in vitro*, have proven successful in resolving intractable episodes of CDI where the patient’s own immune response was insufficient (Salcedo *et al.*, 1997; Wilcox, 2004; Abougergi and Kwon, 2011). Studies, using the hamster model for CDI, have also provided strong evidence that exogenous PcAb are capable of preventing the onset of symptoms and treating established infections (Lyerly *et al.*, 1991; Kink and Williams, 1998; Roberts *et al.*, 2012). These findings merit further investigation into the use of immunotherapy for prevention or treatment of CDI.

The routine use of normal pooled human plasma, as a source of toxin neutralising PcAb, is unrealistic due to factors such as donor screening, product availability and specific antibody concentrations which are likely to be variable or low. Active vaccines, such as those being developed by Sanofi Pasteur and Merck, whilst having potential value in some circumstances, are unlikely to be of any benefit as a therapy in CDI management due to reasons discussed in section 1.10.4. Fully human mAbs have been shown to neutralise toxins and exhibit a long circulating half life *in vivo*. However, their use for CDI management is unlikely to be successful primarily because of the many toxinotypes of *C. difficile* that exist, with each likely to present different epitopes on their toxin proteins.
which may limit the use of a successful mAb to a single strain. Moreover, as toxigenic strains of *C. difficile* evolve there are likely to be slight alterations in toxin protein structure over time, hence PcAb able to effectively bind many different epitopes of TcdA and TcdB are likely to be required. Further benefits of using PcAb over mAb include a greatly reduced cost of manufacture and increased production scale potential (Landon and Chard, 1995). Ideally, PcAb must be capable of binding and neutralising toxins present within the colon and in the systemic circulation. In mild, moderate or recurrent cases, PcAb may be administered orally as a treatment or, potentially, as prophylaxis for patients at high risk of contracting CDI. Systemic administration of PcAb would be the preferred method of delivery in severe, refractory or complicated cases because an orally administered product would be unlikely to reach the infected colon due to paralytic ileus. Also, once CDI becomes severe it is likely that toxins would have already translocated into the patient’s circulation where systemic administration of PcAb would be required to neutralise them. Given that fluids infiltrate the intoxicated colon in severe CDI, systemically administered circulating PcAb are also likely to permeate the site of infection and neutralise toxins at their source.

1.12 Ovine Antibody-Based Platform for Treatment of *Clostridium difficile* Infection

Great efforts are being made by researchers to find more effective means to eradicate *C. difficile*. However, elimination of *C. difficile* from an infected patient, either by antibiotics or displacement by microbiota, does not eliminate its toxins. Being the main virulence factor of the organism, residual active toxin can continue to damage colonic epithelial cells and may pre-dispose the patient to *C. difficile* re-colonisation and subsequent CDI relapse. The long term successful treatment of CDI may ultimately require a bilateral approach, whereby the organism is eradicated and its potent toxins are
neutralised. This could potentially be achieved by the co-administration of a suitable narrow spectrum antimicrobial agent along with efficacious toxin neutralising PcAb.

MicroPharm Ltd (MPh) have developed and marketed several immunotherapeutic drugs based on their well established ovine PcAb platform. Their product portfolio includes snake antivenins and drug antitoxins, all of which contain PcAb that neutralise their respective antigen resulting in positive clinical outcomes. The use of ovine PcAb, and their fragments, for use in immunotherapeutic applications has been well documented and shown to have a good safety profile (Dart and McNally, 2001; Pizon et al., 2007; Schaeffer et al., 2010). MPh, in partnership with Public Health England (PHE, formerly Health Protection Agency), have been investigating the application of this platform to produce a viable C. difficile antitoxin.

Recent studies published by the MPh, PHE partnership have demonstrated the efficacy of ovine derived PcAb in neutralising both TcdA and TcdB (Roberts et al., 2012; Maynard-Smith et al., 2014). In the first of two published studies, ovine derived IgG based PcAb, raised against formaldehyde inactivated natural TcdA and TcdB, were shown to either prevent or treat CDI in the hamster model (Roberts et al., 2012). Hamsters of between 80 g and 100 g were given either 2.5 mg or 25 mg of IgG, by intraperitoneal injection, two days before being experimentally infected with a hyper-virulent strain of C. difficile. Two further injections were given 1 and 4 days post infection. As found in previous studies, control animals (not receiving passive immunisation) developed severe CDI within 5 days of the infectious challenge. Of the animals receiving the 2.5mg dose, 40% survived compared to a 90% survival in the 25 mg group. The surviving animals remained asymptomatic for 21 days post challenge (i.e. the end of the experiment), supporting the results of Kink and Williams (1998). This study also demonstrated that hamsters given only anti-TcdA developed CDI, but at a slower rate than the control group, and animals given only anti-TcdB developed CDI at a similar rate to the control group (Roberts et al., 2012). This mirrors the findings of Babcock et al. (2006), but seems to
contradict those of Kink and Williams (1998). The reason for this discrepancy may be due to the method of antibody administration. Thus, Kink and Williams (1998) used orally administered IgY compared to the intraperitoneal injection of mAb by Babcock et al. (2006), and ovine PcAb by Roberts et al. (2012). This may indicate that systemic anti-TcdA IgG does not translocate easily into the intestine and that TcdA must be neutralised within the intestines, by orally administered immunoglobulins, to prevent the onset of CDI if anti-TcdA is given alone. It seems likely that the TcdA within the intestines would damage the mucosa leading to leakage of TcdA and TcdB from the intestine where TcdA, but not TcdB, would be neutralised. The fact that animals given only anti-TcdB developed CDI at a similar rate to the control group indicates that TcdA alone is capable of causing severe disease, probably once the intestinal mucosa is damaged and the toxin enters the circulation. The apparent difference in disease outcome between the two methods of antibody administration merits further investigation and may indicate an optimum method for delivery of an immunotherapy, based on the desired outcome, i.e. oral administration for prophylaxis and treatment of mild CDI or systemic administration for the treatment of more severe cases.

During the initial study (Roberts et al., 2012) it was discovered that immunogens prepared using active natural toxins did not result in the production of toxin neutralising PcAb, although toxin binding PcAb were present in high titres. When the natural toxins were inactivated, by treatment with formaldehyde, the resulting immunogens produced an antibody response with much greater toxin neutralising activity (Roberts et al., 2012). Recombinant fragments, based on the C-terminus region of TcdA and TcdB along with the N-terminus region of TcdB, also failed to stimulate a toxin neutralising immune response, although binding PcAb were again present in the resulting antisera (Roberts et al., 2012).

Due to the high cost of the natural toxins, it is preferable to utilise appropriate recombinant fragments in the formulation of immunogens. Thus, further studies were conducted into the development of recombinant fragments based on a variety of regions of
the natural toxins. Recombinant constructs were created (Figure 1.5) and separate immunogens were prepared using the purified fragments or their formaldehyde treated forms (Maynard-Smith et al., 2014). The highest neutralising titres were again observed from animals immunised using the formaldehyde treated fragments, although all immunogens were capable of stimulating high PcAb titres which could bind to their respective natural toxins. This study determined that formaldehyde treated recombinant fragments TxA4 and TxB4, when used to actively immunise sheep, produced the highest titres of toxin neutralising PcAb (Maynard-Smith et al., 2014).

Building on these two published studies, the aim of this project is to produce large volumes of antisera containing the highest achievable levels of toxin neutralising PcAb. Once the antibody production methods have been optimised, the resulting PcAb will ultimately be used commercially to produce an immunotherapy for patients with CDI. As with the previous study, sheep will be used as hosts for raising PcAb due to their relative ease of handling, availability and cost. To successfully achieve the desired aim, the following objectives were identified:

Objective 1 - to establish assays to measure the specific antibody response of sheep immunised with C. difficile toxins

Serum samples (antisera) from individual sheep immunised with C. difficile toxoids fragments TxA4 and TxB4 will be assessed at regular intervals for the presence of toxin specific PcAb. An enzyme immunoassay will be established to detect, semi-quantitatively, anti-TxA4 or anti-TxB4 which binds to its respective natural toxin. Levels of binding PcAb will be monitored in sheep over time.

A cell based cytotoxic neutralisation assay will also be established (and enhanced) to measure the neutralising activity of collected antisera samples. This quantitative assay will be used to determine the potency of each sample, in terms of weight of natural toxin neutralised per volume of antisera in order to monitor the neutralising potency over time.
Figure 1.5  
*C. difficile* TcdB structure and recombinant fragment map (Modified from Rupnik et al. (2009), Davies et al. (2011) and Maynard-Smith et al. (2014))
Objective 2 - to initiate a sheep immunisation programme and maximise the levels of specific neutralising antibody attained

Sheep will be immunised using the antigens identified by Maynard-Smith et al. (2014) as these have been previously shown to elicit a high level of toxin neutralising PcAb. Thus, formaldehyde treated recombinant fragments TxA4 and TxB4 will be utilised as immunogens in the present study. The immunisations will be mono-specific; therefore, two separate groups of sheep will be used, each group receiving one of the antigens.

The two flocks of sheep will each be sub-divided into five groups. The immunogen will then be administered over a range of doses, one dose per sub-group. Using the assessment techniques established in Objective 1, the PcAb response of each dose group will be monitored over time for both binding titre and neutralising potency. Any dose dependant response will be observed and these data will be used to determine the optimum dose of each antigen required to elicit the highest achievable level of neutralising PcAb. This work will underpin the large scale commercial production of C. difficile anti-toxins.

Objective 3 - to investigate the development of a novel cell-based assay to model the bowel barrier

The development of a sensitive in vitro procedure, which models the epithelial cell barrier between the colonic lumen and systemic circulation (bowel barrier), would provide a valuable research tool and aid investigations into toxin translocation and development of potential treatment strategies. Currently, these investigations are routinely performed in vivo using the hamster model. A sensitive and reproducible cell based procedure would have the potential to replace animal models for CDI infection, providing both ethical and cost saving benefits. Experiments will be conducted using a novel tissue co-culture system to explore the feasibility of this in vitro bowel barrier model.
CHAPTER TWO

MATERIALS AND GENERAL METHODS
2.1 Materials

2.1.1 General Materials and Major Items of Equipment

High protein binding plates for enzyme immunoassay were purchased from Fisher Scientific (Loughborough, UK). Tissue culture flasks and plates were obtained from VWR (Leicestershire, UK). Miscellaneous items of plastic-ware, including centrifuge, bijoux and universal tubes, were purchased from BD Falcon (Oxford, UK). Filters were supplied by Sartorius (Surrey, UK), ultrafilter cassettes by Millipore (Hertfordshire, UK) and centrifugal ultrafiltration tubes by VWR (Leicestershire, UK). Cyanogen bromide-activated Sepharose® 4B was sourced from GE Healthcare Life Sciences (Buckinghamshire, UK). Sterile water for irrigation (WFI) was purchased from Baxter (Berkshire, UK). Foetal calf serum was sourced from Invitrogen (Paisley, UK). All other general chemicals and solvents were of ‘Analytical’ grade, or better, and were supplied by Merck (Poole, UK) or Sigma-Aldrich (Dorset, UK) unless stated otherwise. Major items of equipment are listed in Table 2.1 and Table 2.2.

2.1.2 Toxins and Recombinant Fragments

2.1.2.1 Native TcdA and TcdB

Purified natural C. difficile toxins TcdA and TcdB were supplied by the Toxins Group at PHE (Salisbury, UK) and kept frozen at - 80°C. The toxins, derived from C. difficile reference strain VPI 10463, had been produced and purified by PHE according to the method described by Roberts and Shone (2001) using the modifications described by Roberts et al. (2012). Prior to use and as part of this project, the purified toxins were assessed by size exclusion chromatography to verify their purity.
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Table 2.1       Major Items of Equipment (Analytical Laboratory)
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<td>Bibby</td>
</tr>
<tr>
<td>Vortex Mixer</td>
<td>F202A0173</td>
<td>Velp Scientific</td>
</tr>
<tr>
<td>Water Bath</td>
<td>JB Aqua 18 Plus</td>
<td>Grant</td>
</tr>
</tbody>
</table>

Table 2.2  Major Items of Equipment (Cell Culture Laboratory)
2.1.2.2 Recombinant TcdA and TcdB Fragments (TxA4 and TxB4)

Recombinant proteins, based on the central and receptor binding domains of TcdA (aa 770 to 2710, TxA4) and TcdB (aa 767 to 2366, TxB4) were supplied by the Toxins Group at PHE (Salisbury, UK) and stored at 4°C. The recombinant fragments had been produced, purified and formaldehyde treated (toxoided) by PHE according to the methods described by Maynard-Smith et al. (2014). The toxoiding process forms intra-protein methylene bridge cross-linking of lysine residues and stabilises the protein structure (Metz et al., 2004). Some inter-molecular cross-linking also occurs, creating a larger and more stable protein complex, which may enhance immunogenicity (Shone et al., 2009). These toxoided fragments formed the basis of the immunogens against which ovine PcAb were raised for this study.

2.1.3 Tissue Culture Cell Lines

A cryotube containing frozen African green monkey kidney cells (Vero, ECACC-10F019) was supplied by PHE (Salisbury, UK). Human colon carcinoma cells (Caco-2, catalogue no. 86010202-1VL) were purchased from Sigma-Aldrich (Dorset, UK). Low passage populations of both cell lines were stored in vapour phase liquid nitrogen at -196°C until required.

2.1.4 Antisera

Samples of normal sheep serum, donkey anti-sheep IgG (conjugated to horse radish peroxidase), sheep anti-TcdA IgG and sheep anti-TcdB IgG that were used in this study
were obtained from stocks held by MicroPharm (Newcastle Emlyn, UK) and used routinely in their laboratories.

2.2 General Methods

2.2.1 Protein Concentration – Optical density (OD) at 280 nm

The protein concentrations of purified IgG samples were determined using a spectrophotometer at a wavelength of 280 nm. The spectrophotometer was blanked/zeroed with an appropriate buffer before sample measurements and a quartz cuvette with a path length of 1 cm was used for all measurements. Samples were diluted as required to obtain readings, ideally between 0.3 to 1.4, and the protein concentrations were calculated thus:

\[
\text{Protein concentration (g/L)} = \frac{\text{Optical density @ 280 nm}}{\text{Extinction coefficient}} \times \text{dilution factor}
\]

The extinction coefficient of a protein is equal to its OD280 at a concentration of 1 g/L when measured through a path length of 1 cm. For ovine IgG, an extinction coefficient of 1.4 (Johnstone and Thorpe, 1996) was used throughout. For proteins with unknown extinction coefficient, a value of 1 was used.

2.2.2 Size Exclusion Chromatography (SEC)

A Superose 12 gel filtration column was used to assess the purity of protein samples. Superose 12 is a cross-linked agarose based medium which can be used to separate proteins based on their molecular weight (MW). This medium comprises a solid phase made up of many porous channels of varying size. Small MW proteins are able to enter these channels resulting in a longer flow path (higher retention volume) relative to that of larger MW proteins (lower retention volume). Filtered samples were loaded into a 100 µL
sample loop and applied to the column at a constant flow rate of 0.5 mL/min and a pressure of 1.2 MPa, according to the manufacturer’s specifications. Sodium citrate buffered saline (SCS, 10 mM sodium citrate, 153 mM sodium chloride, pH 6) was used as the running buffer and the OD of eluted proteins were automatically measured via the integrated UV flow cell at a wavelength of 280 nm. This system was operated electronically, via Unicorn software, and generated a chromatogram including peak integration (Figure 2.1). Each peak represents a protein of specific MW and the integrated peak area indicated the quantity of that protein as a percentage of the total protein detected in the sample. The MW of unknown peaks was determined using an existing in-house (MicroPharm) reference curve.

2.2.3 Affinity Chromatography

The antigen of interest was immobilised by chemical conjugation, via a covalent bond, to a solid phase, packed into a suitable column and used to separate specific antibodies from the much larger amount of non-specific antibodies. Under the conditions of neutral pH, any antibodies in the sample that are specifically directed to the immobilised antigen normally bind. Non-specific proteins were washed from the column using washing buffer (PBW, 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.5), whilst those bound to antigen were retained. By altering the conditions within the column, the antibody-antigen interaction can be disrupted and the bound specific antibodies eluted as a purified fraction. The antibody-antigen dissociation was achieved by altering either the ionic strength or pH of the buffer within the column. Both of these dissociation techniques involve changing the charge of the interacting proteins and, therefore, altering their conformational state. Glycine solution (100 mM, pH 1.5) was used in this project to elute proteins for concentration determination. Once the column had been eluted, the solid phase was re-equilibrated at neutral pH, using washing buffer, and used for subsequent samples.
Figure 2.1  This example of a SEC chromatogram illustrates the elution peaks typically obtained from hyper-immune ovine serum. The ‘Y’ axis measures absorbance at 280 nm (in milli-absorbance units) and the ‘X’ axis represents protein retention volume (in mL). The sample composition can be assessed by comparing the retention volume and integrated peak area % of each eluted protein. In this example, IgG (retention volume, 9.53 mL) makes up 65% of the total protein detected in the sample.

<table>
<thead>
<tr>
<th>No</th>
<th>Ret (ml)</th>
<th>Area (mAU*ml)</th>
<th>Area/Peak area (volume) %</th>
<th>Height (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.30</td>
<td>69.2178</td>
<td>7.51</td>
<td>112.752</td>
</tr>
<tr>
<td>2</td>
<td>9.53</td>
<td>600.8787</td>
<td>65.16</td>
<td>570.429</td>
</tr>
<tr>
<td>3</td>
<td>10.37</td>
<td>252.1160</td>
<td>27.34</td>
<td>372.582</td>
</tr>
<tr>
<td>4</td>
<td>18.23</td>
<td>0.0028</td>
<td>0.00</td>
<td>0.076</td>
</tr>
</tbody>
</table>

Total number of detected peaks = 6
Total area = 922.4952 mAU*ml
Area in evaluated peaks = 922.2153 mAU*ml
Ratio peak area / total area = 0.999697
Total peak width = 6.15 ml
Calculated from: CLFSER000900R01:1_UV1_280nm
Baseline: CLFSER000900R01:1_UV1_280nm@01,BASE
Peak rejection on:
Maximum number of peaks: 20
The affinity chromatography technique was used in the present study for two distinct purposes. Small scale affinity chromatography (SSAC) columns, containing 1 g of solid phase conjugated to either TxA4 or TxB4, were used to quantify the concentration of specific PcAb in the antisera or other samples. Thus, SSAC was used as an analytical method (section 3.2.4) and medium scale affinity chromatography (MSAC) columns, containing 2 g of solid phase conjugated to the required antigen, were used to purify small volumes of specific PcAb from pooled antisera stocks. These fractions were used in subsequent cytotoxic neutralisation experiments. Thus, MSAC was used as a laboratory scale PcAb purification technique (section 5.2.1).

Cyanogen bromide activated Sepharose® 4B was used as the solid phase and was prepared according to the manufactures’ instructions. A vacuum filtration system and sinter funnel were used to remove excess solution from the Sepharose gel between each step. The antigen to be coupled to the solid phase was dissolved in coupling buffer (sodium hydrogen carbonate 100 mM, pH 8.3), if not already in solution, and protein concentration of the antigen was determined (section 2.2.1) before coupling. The required quantity of freeze dried solid phase was swollen for 30 minutes in hydrochloric acid solution (1 mM), drained and then washed three times with the same solution. The swollen solid phase (1 g of solid phase swells to 3.5 mL) was then washed with coupling buffer and mixed with the required antigen at a ratio of 5 mg antigen to 1 g (dry weight) solid phase. This mixture was incubated at room temperature overnight on an end-over-end mixer to allow antigen-solid phase conjugation to occur. The binding efficiency of antigen to solid phase was assessed by measuring the optical density of the supernatant at 280 nm and calculating residual protein concentration. The remaining active groups on the solid phase were blocked with ethanolamine (1 M) and the gel was washed successively with sodium acetate buffer (100 mM, pH 4.0), coupling buffer, sodium acetate buffer and then re-suspended in washing buffer. The conjugated solid phase was then packed into an appropriate column and connected to a BioRad automated chromatography system.
2.2.4 Ultrafiltration / Diafiltration

This method is distinct from ‘dead-end’ filtration, whereby material flows perpendicular to a porous membrane to remove components (e.g. microbes and aggregates) larger than the nominal pore size. Thus the material flows parallel to the membrane (Figure 2.2) and is often referred to as cross-flow or tangential flow filtration. A membrane with a specific molecular weight cut off (MWCO) value was selected so that components of interest (i.e. immunoglobulins) were retained, but lower molecular weight components, often impurities, were removed by passing through the membrane unimpeded (Figure 2.3). As filtrate (permeate) flowed to waste, equal quantities of fresh buffer were added to the feed tank to maintain a constant volume for diafiltration or to exchange one buffer for another. The same system was used to concentrate components in solution by not adding additional buffer.

In this project, small volumes (< 50mL) of material were concentrated using centrifugal concentrator tubes (Vivaspin 20) with a MWCO of 30 kDa. The tubes were processed using a bench top swing-out bucket type centrifuge, according to manufacturer’s instructions. Larger volumes of material (50 mL to 1000 mL) were concentrated or diafiltered, as required, using the Millipore ‘Labscale’ TFF system according to the manufacturer’s instructions. The system was connected in parallel to three Pellicon XL 30 ultrafilter cassettes, each of 30 kDa MWCO. The Millipore system was sanitised prior to use by re-circulating a solution of sodium hydroxide (1 M) for 30 minutes followed by rinsing with water for injection (WFI) to lower the pH to neutral. An appropriate buffer solution was then used to ‘prime’ the membranes before the addition of material to be filtered. Following the filtration, small volumes of fresh buffer were used to chase the remaining ‘dead volume’ from tubing and housings and the system (including membranes) was stored in sodium hydroxide (100 mM).
Figure 2.2  Comparison of “dead-end” filtration and ultrafiltration (adapted from Millipore Corporation, 2003).

Figure 2.3  Schematic ultrafiltration system (adapted from Millipore Corporation, 2003).
2.2.5 Production of Antisera

The production of hyper immune ovine antisera, directed to either TxA4 or TxB4, was contracted out to IG-Innovations (Llandysul, UK) and was performed under full Home Office approval. Ethical approval for the project was also granted by Cardiff Metropolitan University. Formaldehyde toxoided recombinant fragments TxA4 and TxB4 were formulated, separately, into immunogens using Freund’s adjuvant at a ratio of 2 : 2.6 (antigen : adjuvant). Freund’s complete adjuvant was used for the initial immunisation and Freund’s incomplete adjuvant for each subsequent re-immunisation. The volume of toxoid required (in µL) for each dose was calculated by dividing the required dose (in µg) by the toxoid concentration (in g/L). The calculated toxoid volume was dispensed, diluted to 2 mL (if required) with saline (0.9% w/v sodium chloride) and mixed with 2.6 mL of adjuvant. The immunogens were mixed extensively using a mechanical device to form a stable water-in-oil emulsion between the aqueous toxoid and the oil based adjuvant. Pre-immunisation serum samples (~ 5 mL) were taken from each animal prior to receiving their initial dose, which was administered by subcutaneous injection between six sites near the relevant lymph nodes (Figure 2.4). A second immunisation was administered 28 days later, and then a ~ 10 mL blood sample was taken, via the external jugular vein, at 6 weeks post initial immunisation. Further re-immunisations were given and blood samples taken on a 28 day schedule, with these two activities staggered by 14 days. When adequate levels of specific PcAb had been attained, 10 mL / kg body weight of blood was collected every 28 days, in addition to the routine sample. The collected blood samples were rolled slowly for two hours at room temperature, to enhance clotting, before being centrifuged and the serum aspirated from the precipitate. The bulk serum was pooled, sterile filtered and stored frozen at -20°C. Routine samples were kept discretely so that the antibody response of individual sheep could be assessed.
2.2.6 Cell Culture

Sub-cultures, taken from low passage populations, were maintained as described in sections 2.2.6.1 (Vero) and 2.2.6.2 (Caco-2).

2.2.6.1 Maintenance of Vero Cells

Vero cells were cultured in 75 cm$^2$ flasks using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum, 2 mM L-glutamine and 10 mM HEPES buffer. The Vero cells were seeded at a rate of $5 \times 10^5$ per flask in 30 mL of the culture medium and incubated at 37°C in a 5% CO$_2$ humidified atmosphere. Once the cell monolayer had grown sufficiently to cover ~ 90% of the base of the flask (90% confluent), the cells were rinsed twice with 10 mL aliquots of Dulbecco’s phosphate buffered saline (PBSa) and separated from the flask using 4 mL trypsin-EDTA (5 g/L porcine trypsin, 2 g/L EDTA). The trypsin was then inhibited by the addition of 5 mL
DMEM and the cell suspension aspirated into a sterile universal tube. The cell concentration (number of viable cells per mL) was determined using a haemocytometer (section 2.2.6.3).

2.2.6.2 Maintenance of Caco-2 Cells

Caco-2 cells were cultured and maintained in 75 cm² tissue culture flasks using DMEM supplemented with 0.8% non essential amino acids. Cells were seeded at 1 x 10⁶ per flask in 30 mL of the culture medium and incubated at 37°C in a 5% CO₂ humidified atmosphere. When the cell monolayers had grown to ~ 90% confluence, they were detached from the flasks using the methods described in section 2.2.6.1 and the cell concentration was determined using the methods described below (section 2.2.6.3).

2.2.6.3 Cell Concentration Determination by Haemocytometer

A 1/10 dilution of cell suspension was made in Trypan Blue (100 µL in 1 mL) and gently mixed using a vortex mixer. A moistened cover-slip was placed on the haemocytometer and gentle pressure was applied to the slip until Newton’s refraction rings were visible (appearing as rainbow-like rings between the haemocytometer and the cover-slip). Diluted cell suspension was transferred, via a pipette, to fill the chambers of the haemocytometer beneath the cover-slip. Using an inverted light microscope, at 100X magnification, viable cells (those not stained blue) were counted from each of the four corner grids of the haemocytometer (Figure 2.5a). Viable cells falling on the left and lower edge of the grid were counted, but cells on the right and upper edges were not (Figure 2.5b). The concentration of viable cells was calculated using the equation below:

\[
\text{Viable Cell Count (cells / mL)} = \left( \frac{\text{No.Live Cells Counted}}{\text{No.Corners Squares Counted}} \right) \times \text{Dilution Factor} \times 10,000
\]
2.2.6.4 Seeding of 96-Well Tissue Culture Plate

Vero cells were seeded onto 96-well culture plates at 200 µL per well at a concentration of $1.0 \times 10^5$ mL$^{-1}$ (~ 20,000 cells per well). The plates were incubated for 24 hours to allow the cells to adhere and form a confluent monolayer.

Caco-2 cells were seeded onto 96-well culture plates at 200 µL per well at a concentration of $2.5 \times 10^5$ mL$^{-1}$ (~ 50,000 cells per well). The plates were incubated for 24 hours to allow the cells to adhere and form a confluent monolayer.

2.2.6.5 Seeding of 24-Well Tissue Culture Plate

Vero cells were seeded onto 24-well culture plates at 500 µL per well at a concentration of $2.4 \times 10^5$ mL$^{-1}$ (~ 120,000 cells per well). The plates were incubated for 24 hours to allow the cells to adhere and form a confluent monolayer.
CHAPTER THREE

ESTABLISHING ANALYTICAL TECHNIQUES

FOR ANTISERA ASSESSMENT
3.1 Introduction

During the two previous collaborative studies conducted between MPh and PHE, responsibilities were divided such that MPh raised and purified PcAb, whilst PHE supplied antigens and performed all of the analytical assessments of the resulting PcAb. In the present study, the analytical assessments of all the antisera and purified PcAb samples were conducted at MPh, the methods for which were not in the public domain at that time.

Analytical methods, designed to measure PcAb binding titre and cytotoxic neutralisation, had to be established at MPh and were based on those used by PHE in the previous studies (Roberts and Shone, 2001; Maynard-Smith et al., 2014). Binding titre was measured using a direct enzyme immunoassay (EIA) which was optimised to reduce the quantity of natural toxin required to coat each plate, and then validated to demonstrate consistent between-assay performance. The cytotoxic neutralisation assay method was modified to a greater extent to provide a more robust measure of toxin inactivation by the PcAb under test. This modification initially required the establishment of an additional in-house assay to measure the cytotoxicity of each toxin to be used in subsequent assessment assays. Ultimately, the modified cytotoxic neutralisation assay method provided a means to measure PcAb toxin neutralising potency in terms of µg of toxin neutralised by 1 mL of antisera. An additional analytical method, designed to measure specific binding PcAb concentration in terms of grams per litre, was also required. This assay had to be based on a small scale affinity chromatography technique, which is unique to MPh, using recombinant toxin fragments as the immobilised antigen.

Once established, optimised, and validated to ensure acceptable between-assay performance, these analytical methods were planned to be used to facilitate the meaningful comparison of antisera samples taken from individual sheep over time, and between
individual sheep across categories of immunising schedule doses, thus satisfying objective 1 described in section 1.12.

3.2 Methods

3.2.1 Toxin Purity by Size Exclusion Chromatography (SEC)

This chromatographic method was used to assess the purity of TcdA (batch #300112 @ 1.0 g/L) and TcdB (batch #270611 @ 0.45 g/L), which were used in all subsequent EIA, cytotoxicity and cytotoxic neutralisation assays. Each sample was diluted to 1/10 in running buffer (SCS) prior to loading the sample loop, and the assay was performed according to the method described in section 2.2.2. The absorbance peak, associated with the toxin being assessed, was integrated and the % peak area was deemed to be equivalent to the toxin purity of the sample.

3.2.2 TcdA & TcdB Cytotoxicity Assessment

To enable the development and optimisation of a quantitative cytotoxic neutralisation assay, the level of cytotoxicity of both TcdA and TcdB was determined. The Vero cell line was used as an indicator of cytotoxicity, essentially as described by Roberts and Shone (2001). Serial doubling dilutions of toxin (starting at 10 ng/mL for TcdA and 200 pg/mL for TcdB) were made in DMEM (section 2.2.6.1) and, following the removal of 100 µL of media from each test well of a previously seeded plate (section 2.2.6.4), 100 µL of each toxin dilution was added to assigned wells on the plate. Assay controls were included using media without toxin as a negative and media with an excess of toxin as a positive. Following incubation for 24 hours, visual assessment of the monolayer was made using an inverted light microscope, at 100X magnification, to assess each well in terms of
percentage cell rounding (Figure 3.1). Graphs were plotted (% cell rounding against toxin concentration) and the concentration of toxin required to cause cell rounding in 50% of the monolayer was determined. Multiple assays were performed using ten replicates of each toxin dilution series per plate. A total of ten plates were assessed, on different days, to determine the within-assay and between-assay coefficient of variation (%CV). A between-assay %CV of ≤ 30% was deemed acceptable. The concentration of toxin required to cause cell rounding in 50% of the monolayer was determined for each replicate with the between-assay mean value defined as the toxin LC$_{50}$.

3.2.3 Neutral Red Uptake Assay

The neutral red uptake assay, essentially as described by Valdivieso-Garcia et al. (1993), was studied as a possible alternative to visual assessment of the cell monolayer by light microscope. The neutral red assay (NRA) is based on the uptake of a dye by metabolically active cells. A stock solution of neutral red dye (0.33% w/v) was diluted to 1:40 in PBSa and added to each well of the 96 well plates at 50 µL per well. Plates were incubated for 3 hours at 37°C in a 5% CO$_2$ humidified atmosphere. The liquid was removed from the plates and the wells were washed twice with PBSa to remove any residual dye. The stain was then solubilised from the cells using 200 µL per well of destain solution (50% ethanol and 1% acetic acid) and placed on to a plate shaker for 15 minutes. The plates were read in a PolarStar plate reader at a wavelength of 540 nm (test measurement) and 690 nm (background measurement). The absorbance for each well was calculated by subtracting the background measurement from the test measurement. Percentage cell death was calculated as follows:

$$\text{Cell death (\%)} = \left( \frac{\text{absorbance} - \text{negative control}}{\text{positive control} - \text{negative control}} \right) \times 100$$
Figure 3.1  Comparison of percentage cell rounding of a Vero cell monolayer, representing (a) 0 %, (b) 50% and (c) 100% cell rounding.
NRA results obtained from the cytotoxicity plates were used to determine the LC$_{50}$ of TcdA and TcdB (section 3.2.2), and the %CV calculated for the between-assay LC$_{50}$ values. The %CV of the LC$_{50}$ value obtained by NRA was compared to that from visual assessment by light microscopy. In addition to the LC$_{50}$ comparison, %CV values were also calculated for measurements at each toxin concentration between all replicates of the 10 plates.

3.2.4 Specific Antibody Concentration by Small Scale Affinity Chromatography (SSAC)

This analytical method was used to determine the specific antibody concentrations (in terms of g/L) of the reference antibody (anti-TcdA, CDA 000264 #05/05/10 or anti-TcdB, CDB 000266 #08/07/10, purified total IgG at 50 g/L) used in the EIA and cytotoxic neutralisation assays. SSAC columns were prepared using the method described in section 2.2.3 with TxA4 or TxB4 (as required) as the coupled antigen.

The SSAC columns were connected to a BioRad automated chromatography system and used to quantify the specific PcAb concentration from antisera or total PcAb samples according to the following parameters. With the systems UV detector (measuring absorbance at 280 nm) reset to zero and the pump speed set at 1.0 mL/min, a PcAb sample of 0.5 mL was loaded onto the column and allowed to re-circulate for 2 hours. Washing buffer (PBW) was then passed through the column until the UV reading stabilised at baseline and the bound PcAb eluted from the column using glycine solution (100 mM, pH 1.5) and collected. The purified specific PcAb concentration was measured at OD$_{280}$, as described in section 2.2.1, and the specific PcAb concentration of the original sample calculated as follows:

Original sample concentration = Concentration of eluate x \( \frac{\text{volume of eluate}}{\text{loaded sample volume}} \)
3.2.5 Antibody Binding Titre by Enzyme Immunoassay

The specific PcAb binding titres of both reference antibody samples (described in section 3.2.4) were determined by a direct antibody capture EIA, essentially as described by Roberts et al. (2012). Either native TcdA or TcdB (as appropriate) was coated onto 96 well microtitre plates, 100 µL per well at a concentration of 2 mg/L in phosphate buffered saline (PBS, 10 mM phosphate, 0.9% w/v sodium chloride, pH 7.5) and incubated at 4°C for 12 hours, or over-night. The plates were washed three times with phosphate buffered saline containing tween (PBST, 10 mM phosphate, 0.9% w/v sodium chloride, 0.1% v/v tween, pH 7.5) using an automated multi-channel plate washing machine. Plate blocking was achieved by adding 300 µL per well of PBS with 2.5% foetal calf serum (blocking buffer) and incubating at 4°C for 12 hours, or over-night. The plates were washed three times with PBST before use.

Reference antibody (either anti-TcdA or anti-TcdB) and a normal sheep serum (NSS) were diluted to 1:1000 with PBST and 200 µL of each sample dilution was added to designated wells in column 1 of the plate. Serial doubling dilutions were made across the plate for each sample. Following incubation at 37°C for 1 hour, the plates were washed three times with PBST, after which 100 µL per well of secondary antibody (donkey anti-sheep diluted in PBST to 1:500) conjugated to horseradish peroxidase was added. The plates were then incubated again at 37°C for 1 hour and washed three times with PBST. One 10 mg tablet of o-phenylenediamine dihydrochloride (OPD) was dissolved in 100 mL sodium citrate buffer (70 mM, pH 5) and 20 µL of hydrogen peroxide (36% v/v) was added to the solution. A volume of 100 µL OPD solution was added to each well and incubated at 37°C for 20 minutes. The reaction was stopped by adding 50 µL of 3 M sulphuric acid and the plates were read using a PolarStar plate reader at a wavelength of 492 nm. Binding titres were defined as the reciprocal of the dilution factor at 50%
maximum absorbance after correction for the background NSS absorbance. Measured absorbance readings were plotted against the sample dilution factor and the Y axis 50% binding titre for each sample was calculated thus:

\[
Y \text{ axis } 50\% \text{ binding} = \left( \frac{\text{highest absorbance} - \text{background}}{2} \right) + \text{ background}
\]

The X axis 50% binding titre of each sample was then interpolated from the graph.

The concentration of antigen bound to the plate was optimised by coating each of four plates with a different concentration of toxin (0.5, 1, 2 or 4 mg/L) and performing the assay using the reference antibody (six replicates per plate) and normal sheep serum (two replicates per plate). The optimum coating concentration was selected based on the highest absorbance range and lowest variation between replicates. Once the plate coating concentration was optimised, multiple assays were performed using six replicates of each reference antibody dilution series and two of NSS per plate. A total of ten plates (per reference antibody) were assessed, on different days, to determine the within-assay and between-assay %CV. The between-assay mean titre was used in all subsequent EIA assessments to correct for between-assay variation.

3.2.6 Cytotoxic Neutralisation Assay

In contrast to the EIA, which measures specific binding PcAb titres, this cell based assay provides a means to measure specific toxin neutralising PcAb. Vero cell seeded culture plates, as described in section 2.2.6.4, were used as an indicator for residual toxicity. A fixed concentration of toxin (the challenge dose) was used, based on 20 times the LC_{50} for TcdA and 25 times the LC_{50} for TcdB. These concentrations were shown to be sufficient to cause cell rounding in 100% of the Vero monolayer.

Serial doubling dilutions of the reference antibody were made in DMEM culture media on an empty 96 well plate (the reaction plate). Each well of the reaction plate
contained 125 µL of sample dilution (Vab). An equal volume of toxin challenge dose (Vcd) was added to each sample well, resulting in a final well volume (Fv) of 250 µL. The reaction plate was incubated for 1 hour at room temperature. Following the removal of 100 µL of media from each test well of a previously seeded culture plate, 100 µL from each well of the reaction plate was added to the culture plate. Assay controls were included using media without toxin as a negative control and a toxin dilution equal to the challenge dose as a positive control. The plate was then incubated for 24 hours. Visual assessment was made using an inverted light microscope, at 100X magnification, to assess each well in terms of percentage cell rounding. Graphs were plotted (% cell rounding against reference antibody dilution factor) and the dilution required to protect 50% of the monolayer was determined. This value is defined as the ED\textsubscript{50}, or neutralising titre. The toxin neutralising potency, defined as the weight of toxin (in µg) neutralised by 1 mL of reference antibody (or other sample), was expressed quantitatively by using the following equation:

\[
\text{Potency (µg/mL)} = \left( \frac{(\text{CD} \times \text{Vcd}) - (\text{LC}_{50} \times \text{Fv})}{(\text{Vab} \times 1000)} \right) \times \text{ED}_{50}
\]

Where:
- CD – Challenge dose (ng/mL)
- Vcd – Volume of challenge dose (mL)
- LC\textsubscript{50} – 50% lethal concentration (ng/mL)
- Fv – Final well volume
- Vab – Volume of antisera dilution (mL)
- ED\textsubscript{50} – Neutralising titre

This method was intended to be the main antisera assessment technique with up to five assay plates required to process each batch of antisera samples obtained from sheep. Therefore, enhanced validation was performed using five plates (on the same day) to determine the within-assay within-plate and within-assay between-plate %CV. An additional nine assay plates were processed (on different days) to determine the between-
This validation work was performed separately for the anti-TcdA and anti-TcdB reference antibodies, using 10 replicates per plate. The ED$_{50}$ value of each reference antibody was derived from the mean of the between-assay variation results and was used to correct for between-assay variation in subsequent antisera assessment assays before applying the potency calculation.

The principle of the potency calculation is that the dilution of PcAb sample required to protect 50% of the monolayer (ED$_{50}$) neutralises the challenge dose minus a quantity of toxin equal to one LC$_{50}$. Thus, a known volume of PcAb sample will neutralise a known quantity of toxin when a known volume of solution is used (Table 3.1).

<table>
<thead>
<tr>
<th>Neutralisation Assay Parameters (TcdA)</th>
<th>Neutralisation Assay Parameters (TcdB)</th>
</tr>
</thead>
<tbody>
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<td>Challenge Dose (CD)</td>
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<tr>
<td></td>
<td>0.4 ng/mL</td>
</tr>
<tr>
<td>LC$_{50}$</td>
<td>1 ng/mL</td>
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<tr>
<td></td>
<td>0.016 ng/mL</td>
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<tr>
<td>Volume of Antisera Dilution (Vab)</td>
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<td>0.125 mL</td>
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<tr>
<td>Volume of Challenge Dose (Vcd)</td>
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<td>0.125 mL</td>
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<tr>
<td>Final Well Volume (Fv)</td>
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</tr>
<tr>
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<td>0.25 mL</td>
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<tr>
<td>CD in Fv (A)</td>
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<tr>
<td></td>
<td>0.05 ng</td>
</tr>
<tr>
<td>Dose Equivalent to 1 x LC$_{50}$ at Fv (B)</td>
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</tr>
<tr>
<td></td>
<td>0.004 ng</td>
</tr>
<tr>
<td>Toxin Neutralised at ED$_{50}$ (A - B)</td>
<td>2.25 ng</td>
</tr>
<tr>
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<td>0.046 ng</td>
</tr>
</tbody>
</table>

Table 3.1 Fixed parameters for the TcdA and TcdB cytotoxic neutralisation assays
3.3 Results

3.3.1 Toxin Purity by SEC

Each batch of toxin, supplied by PHE, which was to be used in antisera assessment assays was analysed to quantify their percentage purity. TcdA batch #300112 produced a peak on the SEC chromatogram at retention volume 7.68 mL, equivalent to ~ 310 kDa when compared to an in-house (MPh) reference chart, and a peak height of 84.155 mAU (Figure 3.2). The integrated peak area indicated a protein purity of 91.27%, relative to the total amount of detected protein in the loaded sample. TcdB batch #270611 produced a peak at retention volume 7.78mL (Figure 3.3), equivalent to ~ 270 kDa, with a peak height of 17.672 mAU. The integrated peak area indicated a protein purity of 64.38%, relative to the total amount of detected protein in the loaded sample. Several additional protein peaks were detected in both toxin samples. In the chromatogram for TcdA, all of the additional peaks were of lower MW than the target protein. However, a protein of higher MW than the toxin was detected in the TcdB sample.

3.3.2 TcdA & TcdB Cytotoxicity Assessment

The concentration of toxin required to cause 50% cell rounding of the monolayer was determined for each replicate of a toxin dilution series (Figure 3.4). The within-assay %CV was 12% and 8% for the TcdA and TcdB cytotoxicity assays, respectively. The between-assay %CV of 10 replicates across 10 assay plates was 16% and 23% for the TcdA and TcdB assay, respectively.

The LC\textsubscript{50} values were determined to be 1.0 ng/mL for TcdA (batch #300112) and 16 pg/mL for TcdB (batch #270611). Thus, TcdB was ~ 60 times more potent than TcdA.
Figure 3.2  Size exclusion chromatogram of TcdA #300112.

The protein peak at retention volume 7.68 mL represents the TcdA toxin and the integrated peak area indicates a protein purity of 91.27%, relative to the total detected protein in the sample.
Figure 3.3 Size exclusion chromatogram of TcdB #270611.

The protein peak at retention volume 7.78 mL represents the TcdB toxin and the integrated peak area indicates a protein purity of 64.38%, relative to the total detected protein in the sample.
Figure 3.4  Cytotoxicity curves for TcdA #300112 (a) and TcdB #270611 (b). These plots represent the percentage cell rounding of confluent Vero monolayers, within-assay, after exposure to 10 replicates of a TcdA or TcdB dilution series for 24 hours.
3.3.3 Neutral Red Uptake Assay

The cytotoxicity assay plates used in section 3.3.2 were also assessed, in parallel, using the neutral red uptake assay. The within-assay %CV of the LC$_{50}$ values were 60% and 29% for the TcdA and TcdB cytotoxicity assays, respectively. The between-assay %CV of the LC$_{50}$ values of 10 replicates across 10 assay plates were 87% and 37% for the TcdA and TcdB assay, respectively. Using this neutral red assay method, the LC$_{50}$ values were determined to be 3.3 ng/mL for TcdA (batch #300112) and 58 pg/mL for TcdB (batch #270611). Thus, TcdB was ~ 60 times more toxic than TcdA, which concurred with the cytotoxicity data derived by visual assessment.

The calculated %CV across all replicates at each toxin concentration were plotted and compared to those calculated from visual assessments (Figure 3.5). The TcdA cytotoxicity replicate %CV ranged from 10% to 172% by neutral red assay measurement compared to 0% to 16% by visual assessment. Values for the TcdB plate replicates ranged from 9% to 99% and 0% to 23% for neutral red and visual assessment derived data respectively.

3.3.4 Specific Antibody Concentration by Small Scale Affinity Chromatography

Affinity matrix solid phase media was conjugated to recombinant antigen fragments with coupling efficiencies calculated at 96.4% (TxA4) and 92.8% (TxB4). Non-specific binding of PcAb from NSS to both columns was measured at 0.3 g/L. The maximum binding capacity of both columns was determined as 40 mg of specific PcAb per gram of solid phase. Reference antibody samples, anti-TcdA (CDA 000264 #05/05/10) and anti-TcdB (CDB 000266 #08/07/10) were analysed and found to contain specific binding PcAb concentrations of 7.0 g/L and 6.1 g/L, respectively, after subtraction of the background non-specific binding. The %CV between 10 replicates was calculated at 8% (anti-TcdA) and 12% (anti-TcdB).
Figure 3.5 Comparison of percentage coefficient of variation against toxin concentration between measurements made by visual and by neutral red assessments for TcdA #300112 (a) and TcdB #270611 (b) cytotoxicity plates.
3.3.5 Antibody Binding Titre by Enzyme Immunoassay

The optimum EIA plate coating concentration was determined to be 2 mg/L, with an absorbance range of 0.98 to 0.22 and a maximum %CV between replicates of 2.5% (Figure 3.6). The within-assay %CV was 14% for both the anti-TcdA and anti-TcdB reference antibodies. The between-assay %CV of six replicates across 10 assay plates was 20% and 25% for the TcdA and TcdB assay, respectively.

The mean 50% binding titre values were determined to be ~1 : 220,000 for anti-TcdA and ~1 : 160,000 for anti-TcdB. These values were used to correct for between-assay variations in all subsequent EIA plates.

3.3.6 Cytotoxic Neutralisation Assay

The within-assay within-plate %CV for the calculated ED$_{50}$ values of 10 replicates was 0% for both reference antibodies. Within-assay between-plate %CV was 4% and 8% for anti-TcdA and anti-TcdB respectively. When comparing the ED$_{50}$ values of 10 replicates across 10 assay plates, each performed on a different day, the between-assay %CV was 29% and 13% for the TcdA and TcdB cytotoxic neutralisation assays, respectively (Figure 3.7).

The mean ED$_{50}$ values were determined to be 110,000 for anti-TcdA and 50,000 for anti-TcdB. These values were used to correct for between-assay variation in all subsequent cytotoxic neutralisation assays.
Figure 3.6  
EIA plate coating optimisation.
Each of four EIA plates was coated with a different concentration of toxin (4, 2, 1 or 0.5 mg/L). On each plate, six replicates of reference antibody and two replicates of normal sheep serum dilution series were assayed. The mean of the replicates are plotted with error bars indicating standard deviation.
Figure 3.7  Anti-TcdB cytotoxic neutralisation curve plotted using 4-parameter non-linear regression. Visual assessment data from the between-assay variation plates are plotted as mean values at each dilution with error bars to indicate standard deviation.
3.4 Discussion

Each batch of TcdA and TcdB to be used in subsequent assays was assessed for purity by SEC. Although the percentage purity of each toxin was not used in any downstream calculations, it was intended to be used to assess batch to batch variation in the toxins supplied by PHE. Both of the toxin samples were diluted to 1/10 with SCS buffer prior to loading and running of the assay. This dilution is in accordance with MPhe standard operating procedure for use of the Superose 12 SEC column and ensures that the column is not overloaded with protein. The dilution factor used for the TcdA sample was appropriate and resulted in a chromatogram with the peak height of the target protein reaching 84.2 mAU. However, as the total protein concentration of the original TcdB sample was already relatively low (0.45 g/L), the 1/10 dilution resulted in a chromatogram with the peak height of the target protein reaching only 17.7 mAU and the presence of background ‘noise’. Considering the high cost of the purified *C. difficile* toxins and the volume of sample required for flushing and loading of the SEC column sample loop, the 1/10 dilution of both samples was deemed appropriate and the resulting peaks acceptable.

The additional detected peaks at retention volumes greater than that of the relevant toxin (smaller MW) represent either impurities remaining from the toxin purification process or degradation of the toxin proteins. In the case of the TcdB sample, the many additional small MW peaks are indicative of background ‘noise’ resulting from the low total protein concentration of the sample. Protein peaks at retention volumes lower than the relevant toxin (larger MW) are likely the result of either toxin aggregation or residual cell debris remaining from the toxin purification process.

The level of cytotoxicity of both batches of toxin was determined using confluent Vero cell monolayers as an indicator. The within-assay and between-assay %CV for both toxins were below 25% and, therefore, the cytotoxicity assay method was considered to be
valid. As expected, the within-assay %CV were lower than those of the between-assay variation. This is likely to be due to many different factors such as slight variations in cell seeding density of the plates and pipetting accuracy. There was a larger difference in the TcdB within-assay and between-assay %CV (8% and 23% respectively) compared to those of the TcdA assessment (12% and 16% respectively). This difference may reflect the greater cytopathic effect induced by TcdB, which appears to be ~60 times more potent than TcdA. Thus, slight variations in pipetting accuracy of TcdB, compared to TcdA, are likely to result in a greater variation in LC50 between assays. An additional factor that may increase the between-assay %CV is the storage of the toxin. Routinely, both toxins were kept frozen at -80°C for long term storage or refrigerated at 4°C for short term use. As no stability data was available for either TcdA or TcdB, it is now thought to be more appropriate for each batch to be permanently stored at -80°C. Thus, toxins were divided into small aliquots (15 µL) for storage. These small aliquots can be removed for use as required with any residual volume discarded, thus eliminating the risk of repeated freeze/thaw cycles affecting the protein. The LC50 values determined from these cytotoxicity assays were used in all subsequent calculations.

The neutral red uptake assay was trialled as an alternative method to visual assessment of the Vero cell monolayers. It was hoped that this colourimetric method would provide a less subjective and more robust determination of % cell rounding. However, the %CV values measured for the calculated LC50 and the replicates at each toxin concentration were considerably higher than those of the visual assessments. The LC50 values for TcdA and TcdB, as determined by the neutral red method, were approximately 300% higher than those derived by visual assessment. This could be due to the fundamental difference between the two methods, thus visual assessment of the cell monolayer measures cell rounding (cytopathic effect) whereas the neutral red method measures cell death. It is likely that some toxin concentrations that cause cytopathic effects may not also cause cell death. The %CV of the TcdA dilution series replicates started
relatively low (10%) at the lowest toxin concentration, but rose sharply (maximum 172%) as the toxin concentration increased. Conversely, %CV of the TcdB dilution series replicates started high (99%) at the lowest toxin concentration, but reduced (minimum 9%) as the concentration increased. The reason for this difference is unclear, but may be due to TcdA being enterotoxic whilst TcdB is a more potent cytotoxin. Thus, TcdA may cause cytopathic effects with either no loss or a uniform loss of cell metabolic function at lower concentrations (resulting in low %CV), with a varying loss of cell metabolic function as toxin concentration increases (high %CV). TcdB may cause a varying loss of cell metabolic function at low concentration (high %CV) but result in a more uniform loss of metabolic function as the toxin concentration increases (low %CV). Considering the unacceptably high %CV values derived from the neutral red uptake assay, this assessment technique was not considered to be appropriate for this study in its current form. Therefore, it was decided that all monolayer assessments would be made visually and measured in terms of % cell rounding. Thus, the values derived from all cell assays would be based on the cytopathic effect rather than cell death.

Covalent conjugation of TxA4 and TxB4 recombinant fragments to cyanogen bromide-activated Sepharose was successful with coupling efficiencies > 90%. Non-specific binding of PcAb from normal sheep serum was measured as 0.3 g/L. It is unclear whether this was due to non-specific PcAb binding to immobilized antigen, exposed Sepharose media or to other column components. It is possible that non-immunoglobulin protein components present in the NSS, such as albumin, may bind non-specifically to the glass or plastic components of the column and be eluted under the same low pH conditions as for PcAb bound to antigen. It is also possible that all sheep sera contains low levels of specific PcAb directed against TcdA and TcdB since C. difficile is ubiquitous in the soil and sheep may, therefore, develop an immune response in early life.

During initial development work using the SSAC columns the glycine elution buffer was formulated to pH 2.5, as recommended by the manufacturers of the solid phase media.
This elution buffer, however, proved to be unreliable and resulted in inconsistent measurements of specific PcAb concentration. The glycine buffer was adjusted to pH 1.5, which improved the consistency of the measurements. It is likely that the avidity of the specific PcAb to the antigen was too high to be dissociated by the buffer at pH 2.5, but the tertiary structure of the antibody-antigen complex was altered sufficiently at pH 1.5 for dissociation to occur. This elution buffer is acceptable where only the specific PcAb concentration is to be measured. However, the lower pH is likely to damage the eluted antibodies and is therefore not appropriate for elution of PcAb to be used in toxin neutralising applications. Where specific PcAb are to be collected for subsequent assessment, an elution buffer at pH 2.5 should be used followed by a column regeneration step using elution buffer at pH 1.5 (collected as waste) to dissociate all remaining PcAb and restore column binding capacity.

Although the SSAC for specific PcAb measurement is a robust technique (%CV of 8% and 12% for anti-TcdA and anti-TcdB respectively), the method is protracted and offers a low sample throughput potential. It was, therefore, not ideal as a routine assessment method for the individual sheep serum samples to be processed in the present study.

The cytotoxic neutralisation assay demonstrated low variability between replicate samples in the within-assay within-plate experiment with no measurable %CV observed in the ED$_{50}$ values of either the TcdA or TcdB assays. The within-assay between-plate %CV, calculated from the replicate ED$_{50}$ values of 10 replicates across five plate (performed on the same day), were also relatively low (4% and 8% for TcdA and TcdB assays, respectively). The low variability may be related to the use of ‘reaction plates’ in-which all PcAb dilutions are made and the fixed concentration of toxin added prior to transfer to the cell culture plate. When using the ‘reaction plate’ each set of replicate dilutions/toxin additions are made in a single step across the plate using a multichannel pipette, as opposed to the many individual manipulations performed using a single channel instrument.
as was used for the cytotoxicity assay dilutions. The between-assay %CV was higher for both the TcdA and TcdB cytotoxic neutralisation assays (29% and 13% for each, respectively). However, due to the many variable factors that can affect such a bio-assay, a between-assay %CV of ≤ 30% is acceptable. Considering the low %CV observed within-assay, it is reasonable to suggest that a shift in the reference antibody ED₅₀ value between-assay will result in a shift of equal proportion in the measured values of other samples within the same plate. Based on this assumption and to normalise ED₅₀ measurements between plates, the mean ED₅₀ values derived from the between-assay validation (110,000 and 50,000 for anti-TcdA and anti-TcdB, respectively) will be used to correct all subsequent sample measurements. Correction factors will be calculated for each assay plate by dividing this established mean ED₅₀ value by that derived from the assay reference ED₅₀ value. Normalised/corrected ED₅₀ values for all samples will be obtained by multiplying their neutralising titre by the correction factor. Normalisation in this manner will facilitate the meaningful comparison of antisera samples from individual sheep over time and between sheep over categories of immunising schedule doses.

The PcAb cytotoxic neutralisation potency calculation enables the expression of the ED₅₀ values in terms of mass of toxin (µg) neutralised per volume (mL) of antisera, or other sample. Given that the parameters of the TcdA and TcdB cytotoxic neutralisation assay are fixed (section 3.2.6, Table 3.1), the potency calculation can be simplified as follows:

\[
\text{TcdA Neutralising Potency (µg/mL)} = \frac{(20 \times 0.125) - (1 \times 0.25)}{(0.125 \times 1000)} \times \text{ED}_{50}
\]

\[
= \frac{(2.5) - (0.25)}{125} \times \text{ED}_{50}
\]

\[
= \frac{2.25}{125} \times \text{ED}_{50}
\]

\[
= 0.018 \times \text{ED}_{50}
\]
TcdB Neutralising Potency (µg/mL) = \( \frac{(0.4 \times 0.125) - (0.016 \times 0.25)}{0.125 \times 1000} \times \text{ED}_{50} \)

= \( \frac{(0.05) - (0.004)}{125} \times \text{ED}_{50} \)

= \( \frac{0.046}{125} \times \text{ED}_{50} \)

= \( 0.000368 \times \text{ED}_{50} \)

Thus, to express toxin neutralising potency, in terms of µg of toxin neutralised per mL of sample, the constants calculated above (0.018 for TcdA or 0.000368 for TcdB) were multiplied by the measured ED\(_{50}\) titre as determined for samples from each assay.

No cross reactivity was detected in either the EIA or cytotoxic neutralisation assays between anti-TcdA or anti-TcdB (data not shown). Thus, anti-TcdA was specific only to TcdA and anti-TcdB was specific only to TcdB.

Hence, EIA and cytotoxic neutralisation assays were established at MPh and were utilised for the routine assessment of the antisera samples obtained throughout the immunogen dose optimisation study.
CHAPTER FOUR

IMMUNOGEN DOSE OPTIMISATION STUDY
4.1 INTRODUCTION

The primary aim of the present study is to produce large volumes of antisera containing high levels of toxin neutralising PcAb. The results presented here will underpin the larger scale production of *C. difficile* antitoxin which will require the immunisation of, possibly, thousands of sheep. Ultimately, ovine derived PcAb will be purified and used commercially to produce an immunotherapy for administration to patients with CDI.

Due to the high costs associated with producing the immunogens, it was important to determine the immunising dose of each antigen required to elicit an optimum neutralising PcAb response. An optimum dose was defined as the minimum quantity of antigen required to produce antisera of the highest neutralising potency. Antisera assessment assays, established and validated in Chapter 3, were used to determine specific PcAb binding titres and toxin neutralising potency.

Prior to the commencement of the immunisation schedule detailed in section 4.2.1, an initial flock of sheep (CF1551 to CF1565) was immunised with TxB4 according to the same dose and schedule parameters. This initial flock developed adverse reactions to the immunogen which, upon investigation by PHE, was attributed to the presence of residual formaldehyde in the toxoided recombinant fragments. Consequently, the flock was removed from the immunisation schedule and rested to allow for a complete recovery. The recombinant fragment toxoiding method was amended by PHE to include an additional diafiltration step, which reduced the residual formaldehyde level by a further 100 fold (communication from PHE Toxin Group, Porton Down). The immunisation schedule was restarted using two new flocks (section 4.2.1) and no further adverse reactions to the immunogen where observed.

Following a resting period of six months and restoration to full health, the original flock was restarted on the TxB4 immunisation schedule and their PcAb monitored over a
period of 30 weeks. This rest-restart schedule was repeated for the TxB4 dose optimisation flock (sheep CF1600 to CF1614) following completion of their dosing study.

The objectives required to achieve the primary aim of this study are described in section 1.12 (objective 2) and this chapter documents the immunisation schedule, dose optimisation, and immunisation restart studies.

4.2 METHODS

4.2.1 Sheep Immunisation and Dose Optimisation

Antisera were raised according to the methods described in section 2.2.5. Two separate flocks of 15 sheep (one flock per antigen) were sub-divided into five dose groups, each comprising three sheep. Baseline serum (pre-immune) samples were taken from all individual animals prior to primary immunisation. Each group of three sheep was immunised with a specific dose of formaldehyde treated (toxoided) recombinant fragments (Table 4.1 and 4.2) according to a pre-defined schedule (Table 4.3). Primary immunisation was undertaken at week 0, re-immunisations at week 4 and every 4 weeks thereafter. Serum samples were taken every 4 weeks from weeks 6 to 30. Thus, serum samples were obtained from each animal at 6, 10, 14, 18, 22, 26 and 30 weeks post initial immunisation. The 30 week sample point was deemed to be the end of the dose optimisation study.

Immunisation of the TxB4 flock was stopped at week 30 and the sheep were rested for six months. Following this, the re-immunisation schedule was initiated and was continued for an additional 30 weeks (section 4.2.4)
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<thead>
<tr>
<th>Sheep ID No.</th>
<th>No. Of Sheep</th>
<th>Dose</th>
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</thead>
<tbody>
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<td>CF1615 CF1616 CF1617</td>
<td>3</td>
<td>50 µg</td>
</tr>
<tr>
<td>CF1618 CF1619 CF1620</td>
<td>3</td>
<td>100 µg</td>
</tr>
<tr>
<td>CF1621 CF1622 CF1623</td>
<td>3</td>
<td>250 µg</td>
</tr>
<tr>
<td>CF1624 CF1625 CF1626</td>
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<td>500 µg</td>
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<tr>
<td>CF1627 CF1628 CF1629</td>
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Table 4.1  Toxoid A4 Sheep Identification and Dose Groups

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<th>Sheep ID No.</th>
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<th>Dose</th>
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<td>250 µg</td>
</tr>
<tr>
<td>CF1606 CF1607 CF1608</td>
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<td>CF1609 CF1610 CF1611</td>
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<td>1 mg</td>
</tr>
<tr>
<td>CF1612 CF1613 CF1614</td>
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<td>2 mg</td>
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Table 4.2  Toxoid B4 Sheep Identification and Dose Groups
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<th>Weeks Post Primary Immunisation</th>
<th>Scheduled Activity</th>
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<tbody>
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<td>0</td>
<td>Pre-Immune Sample &amp; Primary Immunisation</td>
</tr>
<tr>
<td>4</td>
<td>Re-Immunisation</td>
</tr>
<tr>
<td>6</td>
<td>Sample</td>
</tr>
<tr>
<td>8</td>
<td>Re-Immunisation</td>
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<td>28</td>
<td>Re-Immunisation</td>
</tr>
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<td>30</td>
<td>Sample</td>
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</table>

Table 4.3  Immunisation schedule for dose optimisation study
4.2.2 Binding Titre Assessment

The established EIA (section 3.2.5) was used as a semi-quantitative method to compare binding titres from individual sheep over time and between individual sheep across varying dose groups, after correction for between assay variations using the reference antibody.

4.2.3 Cytotoxic Neutralisation Assessment

Samples were assessed according to the methods established in section 3.2.6. The calculated antisera potency was used as a quantitative method to measure and compare samples from individual sheep over time and between individual sheep across categories of immunising dose group. Statistical analysis was performed, according to the methods described in section 4.2.5, to determine whether potency results were significantly different between categories of immunising dose group.

4.2.4 TxB4 Flock Immunisation Rest-Restart Study

Following a resting period of six months, sheep from the original TxB4 flock (CF1551 to CF1565) were assessed by a veterinarian and those in good health were enrolled on a re-immunisation schedule, identical to that detailed in section 4.2.1, Table 4.3 with the addition of a sample point at 2 weeks post restart. To assess this rest-restart approach further, the sheep in the TxB4 dose optimisation study flock (CF1600 to CF1614) were also rested for six months, following the completion of their initial 30 week dosing study, and those in good health were again enrolled on the re-immunisation schedule. The toxin neutralising potency of the antisera from each individual sheep was monitored over the 30 week study and compared to the potency of samples prior to the resting period. All
sheep enrolled in the rest-restart programme (Tables 4.4 and 4.5) were immunised with a
dose of 250 µg TxB4. This dose was selected based on a limited supply of antigen, at the
time, and its high cost.

4.2.5 Statistical Methods

The results generated in this study were analysed statistically using GraphPad Prism
V5.03 or SPSS V20 statistical software. Distributions of the binding titre and potency data
from all individual sheep at each sample point were first analysed for normality, relative to
Gaussian distribution, using the Kolmogorov-Smirnov test. Although the distribution was
found to be normal, non-parametric analysis was judged to be more appropriate and robust
compared to parametric tests due to the low number of samples per immunising dose group
(n=3) and the high variability of results within some groups. Thus, data are described using
median and inter quartile range (IQR) values with statistical significance calculated using
Kruskal-Wallis (K-W) including Dunns post-hoc test for multiple comparisons, or Mann-
Whitney (M-W) tests. Where a significant result was indicated (defined as P = ≤ 0.05)
using the Dunns post-hoc test, this was confirmed using M-W.

4.3 RESULTS

4.3.1 Effects of Immunisation Dose on Antibody Binding Titre

Antisera samples from each individual animal were measured by EIA, to determine
the 50% binding titre. The median binding titre of each dose group was plotted at each
sample point from 6 to 30 weeks post initial immunisation (Figure 4.1). No toxin binding
PcAb were detected in the pre-immune samples of any individual sheep. However, toxin
binding PcAb were detected in all samples at 6 weeks post initial immunisation, with
median titres of 74,000 (IQR = 111,000) and 90,000 (IQR = 92,000) for the TxA4 and
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<th>Re-start Dose</th>
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</tr>
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<tr>
<td>CF1556</td>
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<tr>
<td>CF1558</td>
<td>500 µg</td>
<td>250 µg</td>
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<tr>
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<tr>
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<tr>
<td>CF1563</td>
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Table 4.4  TxB4 Flock 1 Re-start Sheep Identification and Immunogen Dose

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<th>Original Dose</th>
<th>Re-start Dose</th>
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<tbody>
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Table 4.5  TxB4 Flock 2 Re-start Sheep Identification and Immunogen Dose
Figure 4.1  Dose optimisation study, binding titre results.
The median 50% binding titres, assessed by EIA, for each dose group of TxA4 (a) and TxB4 (b) are plotted against sampling point (weeks post primary immunisation). There was no significant difference in immune response between categories of immunising dose at any sample point for either the TxA4 (P = 0.7) or TxB4 (P = 0.9) flocks (K-W). The titres of the TxA4 flock were significantly higher than those of the TxB4 flock (P = < 0.01) at week 30 (M-W).
TxB4 flocks respectively. Measured at 10 weeks, the median titres had increased to 194,000 (IQR = 71,000) and 158,000 (IQR = 87,000) respectively and did not increase considerably beyond this point, with the exception of an unusually high measurement recorded from the TxB4 250 µg group at week 18. This high measurement (250 µg group median = 285,000, IQR = 372,000) was confirmed by a subsequent repeat assay and was, therefore, thought to be a true result. Statistical analysis of all 15 antisera samples at each sample point revealed no significant difference in immune response, as assessed by binding titres, between categories of immunising doses at any time during the study in either the TxA4 (P = 0.7) or TxB4 (P = 0.9) flocks, although a high variation in range was observed within the dose groups (Figure 4.2).

At the end of the dose optimisation study (30 weeks post initial immunisation) the PcAb binding titre of the TxA4 flock (median = 164,000, IQR = 79,000) was significantly higher than that of the TxB4 flock (median = 112,000, IQR = 47,000) (P = < 0.01). Thus, the TxA4 antigen appeared to have a greater immunogenicity than TxB4.

4.3.2 Effects of Immunisation Dose on Neutralising Potency

The neutralising potency (expressed as µg of toxin neutralised per mL of antisera) of each individual sheep sample over the 30 week dose optimisation study was assessed and the median of each dose group was plotted (Figure 4.3), together with the variation within the individual dose groups at week 30 (Figure 4.4). No neutralising activity was detected in the pre-immune samples from any of the individual sheep. However, toxin neutralising PcAb were present in all samples 6 weeks post initial immunisation.

Within the TxA4 flock, toxin neutralising potency in all dose groups peaked at week 26 and thereafter appeared to stabilise. The greatest rise in median potency was observed at the highest dose (1 mg) which increased from 200 µg mL⁻¹ (IQR = 640) at week 6 to 2850 µg mL⁻¹ (IQR = 4870) at week 30. The trend, illustrated in Figure 4.3, seems to show that antisera potency increased in a dose dependant manner with the 1 mg dose eliciting the
Figure 4.2  Comparison of 50% binding titre between dose optimisation study groups at week 30.
The median binding titre and inter quartile range for each dose group of TxA4 (a) and TxB4 (b) are plotted.
Figure 4.3  Dose optimisation study, toxin neutralising potency results.

The median toxin neutralising potency (µg mL$^{-1}$) for each dose group of TxA4 (a) and TxB4 (b) are plotted against sampling point (weeks post primary immunisation). There was no significant difference in toxin neutralising potency between categories of immunising dose at any sample point for either the TxA4 ($P = 0.6$) or TxB4 ($P = 0.7$) flocks (K-W).
Figure 4.4  Comparison of toxin neutralising potency between dose optimisation study groups at week 30.

The median toxin neutralising potency (µg mL$^{-1}$) and inter quartile range for each dose group of TxA4 (a) and TxB4 (b) are plotted.
highest neutralising PcAb response. However, statistical analysis of the 15 individual measurements between the TxA4 dose groups at week 30 revealed no significant difference in toxin neutralising potency ($P = 0.06$).

Toxin neutralising potency measured for the TxB4 dose groups increased from a median of $2 \, \mu g \, mL^{-1}$ (IQR = 3) at week 6 to $10 \, \mu g \, mL^{-1}$ (IQR = 16) at week 18, where all but the 2 mg group appeared to stabilise. The potency measurements of all dose groups began to trend upwards again from 22 weeks post initial immunisation, which continued to the end of the study. A transient sharp increase in neutralising potency recorded from the 500 µg group at week 18 (500 µg group median = 20 µg mL$^{-1}$, IQR = 35) was confirmed by a repeat assay and must therefore be deduced to be a valid result.

Statistical analysis of the TxB4 flock potency data, at each sample point, revealed no significant difference in toxin neutralising potency between the categories of immunising doses. However, a trend towards significance was evident, thus the calculated $P$ value between the dose groups decreased incrementally from $P = 0.95$ at week 6 to $P = 0.07$ at week 30, with the 2 mg group showing the greatest potency (median = 25 µg mL$^{-1}$, IQR = 10).

A striking difference was observed between the toxin neutralising potencies of the TxA4 (highest median = 2850 µg mL$^{-1}$) and TxB4 (highest median = 25 µg mL$^{-1}$) flock antisera. The reason for this is unclear, but further indicates the greater immunogenicity of the TxA4 antigen.

4.3.3 Immunisation Rest-Restart Study

TxB4 flock 1 pre-immune samples contained no detectable toxin neutralising potency (Figure 4.5). The 6 and 10 week samples, post initial immunisation, contained median potencies of $4 \, \mu g \, mL^{-1}$ (IQR = 2) and $8 \, \mu g \, mL^{-1}$ (IQR = 3), respectively, with no dose dependant response evident between categories of immunising doses. Immunisations were stopped due to the problems described in section 4.1 and the sheep were rested for 6
Figure 4.5 TxB4 Flock 1 rest-restart study data.

Graph (a) represents potency results from all sheep enrolled in the study including pre-immune, 6 to 10 weeks post initial immunisation, rested sample (prior to restart after 6 month rest) and samples from 2 to 30 weeks post restart (R2 to R30). Graph (b) presents the same data displayed in terms of min, max, median and inter quartile range of the 8 individual antisera potency results per sample point.
months. Following restoration to full health and prior to restarting the immunisation schedule, resting samples were taken and found to contain low levels of residual toxin neutralising potency (median = 1 µg mL\(^{-1}\), IQR = 1). Antisera samples taken 2 weeks post restart contained significantly higher toxin neutralising potency compared to the 10 week post initial immunisation sample (P = < 0.001), with a median of 26.1 µg mL\(^{-1}\) (IQR = 13). No significant difference was observed between median potency from R2 to R30 (P = 0.13). However, at week R22 (22 weeks post restart) there appeared to be a decreasing trend in potency which continued to the end of the study (R30).

The original immunising dose (before the rest-restart study) seemed to influence the antisera potency of some sheep during the immunisation restart schedule, even though a common dosage of 250 µg was used in all sheep at this stage. Thus, some individual sheep who originally received a higher immunising dose produced antisera containing higher toxin neutralising potency (highest measurement = 88 µg mL\(^{-1}\)) compared to some that received a lower dose and produced antisera containing lower toxin neutralising potency (highest measurement = 46 µg mL\(^{-1}\)).

Following the completion of the 30 week TxB4 dose optimisation study, this flock (TxB4 flock 2) was removed from the immunisation schedule and rested for 6 months. Due to an error at the contracted antisera production site, serum samples were not taken prior to re-starting the immunisation of these sheep and, therefore, residual toxin neutralising potency of antisera from these sheep in their rested state was not assessed. Toxin neutralising potency was detected in all antisera samples 2 weeks post restart (R2) with a median value of 13 µg mL\(^{-1}\) and an IQR of 11 (Figure 4.6). Compared to the potencies measured from the 30 week, post initial immunisation, samples (median = 11 µg mL\(^{-1}\), IQR = 6), there was no significant difference in toxin neutralising activity between the 30 week and R2 samples (P = 0.46). However, sheep CF1609 (1 mg original dose), CF1613 and CF1614 (both 2 mg original dose) produced potencies of 39, 30 and 64 µg mL\(^{-1}\), respectively, 2 weeks post restart. These potency values are considerably higher than those
Figure 4.6 TxB4 Flock 2 (ex-dose optimisation flock) rest-restart study data.
Graph (a) represents potency results from all sheep enrolled in the study including 26 to 30 weeks post initial immunisation and samples from 2 to 30 weeks post restart (R2 to R30). Graph (b) presents the same data displayed in terms of min, max, median and inter quartile range of the 14 individual antisera potency results per sample point.
measured at 30 weeks from these individuals (19, 25 and 25 µg mL$^{-1}$ for CF1609, CF1613 and CF1614, respectively) and this presents further evidence of a relationship between original immunising dose and antisera potency post rest-restart. A significant increase in potency was measured between the 30 week and R6 samples ($P = 0.025$), with R6 median potency of 21 µg mL$^{-1}$ (IQR = 15). This increase in antisera potency was not sustained and had significantly decreased at R22 ($P = 0.001$). At the end of the rest-restart study (R30), the antisera potency measured from all individual sheep was either equal to or lower than those measured at 30 weeks post initial immunisation.

4.4 DISCUSSION

Due to a loss in solubility of the TxA4 antigen at concentrations above 0.5 g/L and given the maximum allowable injection volume of 4.6 mL for each sheep, it was not possible to formulate the TxA4 immunogens to a dose greater than 1 mg per sheep. The TxB4 antigen was supplied at a concentration of 1.0 g/L which allowed for a maximum immunising dose of 2 mg per sheep.

Sample populations within the dose optimisation study groups were low (n=3), therefore robust statistical analysis was difficult. However, the data collected was analysed using non-parametric statistical tests and assessed for any evidence of trends. Antisera samples from both dose optimisation flocks contained high levels of specific binding PcAb at 10 weeks post initial immunisation, compared to the reference antibody controls (220,000 and 160,000 for anti-TcdA and anti-TcdB controls, respectively). The binding antibody titres did not increase in a dose dependant manner in either of the dose optimisation flocks and appeared to stabilise from the 10 week sample point. The specific PcAb concentration (in terms of g/L) of the antisera samples were not measured in this study, due to the relatively low throughput potential of the established SSAC assay and the high number of antisera samples to be assessed. However, as specific PcAb binding
concentration and titre must correlate, it is reasonable to assume that the concentration of specific binding PcAb would follow the same trends as those of binding titre. Therefore, it is also reasonable to conclude that the specific PcAb binding concentrations peaked and stabilised from the 10 week sample point. No significant difference was found between the median binding titres between categories of immunising doses at the end of the study.

The neutralising potency showed a general upward trend in the median of each group up to 26 weeks post initial immunisation. The median potency of the higher dose groups (1 mg and 2 mg) in the TxB4 flock continued to trend upwards to the end of the study. At the end of the 30 week dose optimisation study there was no significant difference in median potency values between categories of immunising doses, as analysed by non-parametric tests, although a trend towards significance was noted in the TxB4 flock. It is likely that continuation of the study beyond 30 weeks would have resulted in the median potency value measured from the TxB4 2 mg group being significantly higher than those of the lower immunising dose groups. The 30 week sample data were also analysed using parametric tests as a comparison and a statistically significant difference was found between the TxB4 2 mg and 250 µg groups (P = 0.02). However, this test is known to be less robust than the non-parametric analysis methods, given the low sample size and relatively high variance of the measured potency data within each dose group. Therefore, the significant figure calculated using the parametric analysis method is not deemed to be valid.

The experimental data from the binding and neutralising studies strongly indicate that the avidity of the specific PcAb continued to increase for at least 16 weeks after the specific binding PcAb concentration of the antisera stabilised. This is evidenced by antisera samples of relatively constant specific PcAb concentration showing incremental increases in toxin neutralisation potency. Continued exposure of the sheep to the immunogens and ongoing B cell affinity maturation are likely to be the reason for this increase in PcAb avidity.
Large variations in both binding titre and neutralising potency were seen between sheep, even within individual dose groups. This is probably attributable to natural variation between the immune response of individual sheep with some animals capable of mounting a stronger response than others. The soil acts as a natural reservoir for *C. difficile* (Madigan et al., 2009), and it is possible that some sheep may have been exposed to TcdA or TcdB antigens prior to this study. Thus, any sheep with previous exposure to relevant antigens would mount a greater immune response to the immunogens used in this study.

An unexpectedly large increase in neutralising potency was measured in one sample from within the TxB4 500 µg group at week 18. This result was confirmed by repeat analysis of the sample, but was not seen in any subsequent samples taken from the animal. The cause of this result is unclear, but an increase in binding titre was also detected in all dose groups, most notably in the 250 µg group. This indicates that an enhanced immune response occurred at this point, possibly as a result of exposure to natural *C. difficile* antigens from the environment, although the exact cause is unknown.

Based on the statistical analysis of the data generated in this study, the optimum doses of antigen required to produce the highest quantity of toxin neutralising PcAb are difficult to confirm. Considering the high cost and currently limited supply of antigens together with the lack of any statistically significant difference between the dose group median values, it would seem reasonable to select the lowest immunising dose. However, based on the evidence of an upward trend in antisera potency at the higher doses, particularly in the TxB4 flock, it may be more appropriate to select the highest immunising dose of each antigen. A more robust approach to this study would have been to use a lower number of immunising dose groups with a higher number of sheep in each group. The study could also be extended beyond 30 weeks to allow any increasing trends to stabilise. These altered study parameters would enable a more robust statistical analysis.

The rest-restart approach to immunisation appeared to be an effective method to boost antisera potency judging by the initial TxB4 flock 1 (sheep no. CF1553-CF1563)
study. However, the enhanced antisera potency could not be replicated using this method with TxB4 flock 2 (sheep no. CF1601-CF1614). The toxin neutralising potency measured from TxB4 flock 1 at weeks 6 and 10 post initial immunisation were notably higher than those measured from TxB4 flock 2 at the same sample points. The only known difference between the two flocks was that flock 1 received immunogens containing residual formaldehyde, suffering localised adverse reactions, whereas flock 2 received a more purified immunogen and suffered no ill effects. It is possible that the localised adverse reactions experienced by flock 1 facilitated a greater uptake of immunogen via an enhanced recruitment of phagocytic cells to the immunisation sites. These cells may have increased the presentation of relevant antigen to B lymphocytes, thus producing a higher antibody titre and forming a greater number of memory cells. This may also explain why the flock 1 potency measurements, post restart, were also considerably higher than those of flock 2 as they would be likely to have a greater number of memory cells, relative to flock 2.

The relationship observed between original immunising dose and subsequent antisera potency, post rest-restart, is of potential value. Sheep who originally received high immunising doses (TxB4 1 mg and 2mg) and were then re-immunised with a lower dose (250 µg) produced antisera with remarkably high toxin neutralising potency, relative to sheep who originally received a lower dose. This potency was not sustained, but remained relatively high for up to four months. The decreasing trend in potency observed from R22 is likely to be related to the 250 µg immunising dose used for the restart study and could possibly be reversed by the periodic administration of a higher immunising dose. For the purpose of raising large volumes of high potency antisera using the lowest possible quantities of antigen, this approach to immunisation may have the potential to reduce commercial antisera production costs considerably and warrants further study.

During the development of the analytical techniques (described in Chapter 3) it was noted that TcdB was ~ 60 times more cytotoxic to Vero cells than TcdA. Thus,
approximately 60 times less TcdB was required as a challenge dose for the cytotoxic neutralisation assay. During the dose optimisation study, the toxin neutralising activity of the anti-TxA4 sera were found to be ~ 60 times more potent than the anti-TxB4 sera. This relationship between cytotoxicity and neutralising potency would seem to be a product of the previously described potency calculation (Chapter 3, section 3.2.6). However, given that TcdA and TcdB are reported to be produced in a 1:1 molar ratio \textit{in-vivo} (Voth and Ballard, 2005), if the challenge dose of TcdB was increased to match that of TcdA, a proportionally greater quantity (less dilute antisera sample and proportionally lower ED$_{50}$ value) of anti-TxB4 serum would be required to achieve the same level of toxin neutralisation. In effect, the toxin neutralising potency of anti-TxB4 would remain at ~ 60 times less than that of anti-TxA4. In a clinical setting this may mean that a therapeutic product would need to contain ~ 60 times more anti-TxB4 than anti-TxA4 to neutralise both TcdA and TcdB, given a 1:1 molar ratio of the toxins. However, the relationship between PcAb potency and clinical relevance has not been established at this time.

The specific PcAb binding titres of the anti-TxA4 and anti-TxB4 flocks and, therefore, their concentrations present in the antisera were similar. However, the toxin neutralising potency of anti-TxB4 was ~ 60 lower than that of anti-A4, demonstrating that specific PcAb that are capable of binding to natural toxins do not necessarily render the toxin inactive. Moreover, as toxin inactivation is most likely achieved by steric hindrance, whereby toxin-cell interactions are impeded by a molecular barrier, PcAb binding to specific regions of the protein may be an important factor and these specific regions may be quite distinct between natural TcdA and TcdB. Based on the experimental evidence gained through this study, anti-TxA4 sera must contain a higher proportion of PcAb that both bind and inactivate their corresponding natural toxin compared to those from anti-TxB4 sera of equal specific PcAb concentration.
CHAPTER FIVE

TOXIN FRAGMENT INVESTIGATION
5.1 Introduction

Sheep immunisation studies have used a variety of recombinant protein constructs, based on TcdA and TcdB (Figure 5.1). All elicited PcAbs that bound to natural *C. difficile* toxins, but not all the PcAbs neutralised toxicity *in-vitro* (Roberts *et al.*, 2012; Maynard-Smith *et al.*, 2014). This phenomenon indicated that binding of PcAbs to specific regions of their respective natural toxin had differential importance in achieving toxin inactivation. Interestingly, the PcAb binding sites involved in toxin neutralisation appeared to differ between TcdA and TcdB. Thus, PcAb raised against the C-terminal receptor binding domain of TcdA (TxA2) exhibited a toxin neutralising potency more than 5 times that of PcAb directed to the central domain (TxAcen). In contrast, PcAb directed to the central domain of TcdB (TxBcen) demonstrated a neutralising potency ~ 150 times greater than those directed to the receptor binding domain (TxB2) (Maynard-Smith *et al.*, 2014). Antisera raised against the N-terminal enzymatic domain of TcdB (TxBN1) failed to neutralise natural toxin *in-vitro*, despite the presence of high binding PcAb titres (Roberts *et al.*, 2012). However, unlike the antigens used by Maynard-Smith *et al*, (2014), the TxBN1 antigen used by Roberts *et al*, (2012) had not been formaldehyde treated and, therefore, may not have been adequately presented to the cells of the ovine immune system.

Similar studies, conducted by Leuzzi *et al*. (2013), used a variety of recombinant protein fragments, based on TcdA and TcdB, to actively immunise mice. The resulting PcAb were shown to bind to their respective natural toxin, but those raised against fragments corresponding to the C-terminal receptor binding domain of TcdA (analogous to TxA2), the N-terminal enzymatic domain of TcdB (analogous to TxBN1) and the C-terminal receptor binding domain of TcdB (analogous to TxB2) demonstrated the highest toxin neutralising potency *in-vitro*. When used as a vaccine, these constructs demonstrated
Figure 5.1 Recombinant fragment construct map.
The relative positions of all recombinant fragments are represented with amino acid residue numbers corresponding to those of natural TcdA and TcdB. Adapted from Maynard-Smith et al. (2014).
efficacy in preventing CDI in the hamster model, but no recombinant protein constructs corresponding to the central domain of either TcdA or TcdB were used in this study (Leuzzi et al., 2013).

The ovine antisera produced in Chapter 4, against formaldehyde treated TxA4 and TxB4 antigens, contained high titres of PcAb which were shown to bind to their respective natural toxin and demonstrated relatively high toxin neutralising potency in-vitro. However, although the specific binding PcAb concentrations of the individual sheep were similar, toxin neutralising potency varied considerably between animals, particularly in the TxB4 flock. It seems likely that through continued re-immunisation and ongoing B cell affinity maturation, the immune response of each sheep produced high avidity PcAb populations directed to specific and distinct amino acid residues which varied between individuals. The TxA4 and TxB4 antigens encompassed the entire C-terminal (receptor binding) and central (transmembrane) domains of their respective natural toxin and it is possible that the amino acid residues that form the receptor binding domain are either more immunogenic or better presented to cells of the immune system than those of the central domain. If this is true, the majority of the PcAb produced against TxA4 and TxB4 may be directed to the receptor binding domains of their corresponding toxins and this could help explain the considerable difference in neutralising potency seen between anti-TxA4 and anti-TxB4. If some individuals within the TxB4 flock produced PcAb populations directed predominantly to the central domain whilst those of other individuals were directed predominantly to the receptor binding domain, this may also explain the disparity in toxin neutralising potency between sheep, despite similar binding PcAb concentrations.

Although anti-TxB4 PcAb samples were shown to neutralise natural TcdB with potencies equal to or greater than the anti-TcdB reference antibody, it now seems likely that a large proportion of these specific PcAbs to TcdB do not neutralise the toxin. The presence of these superfluous PcAb in a pharmaceutical anti-toxin formulation would be undesirable due to their diluting effect on the active component and the potential
immunogenicity of the exogenous proteins to the patient, particularly if the immunotherapy is administered systemically and repeated over a period of time. Therefore, fractionation of specific PcAb populations that effectively inactivate the natural toxin will be important in the development of a potential immunotherapeutic product for CDI.

The aim of the investigation documented in this chapter is to further localise the region of the TcdB protein to which PcAb must bind in order to effectively inactivate toxicity and, thus, maximise the levels of specific neutralising PcAb attained. It is, therefore, a continuation of the aim described in section 1.12 (objective 2), with the following additional specific objectives:

Objective 1 - to prepare affinity purification columns to separately fractionate PcAb populations directed to each of the recombinant fragments TxBN1, TxCBcen and TxB2

Whilst the previous studies have sought to produce toxin neutralising PcAb by immunising host animals with recombinant fragments corresponding to distinct toxin domains (Roberts et al., 2012; Leuzzi et al., 2013; Maynard-Smith et al., 2014), here a novel approach will utilise PcAb raised against formaldehyde treated whole natural TcdB and fractionate the specific binding antibody populations based on their affinity to the immobilised recombinant fragments. Thus, recombinant fragments TxBN1, TxCBcen and TxB2 will be coupled separately to Sepharose media and affinity chromatography columns prepared for each, respectively.

Objective 2 - to characterise each affinity purification column for non-specific binding and determine the specific PcAb concentrations directed to each fragment from anti-TcdB and anti-TxB4 samples
Each of the three columns (one for each fragment) will be assessed initially for non-specific binding using antisera directed to an irrelevant antigen. Samples of anti-TcdB reference antibody and pooled anti-TxB4 antisera will then be assessed separately using each of the three columns and the concentration of specific binding PcAb directed to each distinct toxin domain of each sample calculated. The outcome of this investigation will reveal the proportion of PcAb (in terms of percentage of total toxin binding PcAb) in each sample directed to each of the three toxin domains under scrutiny.

Objective 3 - to purify volumes of specific PcAb directed to each of the three TcdB domains under investigation and assess them for toxin neutralising potency

Larger volumes of non-specific antisera, anti-TcdB reference and anti-TxB4 sera will be loaded, separately, to each column and the eluted fractions of fragment specific PcAb concentrated, dialysed and assessed for toxin neutralising potency using the Vero cytotoxic neutralisation assay established in section 3.2.6. The results of these assays will reveal which fragment specific PcAb population possesses the highest toxin neutralising potency.

Ultimately, the results obtained on completion of objectives 1, 2 and 3 will determine the percentage of toxin binding PcAb, from both the anti-TcdB reference and pooled anti-TxB4 sera, directed to each of the three TcdB domains under test and will establish the toxin neutralising potency of each PcAb population. These findings may enable refinement of the immunogen used in future to raise TcdB neutralising PcAb and determine which specific population to fractionate for use in a potential therapeutic treatment for CDI.
5.2 Methods

5.2.1 Determination of Specific Antibody Concentration

Three MSAC columns, containing 2 g solid phase (equivalent to 7 mL column volume) were prepared using the method described in section 2.2.3 with the solid phase conjugated to TxBN1, TxBcen or TxB2, as required. Each MSAC column was connected to a BioRad automated chromatography system and the specific PcAb concentrations of samples were determined using the following parameters: with the systems UV detector (measuring absorbance at 280 nm) reset to zero and the pump speed set at 1.0 mL/min, a PcAb sample of 1.0 mL was loaded onto the column and allowed to re-circulate for 2 hours. Washing buffer (PBW) was then passed through the column until the UV reading stabilised at baseline and the bound PcAb was eluted from the column using glycine solution (100 mM, pH 1.5) and collected. The eluted specific PcAb concentration was measured at OD280, as described in section 2.2.1, and the specific PcAb concentration of the original sample calculated as follows:

Original sample concentration = (Concentration of eluate) \times \left( \frac{\text{volume of eluate}}{\text{loaded sample volume}} \right)

Each MSAC column was first assessed for non-specific binding by loading antisera, directed towards *Echis ocellatus* snake venom (batch no. M0113 #270601), and the protein concentration of the eluted fraction measured. The mean of three replicate assays for each column was defined as the non-specific binding value, with a mean of ≤ 0.5 g/L considered acceptable. Specific PcAb concentrations, directed to the three distinct fragments, were then determined for the anti-TcdB reference (BN: CDB000266 08/07/10) and pooled anti-TxB4 sera (batch no M0832 #080513 – taken from TxB4 flock 1 at 10 weeks post restart) with each assay performed in triplicate. The results of the three assays were corrected for non-specific binding before calculation of the mean and standard deviation.
5.2.2 Antibody Fractionation by Affinity Chromatography

The MSAC columns, as prepared in section 5.2.1, were used to purify small quantities of specific PcAb from the anti-TcdB reference and pooled anti-TxB4 sera samples. The chromatography system was operated as per section 5.2.1 with the following amendments:

- 25 mL of sample was loaded onto each column.
- The bound PcAb were eluted from the column using glycine solution (100 mM, pH 2.5) and collected.
- The pH of the eluted PcAb was immediately raised to pH 4.0, by the addition of sodium acetate buffer (800 mM, pH 8.0) and the PcAb concentration was measured at OD280, as described in section 2.2.1.

5.2.3 Concentration and Diafiltration of Eluted Fractions

Prior to assessment of the PcAb fractions by cytotoxic neutralisation assay, each sample was concentrated and diafiltered. Sample concentration was achieved using Vivispin 20 (MWCO 30 kDa) tubes processed using a bench top centrifuge programmed to run for 15 minutes at 3500 rpm. Centrifugation runs were continued until all samples were reduced in volume to a maximum of 10 mL and then each was diafiltered against four volumes (40 mL total) of PBSa. The pH of each buffer exchanged fraction was measured prior to sterile filtration and final protein concentration determination, according to the method described in section 2.2.1.
5.2.4 Toxin Neutralising Potency of Eluted Fractions

With the exception of the PcAb samples derived from non-specific sera, the prepared PcAb fractions were diluted in DMEM to a concentration of 1 g/L. The toxin neutralising potency (µg/mL) of each sample was then determined according to the method described in section 3.2.6 using a serial doubling dilution starting at 1/100. The reference antibody was included on all assay plates, at a starting dilution of 1/5000, the ED_{50} of which was used to correct for between-assay variations. In addition to the routine assay controls (section 3.2.6) additional controls were included using dialysis buffer (PBSa) with and without toxin to ensure that the buffer did not adversely affect cell growth or activity of TcdB.

As the samples had been previously diluted to 1 g/L (equivalent to 1 mg/mL) the calculated toxin neutralising potency was expressed in terms of µg of toxin neutralised per mg of antibody.

5.2.5 Statistical Methods

The results generated in these investigations were analysed statistically using GraphPad Prism V5.03 software. Parametric tests were used to compare these data based on the assumption that measurements, taken from fractions derived from a common PcAb source, conform to normal Gaussian distribution. Thus, data are described using mean and standard deviation (SD) values with statistical significance calculated using one–way analysis of variance (ANOVA) including the Bonferroni post-hoc test for multiple comparisons, or unpaired t-test. Where a significant result was indicated using the Bonferroni post-hoc test, this was confirmed using the unpaired t-test.
5.3 Results

5.3.1 Non-Specific Binding Concentrations

Ovine anti-	extit{Echis ocellatus} snake venom sera was used to assess each of the three affinity chromatography columns for non-specific binding. The mean of three assays, per column, defined the non-specific binding concentrations as 0.2 g/L, 0.3 g/L and 0.2 g/L for the TxBN1, TxBcen and TxB2 columns respectively, each with a SD of 0.1. These values were used to correct for non-specific binding in all subsequent PcAb concentration determination assays.

5.3.2 Fragment Specific PcAb Binding Concentrations

The anti-TcdB reference antibody and anti-TxB4 sera were assessed, independently, using each of the three affinity columns to determine the concentration of specific PcAb of each sample directed to the three distinct fragments. Results were corrected for non-specific binding and the mean specific PcAb concentration (in terms of g/L) of three assay replicates were determined (Table 5.1). The SD of each calculated concentration was ≤ 0.1.

<table>
<thead>
<tr>
<th></th>
<th>anti-TxBN1 (g/L)</th>
<th>anti-TxBcen (g/L)</th>
<th>anti-TxB2 (g/L)</th>
</tr>
</thead>
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<tr>
<td>anti-TcdB Reference</td>
<td>1.5</td>
<td>1.3</td>
<td>3.9</td>
</tr>
<tr>
<td>anti-TxB4 Sera</td>
<td>0.2</td>
<td>2.7</td>
<td>3.2</td>
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Table 5.1 Fragment Specific PcAb Concentrations.

The fraction of PcAb directed specifically to each of the three distinct recombinant fragment constructs (TxBN1, TxBcen and TxB2) was determined for the anti-TcdB reference antibody and anti-TxB4 pooled sera.
The sum of the specific PcAb concentrations directed to each fragment for each sample were 6.7 g/L for the anti-TcdB reference and 6.1 g/L for the anti-TxB4 sera. The proportions of binding PcAb, derived from anti-TcdB, directed to the TxBN1, TxBcen and TxB2 fragments (as a percentage of total specific binding PcAb) were 22%, 19% and 58%, respectively. Binding PcAbs, derived from anti-TxB4 sera, were directed to the TxBN1, TxBcen and TxB2 fragments in proportions of 3%, 44% and 53% for each, respectively.

5.3.3 Fractionation of Fragment Specific PcAb

Antibody purifications were performed, independently, using 25 mL volumes of non-specific serum, anti-TcdB reference antibody and anti-TxB4 sera against each of the three immobilised recombinant fragment columns. Purifications were performed in triplicate using a fresh volume of sample for each run. The concentration of specific PcAb eluted was used to calculate the yield of PcAb obtained from each column (Table 5.2).

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<td>45.2 (4.0)</td>
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Table 5.2 Fragment Specific PcAb Yield.
The quantity of PcAb eluted from each of the three recombinant fragment affinity columns (TxBN1, TxBcen and TxB2) was measured for the fractions of non-specific sera, anti-TcdB reference antibody and anti-TxB4 pooled sera. The mean of three replicates is presented with the value in brackets representing the standard deviation.
5.3.4 Toxin Neutralising Potency of Fragment Specific PcAb

All eluted samples were concentrated and then diafiltered against PBSa to lower the ionic strength and raise the pH to a level compatible with the cytotoxic neutralisation assay (physiological pH 7.4, ionic strength ~ 150 mM). The pH was confirmed prior to final sterile filtration and protein concentration determination. The PcAb fractions were then assessed for toxin neutralising potency as described in section 3.2.6 with the modifications detailed in section 5.2.4.

Fractions of anti-TxBN1, anti-TxBcen and anti-TxB2, derived from anti-TcdB reference PcAb, were found to neutralise native TcdB with a mean potency of 0.8 µg/mg (SD 0.15), 1.0 µg/mg (SD 0.2) and 0.4 µg/mg (SD 0.1) for each respectively (Figure 5.2 a). There was no significant difference in potency between the anti-TxBN1 and anti-TxBcen fractions (P = 0.17). However, the potencies of anti-TxBN1 and anti-TxBcen were significantly higher than that of anti-TxB2 (P = 0.03 and P = 0.01 respectively).

Fractions of anti-TxBN1, anti-TxBcen and anti-TxB2, derived from anti-TxB4 sera, were found to neutralise native TcdB with a mean potency of 0.2 µg/mg (SD 0.06), 2.5 µg/mg (SD 0.4) and 1.2 µg/mg (SD 0.5) respectively (Figure 5.2 b). Neutralising potencies of both anti-TxBcen and anti-TxB2 fractions were significantly higher than that of anti-TxBN1 (P = < 0.001 and P = 0.02, respectively). However, anti-TxBcen was also significantly more potent than anti-TxB2 (P = 0.02).

Purified PcAb fractions derived from non-specific antisera had no measureable toxin neutralising potency. The additional assay controls (described in section 5.2.4) confirmed that the PBSa buffer, in which the PcAb were formulated, had no effect on cell growth or TcdB activity.
Figure 5.2 Toxin Neutralising Potency of Fragment Specific PcAb.

The potency results (µg of toxin neutralised per mg of PcAb) are presented for each of the three replicates per fraction, derived from anti-TcdB reference PcAb (a) and anti-TxB4 sera (b), directed against recombinant fragments TxBN1, TxBcen and TxB2.
5.4 Discussion

The level of non-specific binding of anti-*Echis ocellatus* venom to each of the three columns was found to be acceptably low with all values ≤ 0.3 g/L (derived from in-house MicroPharm acceptance criteria of ≤ 0.5 g/L). It is unclear whether this non-specific PcAb binding interaction occurred with the immobilised antigen, solid phase support, glass column or the plastic components of the column. Moreover, the non-specific binding may not be related to PcAb and may instead involve other serum proteins, such as albumin. Ionic interaction is unlikely to account for the measured levels of non-specific binding due to the presence of 0.5 M sodium chloride (NaCl) in the PBW washing buffer, which limits protein adsorption. Further work could be performed to determine the source of the non-specific binding, for example by altering the ionic strength of the washing buffer or adding low levels of detergent, but this is beyond the scope of the current study.

A different elution buffer pH was used between the protein concentration determination MSAC assay and the PcAb fractionation method. Thus, an elution pH of 1.5 was used for protein determination, whereas pH 2.5 was used to elute PcAb fractions to be assessed by cytotoxic neutralisation assay. This difference in pH was due to an observation that elution at pH 2.5 often failed to dissociate the entire bound fraction from the solid phase, leading to inaccurate protein concentration measurements. This problem was resolved by eluting at pH 1.5, which resulted in a complete dissociation of the bound fraction and reproducible protein concentration measurements, but with a considerable risk of denaturing the PcAb and subsequent loss of toxin neutralising activity. A gentler elution at pH 2.5 dissociated the majority of the bound fraction and the toxin neutralising activity of the PcAb was maintained by immediately adjusting the eluted fraction to pH 4 by the addition of 10% v/v 0.8 M sodium acetate at pH 8. The elution of the bound PcAb fractions could be further optimised by experimenting with different elution buffers, such
as 150 mM ammonium hydroxide at pH 10.5 or a chaotropic salt such as 3 M sodium thiocyanate, but this is beyond the scope of the present investigation.

The proportion of PcAb, derived from anti-TcdB reference and anti-TxB4 sera, directed to each of the three TcdB domains under investigation were successfully quantified (section 5.3.3, Table 5.1). The sum of the concentrations directed to each fragment for each sample were 6.7 g/L and 6.1 g/L for the anti-TcdB reference and anti-TxB4 sera, respectively. The measurement of 6.7 g/L for the anti-TcdB reference PcAb is higher than that obtained by SSAC (section 3.3.4) and is likely due to the fact that the SSAC method utilised immobilised TxB4 fragments as the solid phase, which lacked the N-terminus TxBN1 region and, therefore, failed to account for the presence of TxBN1 specific PcAb which are naturally present in the anti-TcdB sample. This did not affect the anti-TxB4 measurements as this antiserum should contain no TxBN1 specific PcAb. However, a small proportion of the anti-TxB4 sera PcAbs were found to be directed to TxBN1 (0.2 g/L or 3% of total specific PcAb). This may be due to a small degree of amino acid sequence homology between TxBN1 and other domains of TcdB or may be due to naturally circulating anti-TxBN1 antibodies from a previous exposure of the sheep to natural *C. difficile* infection.

The proportions of binding PcAb, derived from the anti-TcdB reference, directed to the TxBN1, TxBcen and TxB2 fragments (as a percentage of total specific binding PcAb) were 22%, 19% and 58% for each, respectively. In contrast, the percentage of total neutralising PcAb directed to the TxBN1, TxBcen and TxB2 fragments were 36%, 45% and 18% for each, respectively. This confirms the previous postulation that the majority of PcAb are directed to the C-terminus, but show the least neutralising activity. Thus, with regard to the anti-TcdB reference, 58% of the TcdB specific PcAb bound to the C-terminus and accounted for only 18% of the toxin neutralising potency, whereas 19% of the specific PcAb bound to the central region and accounted for 45% of the neutralising potency. Surprisingly, although TxBN1 binding PcAb were expected, they accounted for 36% of the
total toxin neutralising potency. This discovery is in stark contrast to the findings of Roberts et al. (2012), whereby antisera raised against TxBN1 fragments failed to produce any toxin neutralising PcAb, although binding PcAb were present in high titres. When compared to the potency of the un-fractionated anti-TcdB reference PcAb (anti-TcdB reference potency = 18.4 µg/mL, derived from the potency calculation described in section 3.2.6 using the ED$_{50}$ determined in section 3.3.6, divided by specific binding concentration of 6.7 g/L = 2.7 µg/mg), the total neutralising activity of the eluted fractions (2.2 µg/mg) equated to only 82% of the expected total. This 18% loss of activity may be due to damage sustained by the PcAbs through the purification process, particularly through the relatively harsh conditions associated with the low pH elution step.

The proportions of binding PcAb, derived from anti-TxB4 sera, directed to the TxBN1, TxBcen and TxB2 fragments (as a percentage of total specific binding PcAb) were 3%, 44% and 53% for each, respectively. No binding PcAb directed to TxBN1 were expected in the antisera as the immunogen did not include any antigen fragments specific to this region. However, there is likely to be some amino acid sequence homology between TxBN1 and other domains of TcdB, as previously discussed. The percentage of total neutralising PcAb directed to the TxBN1, TxBcen and TxB2 fragments were 5%, 64% and 31% for each, respectively. Thus, with regard to the anti-TxB4 sera, 53% of the TcdB specific PcAb bound to the C-terminus and account for 31% of the toxin neutralising potency. In contrast, 44% of the specific PcAb bound to the central region and account for 64% of the neutralising potency. These results provide further evidence that the majority of PcAb are directed to the C-terminus, but show the least neutralising activity.

When compared to the potency of the un-fractionated anti-TxB4 sera (anti-TxB4 sera potency = 26.1 µg/mL, as determined in section 4.3.3, divided by specific concentration of 6.1 g/L = 4.3 µg/mg), the total neutralising activity of the eluted fractions (3.9 µg/mg) equated to only 91% of the expected total. This 9% loss of activity may be due to processing losses as described previously.
The TxB4 antisera contained a higher proportion of TxBcen specific binding PcAb compared to the anti-TcdB reference (44% and 19%, respectively). The reason for this is unclear, but may be related to the absence of the TxBN1 region in the TxB4 immunogen therefore potentially enabling a greater immune response to be directed to the central domain. Another possible factor could be the number of individual sheep enrolled in the antisera production flocks. Thus, the TxB4 flock comprised eight sheep whereas the anti-TcdB flock comprised only two. Antisera derived from only two individuals lack the PcAb diversity of that from a larger pool. In addition, the anti-TcdB reference PcAb was a purified total IgG concentrated to 50 g/L, whereby the non-immunoglobulin components of the serum had been removed by caprylic acid precipitation. The anti-TxB4 serum was not purified prior to use. The difference in relative concentration and potency of the anti-TcdB reference derived PcAb fractions compared to those of the anti-TxB4 sera may be associated with the caprylic acid IgG purification step.

Affinity chromatography has been used in the pharmaceutical industry to separate antigen specific binding from non-specific IgG, allowing for the production of therapeutic IgG formulations with high specific binding antibody concentration relative to total protein content. This purification step is likely to reduce the incidence of adverse reactions and side effects in treated patients. In this study, affinity chromatography has been used for the first time to separate neutralising from non-neutralising populations of binding PcAb. Moreover, previous studies have used immunogens composed of recombinant fragments of TcdB to raise neutralising PcAb, whereas here PcAb raised against whole natural TcdB have been fractionated against recombinant protein constructs to isolate specific binding PcAb and identify domains of TcdB which are important with regard to its inactivation. The outcome of this study presents the potential to formulate a *C. difficile* antitoxin using a high concentration of specific toxin neutralising PcAb relative to total binding PcAb content, further reducing the potential for side effects in treated patients.
Specific binding PcAb directed to the C-terminus of TcdA have been shown to possess relatively high toxin neutralising potency (Leuzzi et al., 2013; Maynard-Smith et al., 2014), suggesting that steric hindrance is responsible for inhibiting the toxin from binding to cell surface receptors. The investigations described in this chapter have revealed that PcAbs that bind to the central (transmembrane) domain of TcdB poses toxin neutralising potencies up to 2.5 times that of PcAb directed to the C-terminus (receptor binding domain), confirming the findings of others (Maynard-Smith et al., 2014). Clearly the mechanism of toxin inhibition differs between TcdA and TcdB. However, this difference remains unexplained at the present time.

Each of the three distinct recombinant fragments, TxBN1, TxBcen and TxB2, were successfully conjugated to Sepharose media with coupling efficiencies of > 90%. Affinity chromatography columns were prepared using these immobilised fragments and characterised for non-specific binding. These columns were successfully used to quantify the proportion of specific binding PcAbs from samples directed to each of the three TcdB regions under investigation. Larger volumes of fractionated specific binding PcAb were purified and assessed using the previously established cytotoxic neutralisation assay (section 3.2.6). This assessment revealed which of the three specific binding PcAb populations possessed the highest toxin neutralising potency. The objectives set out in section 5.1 have therefore been achieved.

The methods described in this chapter could also be applied to TcdA to investigate important toxin binding and neutralising domains. The results of such an investigation could be compared to the results of the study described here and may illustrate differences in domains to which PcAb must bind to effectively neutralise the toxin. This further work was not conducted in the present study due to time constraints.
CHAPTER SIX

SIMULATED BOWEL BARRIER
6.1 Introduction

In cases of severe CDI, both toxins TcdA and TcdB have been shown to enter the systemic circulation, as evidenced by their presence in the serum samples of experimental animals (Steele et al., 2012). This demonstrates that both toxins are capable of translocation from within the colonic lumen (i.e. the site of infection) to the circulation, where they may cause systemic disease, organ failure and death. No mechanisms have been described for the absorption or active transport of TcdA or TcdB across the colonic epithelial cell barrier. Translocation across a damaged cell barrier is the most likely means by which they move from the colon to the circulation because the enterotoxic TcdA has been shown to cause considerable intestinal damage in vivo (Lyerly et al., 1991), which is mirrored by cell rounding and loss of tight junctions in confluent Caco-2 (human colonic carcinoma) monolayers in vitro (Banerjee et al., 2009).

For more than two decades the Caco-2 cell line has been used widely as an in vitro model of the human intestinal barrier. When maintained in culture for 21 days, a confluent Caco-2 monolayer spontaneously differentiates and develops microvilli and tight junctions typical of absorptive intestinal enterocytes (Sambuy et al., 2005; Ehlers et al., 2011). This characteristic has facilitated research into absorption by the gastro-intestinal tract of drug, peptide, heavy metal and environmental toxins (Wikman-Larhed and Artursson, 1995; Ehlers et al., 2011; Rossi et al., 1996).

The development of an in vitro simulated bowel barrier to explore the interactions between epithelial cell barrier of the colonic lumen and systemic circulation would provide a valuable research tool to aid investigations into C. difficile toxin translocation and, thereby, the development of potential treatment strategies. Currently, such investigations are routinely performed in vivo using either the hamster or piglet model and often involve externalisation of the colon. A sensitive and reproducible cell based system would have the
potential to replace these animal models providing both ethical and cost saving benefits. Hence a series of experiments were designed to develop and optimise a cell based preparation to model the bowel barrier. It was decided to utilise 24-well tissue culture plates fitted with Transwell® inserts, which effectively separate each well into two compartments by means of a porous (0.4 µm) polycarbonate membrane. By establishing a differentiated Caco-2 cell monolayer on the upper side of the Transwell membrane, the two compartments are essentially isolated, forming an upper apical chamber (simulated colonic lumen) and lower basolateral chamber (simulated systemic circulation). A Vero cell monolayer, established on the base of each basolateral compartment (BL), will be used as an indicator for detection of toxin translocated from the apical compartment (AP) with relative toxin concentrations estimated by percentage cell rounding of the Vero monolayer (Figure 6.1). The aim of this chapter was to develop a working model and comprised of several distinct objectives:

Objective 1 to quantify toxin adsorption onto the surfaces of the tissue culture vessels

The proposed cell co-culture system involves the use of a 24-well tissue culture plate with the addition of Transwell inserts to each well of the plate. The total working volume of each well is 700 µL. When the Transwell inserts are in position the AP and BL compartments contain 200 µL and 500 µL, respectively. As the concentration of toxin translocated from the AP will be diluted in the working volume of the BL, the level of toxin adsorption to the surfaces of the co-culture system must be quantified in order to determine the optimum toxin concentrations required to establish a sufficiently sensitive simulated bowel barrier system. The level of toxin adsorption to the internal well surfaces of an empty 24-well tissue culture plate will be quantified assuming that 100% of the 200 µL working volume from the AP will translocate to the BL.
Figure 6.1  Schematic representation of the simulated bowel barrier layout. A differentiated Caco-2 monolayer, grown on the porous membrane of the Transwell insert, separates the upper and lower compartments providing a barrier composed of a single layer of differentiated intestinal epithelial enterocytes. Diagram modified from Bouike et al. (2011).
Objective 2 to determine whether toxin can move freely from the apical to the basolateral compartment through the Transwell insert and to quantify toxin adsorption to the polycarbonate membrane.

These experiments will utilise the complete co-culture system, without any cells attached, and will include the addition of the required working volumes of DMEM. Toxin dilutions will be introduced to the AP of each well and the volume and quantity of toxin translocated to the BL will be quantified. Residual toxin remaining in the AP will also be quantified. This will determine whether the porous polycarbonate membrane (0.4 µm pore size) of the Transwell insert allows for the free movement of TcdA and TcdB to the BL without the presence of a differentiated cell monolayer.

Objective 3 to determine the concentrations of TcdA and TcdB required in the AP to disrupt an established and differentiated Caco-2 monolayer.

The complete co-culture system including a Caco-2 monolayer on the AP membrane, with and without the presence of Vero cells in the BL, will be used in these experiments. Various toxin dilutions will be introduced to the AP and the volume and quantity of toxin translocation to BL will be determined. Residual toxin remaining in the AP will also be quantified. The outcome of these experiments will help to establish the quantity of TcdA or TcdB required in the AP to provide sufficient translocated toxin in the BL to cause a measureable effect on the Vero monolayer.
Objective 4  to investigate toxic synergy using combinations of TcdA and TcdB

The optimal toxin concentrations, determined in Objective 3, will be used in these experiments along with the complete co-culture system including the Caco-2 cell barrier and Vero indicator cells. Toxin synergy will be investigated using constant concentrations of either TcdA or TcdB together with serial doubling dilutions of the other toxin. The Vero cell monolayer will be assessed visually to determine the percentage cell rounding. The outcomes from this set of experiments will indicate whether the toxic effects of one or both of the *C. difficile* toxins under investigation, as well as their ability to translocate across the cell membrane, are enhanced by the presence of their counterpart.

Objective 5  to investigate toxin neutralising strategies for simulated oral and systemic anti-toxin administration routes

The established simulated bowel barrier system will be used to investigate the therapeutic use of the PcAb produced in Chapter 4. The optimal TcdA and TcdB concentrations, determined in objectives 3 and 4, will be introduced simultaneously to the AP compartments. Neutralising doses of anti-toxin PcAb will be administered to the AP or BL compartments, in various combinations, to determine the most efficacious method of PcAb delivery (systemic versus oral) for the treatment or prophylaxis of CDI.
6.2 Methods

6.2.1 Establishment of Cell Monolayer in Apical Compartment

Individual Transwell inserts, purchased from VWR (Leicestershire, UK), were placed into each well of a 24-well plate (also purchased from VWR, Leicestershire, UK) to allow seeding of the membranes and feeding from both upper and lower compartments. The lower compartments were filled with 500 µL of DMEM. Caco-2 cells were seeded onto the upper compartment membrane at 200 µL per well at a concentration of 2.5 x 10^5 mL^-1 (equivalent to 50,000 cells per Transwell insert). Each seeded Transwell array was maintained at 37°C in a 5% CO₂ humidified atmosphere for a minimum of 21 days, to allow cell differentiation, with media changes made every second or third day to ensure that the cells remained nourished.

6.2.2 Cytotoxicity Assay

Cytotoxicity assay plates were prepared in 96 well format according to section 2.2.6.4. Immediately prior to use, 100 µL of DMEM was removed from each well and 100 µL of each test sample was added to assigned wells (Table 6.1). Toxin standards were prepared separately in bijou tubes by making serial doubling dilutions of TcdA (20 ng/mL – 160 pg/mL) and of TcdB (400 pg/mL – 3.13 pg/mL) in DMEM. Assay controls included were media without toxin as a negative, and media with sufficient toxin to cause 100% rounding of the monolayer as a positive. Where appropriate, test samples were diluted to bring their expected concentrations to within the linear range of the assay. Following incubation for 24 hours, visual assessment of the monolayer of
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Table 6.1 96-well cytotoxicity plate layout.

All wells, except A11 – A12 and B11 – B12 (media controls), contained confluent Vero cell monolayers.

Assay controls and toxin standards (for construction of standard curves) were included as indicated.

Rows A1 – A8 to F1 – F8 were used for analysis of unknown samples with the results interpolated against the relevant standard curve.
each well was made using an inverted light microscope, at 100X magnification, to assess the percentage cell rounding. Standard curves were plotted, using Graphpad Prism software, (% cell rounding against toxin concentration) and the concentrations of toxin present in the test samples were determined by interpolation to the standard curve.

6.2.3 Toxin Adsorption Assessment (Apical compartment and indicator cells absent)

Using an empty 24-well plate without cells attached, 600 µL of DMEM was added to each well. Serial doubling dilutions of TcdA (400 ng/mL – 12 ng/mL) and TcdB (40 ng/mL – 1.2 ng/mL) were made separately in bijou tubes, using DMEM as the diluent. Aliquots of 100 µL of the toxin dilutions were transferred to assigned wells of the 24-well plate (Table 6.2) providing final toxin concentrations of 57 to 1.8 ng/mL (TcdA) or 5.7 to 180 pg/mL (TcdB) and incubated for 24 hours. Following incubation, 100 µL was aspirated from each well for assessment using the cytotoxicity assay (section 6.2.2). Aspirated samples were diluted, as appropriate, to bring the expected toxin concentration into the linear range of the cytotoxicity assay.

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Table 6.2 24-well plate layout.

No upper (apical) compartments were present in this experiment and toxin dilutions were added directly to the lower (basolateral) compartments. Wells A1 to A6 contain TcdA at final working concentrations from 57 ng/mL to 1.8 ng/mL respectively, duplicated in wells B1 to B6. Wells C1 to C6 contain TcdB at final working volume concentrations from 5.7 ng/mL to 180 pg/mL respectively, duplicated in wells D1 to D6.
6.2.4 Assessment of Toxin Adsorption to Transwell Insert (Apical compartment present, cells absent)

Using an empty 24-well plate without cells attached, 500 µL of DMEM was added to each well. Empty Transwell inserts, without cells attached, were placed into each well and 100 µL of DMEM was added to each AP compartment. Serial doubling dilutions of TcdA (400 ng/mL – 12 ng/mL) and TcdB (40 ng/mL – 1.2 ng/mL) were made separately in bijou tubes, using DMEM as the diluent. Aliquots of 100 µL of the toxin dilutions were transferred to the AP compartments of the co-culture system providing final toxin concentrations of TcdA at 200 to 6.3 ng/mL in wells A1 to A6, duplicated in wells B1 to B6, or 20 to 0.63 ng/mL of TcdB in wells C1 to C6, duplicated in wells D1 to D6. Following incubation for 24 hours, 100 µL was separately aspirated from the AP and BL compartments of each well for assessment using the cytotoxicity assay (section 6.2.2). Aspirated samples were diluted, as appropriate, to bring the expected toxin concentration into the linear range of the cytotoxicity assay.

6.2.5 Toxin Translocation across Cell Barrier (Caco-2 cell barrier present in AP, Vero indicator cells in BL absent)

This experiment was performed using the methods described in section 6.2.4, using a Transwell insert with an established and differentiated Caco-2 monolayer attached (section 6.2.1). No Vero indicator cells were present in the BL compartment. Each differentiated Caco-2 monolayer was assessed visually using an inverted light microscope, at 100X magnification, following the 24 hour incubation.
6.2.6 Toxin Translocation across Cell Barrier with Indicator Cells Present (Complete system)

This experiment was performed using the complete cell barrier and indicator system. A 24-well plate with Vero monolayers (indicator cells) was prepared using the methods described in section 2.2.6.5, and Transwell inserts with differentiated Caco-2 monolayers attached were prepared using the methods described in section 6.2.1. Toxin was introduced to the cell barrier system as described in section 6.2.4. Following incubation with toxin, visual assessments of the Vero monolayers were made using an inverted light microscope, at 100X magnification, to assess the percentage cell rounding.

6.2.7 Toxin Synergy Investigation

This experiment was performed using the complete cell barrier and indicator system (section 6.2.6). The AP compartments of rows A and B contained a constant final concentration of 100 ng/mL of TcdA with serial doubling dilutions of TcdB from 5 ng/mL to 160 pg/mL from wells A1 to A6, respectively, duplicated in wells B1 to B6. The AP compartments of rows C and D contained a constant final concentration of 5 ng/mL of TcdB with serial doubling dilutions of TcdA from 100 ng/mL to 3.1 ng/mL from wells C1 to C6, respectively, duplicated in wells D1 to D6. Following incubation with toxin, visual assessments of the Vero monolayers were made using an inverted light microscope, at 100X magnification, to assess the percentage cell rounding.
6.2.8 Anti-toxin Administration Route Investigation

This experiment was performed using the complete cell barrier and indicator system (section 6.2.6). Anti-toxins were purified separately from anti-TxA4 and anti-TxB4 sera, according to the methods described by Roberts et al. (2012), at a concentration of 25 g/L with toxin neutralising potencies (measured using the methods described in section 3.2.6) of 900 µg/mL and 15 µg/mL for anti-TxA4 (batch #211113) and anti-TxB4 (batch #131113), respectively. The AP compartments of each row of the 24-well plate contained 200µL of media with TcdA and TcdB at final concentrations of 100 ng/mL and 5 ng/mL for each, respectively. A 5µL volume of purified anti-TxA4 was added to the AP compartments of wells A1 to A3, duplicated in wells B1 to B3, and to the BL compartments of wells A4 to A6, duplicated in wells B4 to B6. A 5µL volume of purified anti-TxB4 was added to the AP compartments of wells C1 to C3, duplicated in wells D1 to D3, and to the BL compartments of wells C4 to C6, duplicated in wells D4 to D6.

Following incubation for 24 hours, visual assessments of the Vero monolayers were made using an inverted light microscope, at 100X magnification, to assess the percentage cell rounding.

6.3 Results

6.3.1 Toxin Adsorption Assessment (Objective 1)

The 100 µL aspirated samples taken from each well (no Transwell insert or cells present) were successfully assessed using the cytotoxicity assay and their residual toxin concentrations were determined by interpolation to the standard curves (Figures 6.2a & 6.2b). The quantity of toxin (in terms of weight in ng) added to each well was calculated, based on working well volumes of 700 µL, and compared to the quantity of recovered
Figure 6.2 Typical TcdA and TcdB cytotoxicity standard curve.
Percentage Vero cell rounding versus Log10 of toxin concentration
toxin as calculated from the concentrations determined from the cytotoxicity assay. The quantity of residual TcdA detected varied from 78% at the highest concentration to 50% at the lowest concentration with a mean recovery of 57% (Figure 6.3a). Similarly, TcdB recovery varied from 74% at the highest concentration to 44% at the lowest concentration with a mean recovery of 58% (Figure 6.3b). These data indicate that up to 40% of the initial toxin added to the BL compartment is not recovered, probably due to its adsorption to the well surface. Considering that between 5 ng/mL to 0.5 ng/mL of TcdA or 100 pg/mL to 6 pg/mL of TcdB are required in the BL compartment to have any measureable effect on a Vero cell monolayer, the findings of this experiment will influence the toxin challenge doses selected for further development of this co-culture preparation.

6.3.2 Toxin Translocation across an Empty Transwell Insert (Objective 2)

It was determined that 100 µL of media from the AP compartment moved freely through the porous polycarbonate membrane to the BL compartment in each well of the co-culture plate system. The quantity of toxin recovered from each compartment was calculated using the toxin concentrations, as determined by the cytotoxicity assay, and the final measured volumes of 100 µL in each AP and 600 µL in each BL compartment. Expected toxin recovery was based on the assumption that the concentration added to the AP compartments would reach equilibrium with the larger working volume of the BL compartments over the 24 hour incubation period.

Higher than expected quantities of TcdA were detected in all AP compartment samples (Figure 6.4a). The percentage of toxin recovery ranged from 130% to 257% with a mean value of 205% (SD = 52). These results indicate that TcdA was concentrated in the AP compartment as media moved through the Transwell membrane into the BL compartment.
Figure 6.3  Comparison of percentage toxin recovered from each well (no Transwell insert or cells present), following incubation for 24 hours, relative to the initial quantity of toxin present in each 700 µL volume.
Figure 6.4  Comparison of expected and detected quantities of TcdA from AP (a) and BL (b) compartments of the co-culture system. The ‘Y’ axis columns describe the location on the 24-well plate from where the samples were taken.
Based on the assumption that toxin concentrations of the AP compartments would reach equilibrium with the media of the BL compartments, lower than expected quantities of TcdA were detected in all BL compartment samples (Figure 6.4b). Toxin recovery percentages ranged from 33% to 59% of expected quantities, with a mean value of 44% (SD = 10). Similar results were obtained when TcdB was used as the challenge toxin. The percentage of toxin recovery from the AP compartment ranged from 104% to 225% with a mean value of 189% (SD = 49) (Figure 6.5b). These results support the suggestion that toxins are concentrated in the AP compartment as media moves through the Transwell membrane into the BL compartment. Lower than initially expected quantities of TcdB were detected in all BL compartment samples (Figure 6.5b). Toxin recovery percentages ranged from 45% to 85% of expected quantities, with a mean value of 60% (SD = 16).

The calculated toxin quantities from both the AP and BL compartments were added together and compared to the initial quantity of toxin added to each well of the TcdA (Figure 6.6a) and TcdB (Figure 6.6b) experiments. Total toxin recovery percentages ranged from 62% to 88% (mean = 67%, SD = 11) and 60% to 92% (mean = 77%, SD = 12) for TcdA and TcdB, respectively.

Although toxin concentrations were partially concentrated in the lower volume of the AP compartment by the porous polycarbonate membrane of the Transwell insert, a large percentage of the initial challenge dose did move freely through the membrane into the BL compartment. Given the findings of the previous toxin adsorption experiment, where up to 40% of the initial toxin challenge was not recoverable, probably due to adsorption to the internal surface area of the BL compartment, it is reasonable to assume that the losses in toxin quantity measured in this experiment were due to the same effect rather than being caused by adsorption to the Transwell membrane.
Figure 6.5  Comparison of expected and detected quantities of TcdB from AP (a) and BL (b) compartments of the co-culture system. The ‘Y’ axis columns describe the location on the 24-well plate from where the samples were taken.
Figure 6.6  Comparison of total quantity of TcdA (a) and TcdB (b) measured from both AP and BL compartments of each well against the initial toxin quantity added to the AP compartments.
6.3.3 Toxin Translocation across a Differentiated Caco-2 Monolayer (Objective 3)

With a differentiated Caco-2 monolayer grown on each Transwell porous polycarbonate membrane, smaller volumes of media translocated to the BL compartments following incubation with toxin (Figure 6.7 and 6.8) compared to Transwell inserts without attached cells (Figure 6.4 and 6.5). Translocated volumes of 25 µL or 15 µL of media were measured after the introduction of TcdA or TcdB, respectively. The quantity of toxin recovered from each compartment was calculated using measured toxin concentrations, as determined by the cytotoxicity assay, and the final measured volumes of 175 µL (TcdA) or 185 µL (TcdB) in each AP and 525 µL (TcdA) or 515 µL (TcdB) in each BL compartment. The total percentage of toxin recovered from both AP and BL compartments were calculated relative to the initial quantity of toxin challenge added to the AP (Figure 6.9). Using TcdA at a challenge dose of 40, 20 or 10 ng, approximately 98% of the initial toxin was detectable in the AP following the incubation. At the lower challenge doses of 5, 2.5 and 1.3 ng, the detectable levels of TcdA remaining following incubation were 62%, 50% and 31% for each, respectively. Microscopic visual assessment of the differentiated Caco-2 monolayer revealed that individual differentiated cells did not display cytopathic effect in the same way as their undifferentiated counterparts, grown for only 2 hours. Thus, undifferentiated Caco-2 cells appeared to round up individually when intoxicated with TcdA whereas the differentiated cell sheet developed numerous perforations in the monolayer. Such perforations were visible after incubation with initial TcdA challenge doses of 40, 20 or 10 ng. Detectable TcdA quantities in the BL compartments were calculated as 1.3%, 1.2% and 1.6% of each of these initial challenge doses, respectively. No Caco-2 monolayer perforations were visible at challenge doses of ≤ 5 ng and no TcdA was detectable in the BL compartments at these doses.
Figure 6.7  Comparison of initial and recovered quantities of TcdA from AP (a) and BL (b) compartments of the co-culture system with a differentiated Caco-2 monolayer grown on the polycarbonate membrane. The ‘Y’ axis columns describe the location on the 24-well plate from where the samples were taken.
Figure 6.8  
Comparison of initial and recovered quantities of TcdB from AP (a) and BL (b) compartments of the co-culture system with a differentiated Caco-2 monolayer grown on the polycarbonate membrane. The ‘Y’ axis columns describe the location on the 24-well plate from where the samples were taken.
Figure 6.9 Comparison of total percentage toxin recovered from both AP and BL compartments, following incubation for 24 hours, relative to the initial quantity of toxin challenge introduced to the AP.
When using TcdB as the sole challenge toxin, between 50% and 60% of the initial
challenge dose was detectable, in the AP compartment, at each dose increment following
incubation (Figure 6.9b). No visible disruption to the Caco-2 monolayer was evident and
no TcdB was detectable in any of the BL compartments.

6.3.4 Toxin Translocation across a Differentiated Caco-2 Monolayer with Indicator Cells
Present (Objective 3)

TcdA challenge doses of 40, 20 and 10 ng to the AP compartment containing a Caco-
2 monolayer resulted in cell rounding of the Vero indicator in the BL compartment of 70%,
60% and 10% for each dose, respectively (Figure 6.10). No evidence of Vero cell rounding
was observed following incubation with initial AP compartment TcdA challenge doses of
≤ 5 ng. Also, there was no evidence of Vero cell rounding, following incubation, when the
differentiated Caco-2 monolayers in the AP compartments were challenged with TcdB at
any dose used in this investigation, thus confirming that TcdB did not disrupt Caco-2
monolayers.

6.3.5 Toxin Synergy (Objective 4)

Based on the data collected in section 6.3.4, a constant concentration of 100 ng/mL
(equivalent to 20 ng initial challenge dose) of TcdA in the AP compartment with doubling
dilutions of TcdB starting at 5 ng/mL (equivalent to 1 ng challenge dose) was used in a
toxin synergy investigation to determine the effects on the Vero cells in the BL
compartment. Similarly, the use of constant concentrations of TcdB (5 ng/mL) with
doubling dilutions of TcdA (from 100 ng/mL) were investigated.
Figure 6.10 Percentage Vero cell rounding in the BL compartments following addition of TcdA.
The combined presence of both TcdA and TcdB in the AP compartment increased the measurable cytopathic effect on the Vero indicator cells of the BL compartment, compared to using either toxin individually, and this effect occurred in a dose dependant manner. Vero cell rounding was measured and ranged from 100% to 30% when using a constant concentration of TcdA and doubling dilutions of TcdB, with an association between decreasing TcdB concentration and a decreasing percentage of Vero cell rounding (Figure 6.11a). Synergy was also observed using a constant concentration of TcdB with doubling dilutions of TcdA, with the percentage of Vero cell rounding measured in the range of 100% to 0%. There was a similar dose dependant relationship between decreasing toxin concentration and decreasing Vero cell rounding (Figure 6.11b). However, when the TcdA concentration decreased below 50 ng/mL in the AP compartment (equivalent to 10 ng initial TcdA challenge dose) there was a very sharp decrease in Vero cell rounding. This is in contrast to the more gradual, incremental decreases in cell rounding observed with decreasing concentrations of TcdB. A small amount of cell rounding (10%) occurred in one of the two duplicates at the lowest TcdA concentration. However, this result was possible due to an imperfection or accidental physical disruption to the differentiated Caco-2 monolayer in this particular AP compartment and is not deemed to be a true result because no Vero cell rounding was measured in the duplicate well.

6.3.6 Anti-toxin Administration Route Investigation (Objective 5)

The toxin synergy investigation (section 6.3.5) demonstrated that a constant concentration of 100 ng/mL (equivalent to 20 ng initial challenge dose) of TcdA combined with a constant concentration of TcdB at 5 ng/mL (equivalent to 1 ng challenge dose) was sufficient to effectively disrupt the differentiated Caco-2 monolayer in the AP compartment and cause 100% cell rounding of the Vero indicator cells in the BL compartment. These were selected as the optimal toxin challenge concentrations to be used in this anti-toxin administration route investigation.
Figure 6.11  Toxin synergy investigations. Percentage Vero cell rounding in the BL compartments following addition of constant concentrations of TcdA at 100 ng/mL and doubling dilutions of TcdB from 5 ng/mL (a) or constant concentrations of TcdB at 5 ng/mL and doubling dilutions of TcdA from 10 ng/mL (b).
The anti-toxins to be administered to the established co-culture system were calculated to have neutralising potencies of 900 ng/mL (anti-TxA4) and 15 ng/mL (anti-TxB4). Volumes of 5 µL of the anti-toxins were used, individually, in this investigation, which equated to total toxin neutralising potential of 4.5 µg and 75 ng for toxins TcdA and TcdB respectively. This ensured that an excess of anti-toxin was present in either the AP or BL compartment (depending on the route of administration) relative to the toxin challenge dose in the AP compartment.

When anti-TcdA was administered to the AP compartment, to simulate orally administered PcAb, no cell rounding was observed in the Vero monolayer of the BL compartment. The excess of anti-TxA4 is likely to have neutralised all the available TcdA leaving only the TcdB challenge. This reflects the result found in section 6.3.4, whereby no TcdB was detected in the BL compartment and no Vero cell rounding was observed when only TcdB was used to challenge the AP compartment.

With anti-TxA4 administered to the BL compartment, to simulate systemically administered PcAb, 20% cell rounding was observed in the Vero monolayer. Although an excess of anti-TxA4 was present in the BL compartment, the TcdA challenge dose in the AP compartment was able to partially disrupt the Caco-2 monolayer and allow translocation of a reduced quantity of TcdB to the BL compartment.

It was found through this investigation that an excess of anti-TxB4 administered either to the AP or BL compartments was capable of neutralising a sufficient quantity of TcdB to completely protect the Vero cell monolayer without observable cytopathic effects.
6.4 Discussion

The first four objectives in this study were designed to determine the amount of TcdA and TcdB required in the AP compartment to achieve a measurable cytopathic effect on a Vero cell monolayer established in the BL compartment. Objective 1 (section 6.3.1) determined that a 24-well plate containing the working volume of media, but with no cells attached, adsorbed toxin. Using initial quantities of ≤ 10 ng and ≤ 2 ng, for TcdA and TcdB respectively, approximately 50% of the toxin added to the wells was not detectable by cytotoxicity assay following 24 hours incubation. Initial quantities > 10 ng (TcdA) or > 2 ng (TcdB) resulted in an increasing level of recoverable toxin. The most likely explanation for this finding is that toxin proteins adhered to the internal surface of the wells through physiochemical interactions then, at the point of saturation, toxin was no longer adsorbed and became detectable in incrementally increasing quantities as the level of initial toxin challenge increased.

This experiment did not include the presence of adherent Vero cells grown as a monolayer on the lower internal surface of the well. The presence of a monolayer would reduce the level of toxin adsorption to the bottom of the well, which equates to a surface area of 1.9 cm$^2$. However, a Vero cell monolayer would actively endocytose quantities of toxin, leading to cytopathic effects. This would occur in a dose dependant manner and be measured by visual assessment of the percentage cell rounding, as validated in the cytotoxicity assay (section 3.3.2). Given that the complete co-culture system would include adherent Vero cells, the level of toxin adsorption to the internal surface of the wells is not likely to be relevant. However, these data will be important in any experimental use of the system where no Vero cell monolayer is used in the BL compartment.

The findings of objective 2 (section 6.3.2) determined that 100 µL of media, containing toxin challenge, was able to move freely across the porous polycarbonate membrane of the Transwell insert when no Caco-2 cell barrier was in place. It had been
assumed that the initial toxin challenge dose added to the AP compartments would equilibrate with the working volumes of 200 µL in the AP and 500 µL in the BL compartments during the 24 hour incubation period. The detected quantities of toxin were compared to the assumed equilibrated quantities present following incubation, given that 100 µL of media translocated from AP to BL compartments. Surprisingly, mean quantities of 205% (SD = 52) and 189% (SD = 49) above assumed levels were calculated for TcdA and TcdB, respectively, in the AP compartments. This indicates that although media flowed freely from AP to BL compartments, the 0.4 µm porous polycarbonate membrane had a limiting effect on the movement of both toxins.

As the quantities of detected toxins were higher than expected in the AP compartments, the quantities detected in the BL compartments were naturally lower than expected with recovered values of 44% (SD = 10) and 60% (SD = 16) for TcdA and TcdB, respectively, relative to assumed equilibrated quantities. When the toxin quantities recovered from each individual AP compartment was added to the quantity recovered from its BL compartment and compared to the initial toxin challenge dose, mean recovered values of 67% (SD = 11) and 77% (SD = 12) were calculated for TcdA and TcdB, respectively. This indicates a net loss of 33% (TcdA) and 23% (TcdB) relative to the initial toxin challenge dose and is reasonably consistent with the losses calculated in objective 1 (section 6.3.1), whereby some toxin adsorption to the inner surface of the BL compartment was indicated. Given that the toxin levels in the AP compartment were higher than expected, it is not believed that any measurable adsorption occurred to the internal surface of the Transwell insert. The discovery that the porous polycarbonate membrane acts as a restriction to the movement of toxins from AP to BL compartment is important for the further development of this simulated bowel barrier system as the optimal toxin challenge dose may need to be adjusted to achieve sufficient concentrations of TcdA or TcdB in the BL compartment to cause a measurable effect on the Vero indicator cells.
With a differentiated Caco-2 monolayer established on the porous polycarbonate membrane and media, with toxin challenge, added to the AP compartments, smaller volumes of 25 µL (TcdA) or 15 µL (TcdB) of media translocated to the BL compartments. This equates to 4 times and 6.5 times less translocation of media containing TcdA or TcdB, respectively, compared to the porous polycarbonate membrane with no adherent cells. The reason for this stark difference in translocated volumes is most likely due to the adherent cells of the differentiated Caco-2 monolayer effectively blocking the pores of the membrane. It is unclear why there is a difference between the translocation volumes observed from the TcdA and TcdB challenge experiments, but it may be associated with slightly different cytopathic effects induced on the cells by each of the two toxins. Thus, TcdA may disrupt the tight junctions between differentiated Caco-2 cells to a greater extent than intoxication by TcdB. Moreover, perforations in the established Caco-2 monolayer were observed, by microscopic visual assessment, at TcdA challenge doses of ≥ 10 ng in the AP compartments. Subsequent cytotoxic assessment of the media from the BL compartments of wells exhibiting a disrupted Caco-2 monolayer revealed the presence of TcdA which had translocated from the AP compartment. The quantity of translocated TcdA was calculated to be ~ 1.5% of each initial challenge dose. No translocated TcdA was detected in the BL compartments of wells where the Caco-2 monolayer in the AP compartments were intact. No disruption to the Caco-2 monolayer was observed following challenge with TcdB at any challenge dose used in this study and no TcdB was detected in any of the BL compartments. These findings indicate that the differentiated Caco-2 cells must be exposed to TcdA at initial challenge doses of ≥ 10 ng in the AP compartment before toxin can translocate to the BL compartment. At initial TcdA challenge doses of ≤ 5 ng in the AP compartment, the quantity of recoverable toxin from this compartment, following incubation, decreased incrementally from 62% to 31% (relative to initial quantity) with decreasing challenge dose. With an initial challenge dose of ≥ 10 ng, a consistent quantity of residual TcdA, calculated to be 98% of the initial dose, was detected
in the AP compartments. These findings indicated that the differentiated Caco-2 cells absorb TcdA up to an initial AP compartment challenge dose of between 5 and 10 ng (equivalent to between 25 ng/mL and 50 ng/mL) at which point they either become saturated with TcdA or endocytosis of further TcdA is inhibited due to cellular intoxication. At this point, increasing quantities of TcdA translocate to the BL compartments through the disrupted Caco-2 monolayer. Recoverable TcdB was measured at ~ 50%, following incubation, and was reasonably consistent at each initial challenge dose. The reason for the difference in toxin absorption profiles between TcdA and TcdB remains unclear, but is likely to be related to their different cell surface receptor binding sites and enzymatic substrate specificity. Given that ~ 1.5% of the initial TcdA challenge doses > 10 ng translocated to the BL compartment and the final volumes measured in these compartments were 525 µL, the calculated TcdA concentrations present in the BL compartments were 1.1 ng/mL, 0.6 ng/mL and 0.3 ng/mL for the 40 ng, 20 ng and 10 ng doses, respectively. As there were no Vero cells present in the BL compartments during this part of the investigation, it can be assumed that approximately 50% of the translocated toxin would have adsorbed to the internal surfaces of the BL compartment, as determined in section 6.3.1. It is likely that true concentration values following TcdA translocation were closer to 1.5 ng/mL, 0.8 ng/mL and 0.4 ng/mL for the 40 ng, 20 ng and 10 ng doses, respectively. These concentrations are sufficient to cause Vero cell rounding of approximately 55%, 30% and 5% for each initial challenge dose, respectively, based on typical cytotoxicity calibration curves (section 6.3.1).

These investigative experiments were repeated using Caco-2 monolayers established in the AP, as before, but with Vero cell monolayers included in the BL compartments. Cytotoxic assessment of media from either compartment was not conducted as before and, instead, the percentage of Vero cell rounding was measured by microscopic visual assessment of the monolayer. The results reflected those of the previous experiment, whereby toxin translocation was only evident when the Caco-2 monolayer had been
intoxicated by TcdA at initial doses of ≥ 10 ng. The percentage of Vero cell rounding was measured at 70%, 60% and 10% for the TcdA challenge doses of 40 ng, 20 ng and 10 ng, respectively, and is reasonably consistent with the estimated translocated toxin values determined in the previous experiments.

The outcomes of objective 3 (sections 6.3.3 and 6.3.4) determined that the integrity of the Caco-2 monolayer was reduced using a TcdA challenge dose of between 40 ng and 10 ng with 20 ng providing sufficient translocated TcdA to cause 60% Vero cell rounding in the BL compartment. Therefore, the 20 ng (equivalent to 100 ng/mL) dose was selected for further study in synergy with up to 1 ng (equivalent to 5 ng/mL) of TcdB. The TcdB challenge dose was surmised by assuming that the TcdA challenge would affect the integrity of the Caco-2 monolayer and allow ~ 1.5% of the TcdB challenge dose to translocate to the BL compartment. The value of 1.5% was based on the experimental evidence gained in objective 3 (section 6.3.3), whereby between 1.2% and 1.6% of the initial TcdA challenge dose translocated across a disrupted Caco-2 monolayer. Assuming an equivalent percentage of TcdB would also translocate across a disrupted barrier to the BL compartment, this would equate to ~ 15 pg (equivalent to 30 pg/mL) of TcdB present in the BL compartment which is sufficient to cause a measurable cytopathic effect on the Vero cells of up to 80%. Using a constant TcdA challenge dose of 20 ng and incrementally reduced doses of TcdB, starting at 1 ng, cytopathic effect was observed in the Vero cell monolayer which decreased incrementally with reducing TcdB challenge. The highest doses of TcdB caused Vero cell rounding to a considerably greater extent than using TcdA alone. This demonstrates that when TcdA is present in the AP compartment at a concentration sufficient to affect the Caco-2 barrier, TcdB can readily translocate to the BL compartment and cause cytopathic effect to the Vero cell monolayer in a dose dependant manner. When a constant quantity of TcdB was used in the AP compartment with incrementally reducing quantities of TcdA, it was found that TcdA doses ≥ 10 ng where required to allow the translocation of TcdB to the BL compartment, where Vero cell
rounding was then measurably higher than would be possible with TcdA alone. Below TcdA doses of 10 ng in the AP compartment no Vero cell rounding was evident and therefore no translocation of TcdB to the BL compartment occurred. The experimental evidence gained through these investigations has pointed strongly towards the requirement of a minimum quantity of TcdA in the AP compartment to disrupt the Caco-2 cell barrier before any translocation of TcdA or TcdB to the BL compartment can occur. This demonstrates an important synergy between the two toxins and may indicate a preferred anti-toxin treatment strategy in clinical practice.

The synergistic effect of 20 ng TcdA combined with 5 ng TcdB was found to disrupt an established Caco-2 monolayer in the AP compartment and cause 100% cell rounding of the Vero cell monolayer in the BL compartment. Based on the investigative results gained through the execution of the previous objectives, this co-culture configuration and combined toxin challenge dose was deemed optimal for the investigation of simulated treatment strategies using anti-toxin, in terms of their administration routes. Thus, anti-toxin added to the AP compartment was used to simulate orally administered PcAb and anti-toxin added to the BL compartment simulated systemically administered PcAb. The calculated toxin neutralising potential of the PcAb used in these investigations was 4.5 µg and 75 ng for anti-TxA4 and anti-TxB4, respectively, ensuring a treatment dose sufficient to protect against cytopathic effects elicited by either toxin. Objective 4 (section 6.3.5) determined that TcdA is required to disrupt the simulated bowel barrier before TcdB can translocate into the simulated systemic circulation. Therefore, it can be postulated that orally administered PcAb directed towards TcdA should protect against bowel barrier disruption and subsequent systemic TcdB intoxication. It could also be postulated that once systemic intoxication by TcdB had occurred, the most effective administration route of PcAb directed towards TcdB would be systemic. These hypotheses proved to be the case, based on the results of objective 5 (section 6.3.6), whereby anti-TxA4 PcAb added to the AP compartment provided complete protection against Vero cell rounding in the BL
compartment and, in separate experiments, anti-TxB4 PcAb added to the BL compartment also provided complete Vero cell protection. Interestingly, anti-TxB4 added to the AP compartment also provided complete protection to the Vero cell monolayer. It is likely that the TcdA challenge dose caused disruption to the Caco-2 barrier (although this was not verified by visual assessment), but the TcdB challenge dose had been neutralised before any translocation to the BL compartment could occur. It was also determined that anti-TxA4 PcAb administered via the simulated systemic route, i.e. directly to the BL compartment, did not provide complete protection to the Vero monolayer which exhibited 20% cell rounding. This indicates that the TcdA challenge dose disrupted the Caco-2 barrier to a certain extent (not verified by visual assessment), allowing some TcdB to translocate, but sufficient anti-TxA4 was able to translocate from BL to AP compartments and neutralise remaining TcdA, thus providing protection to the Caco-2 barrier and prevent further disruption.

The outcome of these investigations strongly indicates that orally administered anti-TxA4 PcAb have the potential to prevent or treat systemic intoxication by *C. difficile* toxins. However, the combined oral administration of both anti-TxA4 and anti-TxB4 PcAb may provide enhanced protection. Once disruption to the bowel barrier has occurred, i.e. in a severe clinical case, the systemic administration of anti-TxB4 alone may be beneficial. These findings reflect those of other researches who used animal models of *C. difficile* infection to investigate the effectiveness of toxin neutralising PcAb *in-vivo* (Lyerly *et al.*, 1991; Kink and Williams, 1998; Roberts *et al.*, 2012; Maynard-Smith *et al.*, 2014). Therefore, with further optimisation, the simulated bowel barrier preparation developed in this study could potentially be used in place of animal models in some laboratory experiments, providing clear ethical and cost saving benefits. The preparation could also be useful in the investigation of additional treatment strategies or to refine an approach prior to animal testing, which may increase the success of such experiments.
CHAPTER SEVEN

FINAL DISCUSSION AND CONCLUSIONS
7.1 Final Discussion and Conclusions

The prevalence of CDI continues to ensure that it remains a great burden on healthcare systems throughout the developed world (Dubberke and Olsen, 2012). The clinical manifestations caused by the exotoxins, TcdA and TcdB, trigger healthcare protocols which utilise antibiotics as a first line treatment to combat the bacterial infection (Wilcox, 2006), yet the exotoxins produced in these clinical cases remain unaffected and must be neutralised by the immune system of the patient. As the patients who are most at risk of developing CDI tend to be elderly or immuno-compromised, the ongoing presence of active toxin is likely to contribute to relapse, morbidity and mortality rates. Cost effective and practical alternatives to the conventional therapeutic approach are urgently required.

The primary aim of this study was to raise ovine PcAb capable of binding to and neutralising TcdA and TcdB, with a view to developing potent immunotherapeutic agents for use in humans. In a previous study, two novel recombinant antigens based on TcdA and TcdB were described and shown to evoke a toxin neutralising immune response in sheep (Maynard-Smith et al., 2014). In the present study, a range of concentrations of these formaldehyde treated antigens (TxA4 and TxB4) were administered to groups of sheep to determine the optimal dose required to elicit the highest production of toxin neutralising antibodies.

Antisera samples from individual sheep were assessed at 28 day intervals for the presence of toxin specific PcAb for up to 30 weeks (Chapter 4). A semi-quantitative enzyme immunoassay was established and used to detect levels of anti-TxA4 or anti-TxB4 which bound to their respective natural toxin (Chapter 3). A cell based cytotoxic neutralisation assay was also successfully established to measure the neutralising activity
of each antisera sample (Chapter 2). This method was pioneered as a quantitative assay to determine the potency of each sample in terms of weight of natural toxin neutralised per volume of antisera. All dose response groups in the study produced PcAb which showed high binding titres by immunoassay as well as protecting Vero cells against the two natural toxins \textit{in-vitro}. The PcAb binding titres were found to stabilise from approximately 10 weeks post initial immunisation, whereas the neutralising potency continued to increase until at least week 26. This indicated that even though the serum concentration of specific PcAb remained relatively constant beyond the 10 week sample point, their toxin neutralising activity continued to increase, probably due to ongoing B cell affinity maturation. Although no statistically significant difference in antisera potency was measured between the sheep in different dose groups, a trend towards significance was observed in the toxin neutralising potency of both the TxA4 and TxB4 flocks. This indicated that the highest dose groups (1 mg and 2 mg for the TxA4 and TxB4 immunogens, respectively) elicited the highest achievable levels of toxin neutralising PcAb. The TxA4 immunogen stimulated a greater toxin neutralising immune response than TxB4. Thus, 1 mL of anti-TxA4 sera neutralised 1,800 µg of natural TcdA, whereas 1 mL of anti-TxB4 neutralised only 15 µg of TcdB. However, it should be noted that there is currently no clear evidence that anti-toxin potency and clinical relevance are related.

The reason for the large disparity in PcAb potency was initially unclear, particularly given that the binding titres, and therefore serum anti-toxin concentrations, were similar between the TxA4 and TxB4 groups. However, the investigations documented in Chapter 5 indicated strongly that although the majority of binding PcAb was directed to the C-terminus of native TcdB (53% of total binding PcAb), PcAb directed to the central domain (44% of total binding PcAb) possessed toxin neutralising potency (2.5 µg/mg) twice that of the C-terminus binding PcAb. Future studies might seek to clarify this phenomenon further using the methods described in Chapter 5, applying them to the fractionation of anti-TcdA and anti-TxA4 against their counterpart recombinant fragments that represent the N-
terminus, central domain and C-terminus regions. Evidence obtained from toxin neutralising PcAb raised by immunising host animals with small recombinant fragments corresponding to distinct toxin domains has indicated that antibodies directed to the C-terminus of TcdA are important for its inactivation (Roberts et al., 2012; Leuzzi et al., 2013; Maynard-Smith et al., 2014). Here, however, affinity chromatography has been used for the first time to separate neutralising from non-neutralising populations of binding PcAb. Moreover, previous studies have used immunogens composed of recombinant fragments of TcdB to raise neutralising PcAb, whereas here PcAb raised against whole natural TcdB have been fractionated against recombinant protein constructs to isolate specific binding PcAb and to identify domains of TcdB which are important with regard to its inactivation. The outcome of this study presents the potential to formulate a C. difficile antitoxin using a high concentration of specific toxin neutralising PcAb relative to total binding PcAb content, further reducing the quantity of heterologous protein per treatment and the potential for side effects in treated patients.

The evidence of others has proven that both toxins TcdA and TcdB enter the systemic circulation in CDI (Steele et al., 2012). Systemic intoxication undoubtedly exacerbates CDI and contributes to relapse, morbidity and mortality rates of patients in the healthcare setting. The primary aims of this study have been successful in the generation of toxin neutralising PcAb which could potentially be used to formulate an immunotherapy for CDI. However, the most efficacious route of administration for such an immunotherapy remained to be investigated. Systemic infusion of heterologous PcAb has been proven safe and effective for acute emergencies, such as snake envenomation or drug toxicity, where short term treatment is required (Dart and McNally, 2001; Pizon et al., 2007; Schaeffer et al., 2010). However, the immunotherapeutic treatments of CDIs are likely to be required over a longer term. This would inevitably increase the risk of adverse reactions in the patient to the systemically administered foreign PcAb proteins. Purification by the fractionation of specific toxin neutralising PcAb from those which bind, but do not
neutralise, may be an important step to reduce adverse events in treated patients. However, identifying the most efficacious PcAb administration route (oral versus systemic) may also be of great clinical importance. Moreover, the enterotoxic TcdA has been proven to cause considerable intestinal damage in vivo (Lyerly et al., 1991), and this is mirrored by cell rounding and loss of tight junctions in confluent Caco-2 (human colonic carcinoma) monolayers in vitro (Banerjee et al., 2009). Thus, differentiated Caco-2 monolayers were used as a basis to successfully develop and pioneer a novel simulated bowel barrier model consisting of an upper (apical) simulated colonic lumen and a lower (basolateral) simulated systemic circulation, separated by the established Caco-2 cell barrier (Chapter 6). The simulated bowel barrier model was used to investigate the synergistic relationship between TcdA and TcdB on the simulated bowel barrier, in addition to toxin translocation to the simulated systemic circulation. The separate administrations of oral and systemic anti-toxins were also simulated in this investigation. The presence of TcdA was shown to be a pre-requisite for the translocation of TcdB to occur. Microscopic visual assessment of the TcdA intoxicated Caco-2 monolayer revealed perforations in the differentiated cell barrier, allowing translocation of toxin from apical to basolateral compartments. Translocation was reduced when the TcdA concentration in the apical compartment was below a threshold of 50 ng/mL, and prevented by TcdA concentrations below 13 ng/mL. Anti-TxB4 PcAb administered via either the simulated oral or systemic routes were shown to provide complete protection to the Vero indicator cell monolayer in the basolateral compartment. Anti-TxA4 PcAb administered via the simulated oral route also provided complete protection to the Vero monolayer. However, only partial protection was observed when anti-TxA4 was administered via the simulated systemic route. These important findings indicate that orally administered anti-TxA4 PcAb have the potential to prevent or treat systemic intoxication by C. difficile toxins. However, the combined oral administration of both anti-TxA4 and anti-TxB4 PcAb may provide enhanced protection. The use of orally administered anti-TxA4 may be particularly advantageous as a prophylactic treatment in
‘at risk’ patients in situations where CDI cases have been confirmed in the healthcare environment. In cases of severe or refractory CDI, the systemic infusion of anti-TxB4 alone may be beneficial. An additional advantage to the systemic administration of anti-TxB4 alone is that of further reducing the quantity of heterologous PcAb per treatment dose as the anti-TxA4 would not be included.

The simulated bowel barrier preparation developed here could be enhanced and refined further but transferring the methods to a real time cell analysis system, such as the Xcelligence machine marketed by ACEA Biosciences. This system continuously measures the electrical impedance across the base of each well of a cell culture plate. Cell adhesion, growth and proliferation, as well as cell rounding after intoxication, all affect the electrical impedance and can therefore be measured by the system. Even small, sub visible, changes in cell morphology can be detected by changes in electrical impedance. This would offer a far more sensitive method of measuring cytopathic effects on a Vero cell monolayer, compared to visual assessment alone, and may help to increase the value of the simulated bowel barrier preparation as a research tool.

To summarise, analytical assays were established to assess ovine antisera and flocks of sheep were used successfully to raise potent toxin neutralising PcAb directed against *C. difficile* toxins TcdA and TcdB. Further investigations concluded that the central, transmembrane domain of TcdB is more important than its C-terminus receptor binding domain, in terms of inactivating toxicity. In addition, using a simulated bowel barrier model, it was determined that TcdA is required in the colonic lumen before TcdB is able to translocate to systemic circulation. Moreover, anti-TxA4 administered orally can prevent systemic intoxication, whereas systemically administered anti-TxB4 can prevent cellular damage following toxin translocation to the systemic circulation. These important steps are fundamental to the continued development of effective immunotherapeutic treatments for CDI and their large scale production.
The further development of the anti-toxin PcAb, produced here, into an effective clinical treatment is beyond the scope of this thesis. However, the next steps would be to initiate safety studies in animals followed by efficacy testing, probably using the hamster model for CDI. Assuming positive outcomes from these studies, the next step would be safety studies in healthy human volunteers followed by clinical trials to determine clinical efficacy. Product registration and licensing would then be required prior to product release. The use of a successful immunotherapy in the clinical setting would have a great potential to reduce CDI relapse and mortality rates as well as prevent many cases of infection if used as a prophylactic. This would ultimately reduce costs attributed to CDI as well as reduce the burden on healthcare resources.

7.1.1 Research Limitations

This research was sponsored, in part, by the industrial partner (MicroPharm) and it was an expectation of the company that certain methods and criteria were used. These business requirements gave rise to the following research limitations: only 3 sheep were used in each of 5 immunising dose groups, the immunisation dose response study only extended to 30 weeks, Freund’s adjuvant (either complete or incomplete) was used at each immunisation, binding PcAb levels (measured by EIA) were expressed as the reciprocal of the dilution factor (titre), cytotoxicity and cytotoxic neutralisations assays utilised the Vero cell line.

Given greater funding and time, it would have been useful to investigate only 3 separate immunising doses in groups of 5 sheep, instead of groups of 3. This would allow for a greater increment between immunising doses and provide a more robust data set and, therefore, statistical analysis of the responses of the larger groups. The duration of the dose response study could also be extended to allow for trends to stabilise, thus providing data for a more complete analysis.
Different immunisation adjuvants are available, however only Freund’s was used in this study. It is possible that the use of other adjuvants in the formulation of the toxoided immunogens may give rise to a greater immune response in the antisera production flocks. This postulate remains to be tested.

It was a business requirement that the results of the EIA, used to measure PcAb binding, were expressed in terms of titre. It may have been of greater benefit to use a standard calibration curve on the EIA and calculate the binding titre results in terms of g/L. This would have allowed these results to be more easily compared to results obtained by SSAC.

The Vero cell line was utilised in this research to remain consistent with the methods of Roberts et al. (2012) and Maynard-Smith et al. (2014). Given greater funding and time, it would have been interesting to trial the use of different tissue culture types in an effort to enhance and refine the sensitivity of the cytotoxicity and cytotoxic neutralisation assays. The successful use of an alternative cell line may also enable the use of colourimetric methods (such as the neutral red assay) to assess the level of cytopathic effects, rather than visual assessment of the monolayers. This research study determined that TcdA and TcdB intoxicated Vero cells remained metabolically active, to varying extents, even though cytopathic effects were evident by visual assessment. As many of the available colourimetric methods utilise the cell’s mitochondrial activity to reduce reagents and provide a measurable colour change, residual metabolic activity in the cells interferes with meaningful assessments of cytopathic effect.

7.1.2 Strengths of Research Outcomes

The sheep immunisation rest-restart studies demonstrated that animals, originally receiving high immunising doses that were later restarted on a lower dose, produced antisera with remarkably high toxin neutralising potency relative to sheep that originally
received a lower initial dose. For the purpose of producing large volumes of high potency antisera for the lowest quantities of immunogen, this method may have the potential to considerably reduce commercial antisera production costs.

The cytotoxic neutralisation assay used in this research included a novel potency calculation which expressed the assay results in terms of weight of toxin neutralised per mL of antisera, or other sample. By using pure test samples of known protein concentration, neutralising potency could be expressed in terms of weight of toxin neutralised per weight of protein. This method could be useful for direct comparison of results, possibly between research labs.

The use of affinity chromatography columns, each conjugated of to 1 of 3 different recombinant TcdB domain constructs, enabled the fractionation of PcAb populations raised against whole natural TcdB. This method, followed by subsequent potency assessment, was used to determine that PcAb directed to the central (transmembrane) domain of TcdB demonstrated the highest TcdB neutralising potency. In this research study, affinity chromatography has been used, for the first time, to separate TcdB neutralising from non-neutralising populations of TcdB binding PcAb. This important advance may allow for the clinical administration of immunotherapeutic formulations containing high levels of toxin specific neutralising immunoglobulins, or their fragments, relative to total protein concentration. This would be an important step towards increasing product efficacy and reducing potential side effects from such a treatment.

Some of the greatest outcomes of this research were derived from the ‘Simulated Bowel Barrier’ experiments, detailed in Chapter 6. The successful development and use of the in-vitro, cell co-culture preparation enabled experimental investigations to be conducted, in the lab, which would normally have to be carried out in an animal model. The investigations described here used the co-culture preparation to simulate the colonic epithelial barrier and systemic circulation, providing a valuable research tool with which to investigate the effects of C. difficile toxins and their translocation across the colonic
mucosal barrier. This preparation could also prove valuable to other areas of colonic mucosal barrier research involving bacterial, food-borne or environmental toxins and potentially reduce the need for testing in animal models, thus providing many ethical and cost saving benefits. Moreover, different tissue culture cell lines could be used in place of the colonic epithelial cells, broadening the potential scope of this preparation to include the simulation of other biological interfaces such as the respiratory mucosa and blood-brain barrier.

The findings from the ‘Simulated Bowel Barrier’ investigation determined that TcdA seems to be a pre-requisite for the translocation of TcdB from the colon, where it can then cause systemic intoxication, thus proving a synergy between the toxins. This toxin synergy, supported by further results obtained from anti-toxin administration route experiments, lead to the discovery that different anti-toxin treatment strategies may be indicated based on the stage of CDI. Thus, simulated systemic intoxication was effectively treated by the systemic administration of anti-TxB4, whilst systemic intoxication was completely prevented by the administration of anti-TxA4 by the simulated oral route. These findings have the potential to influence clinical treatment strategies for the management of CDI and offer evidence that orally administered anti-TxA4 could be used as a prophylactic for patients who are at risk of developing CDI, potentially preventing thousands of CDI cases and saving healthcare institutions millions of pounds annually.
References


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