Development of an oral vaccine against *Clostridioides difficile*

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Abstract

Clostridioides difficile (*C. difficile*) is the leading cause of hospital acquired diarrhoeal infection. The major risk factors associated with *C. difficile* infection (CDI) is age (65 and over), use of broad-spectrum antibiotics and prolonged hospital stays. Symptoms range from mild diarrhoea to life threatening fulminant colitis. Clinical manifestation is mainly due to two toxins; TcdA and TcdB. The failure to treat recurrent infection can culminate in death and thus preventative measures are of urgent need. Current vaccine trials in humans are exclusively focused on parenteral delivery of toxin-based formulations. These vaccines elicit toxin-neutralising serum antibody responses, however they fail to provide protection against CDI which occurs in the gut. A more effective way to vaccinate against this gut pathogen would be with a mucosal vaccine via the oral route. This would potentially generate a local mucosal immune response, *i.e.*, secretory IgA (sIgA) that directly targets the site of infection. Mucosal vaccines require adjuvants to elicit potent immune responses. Bacterial lipoproteins harbour intrinsic adjuvant properties due to their lipid moiety interactions with Toll-like receptor 2 (TLR2) found on antigen presenting cells (APCs) which leads to APC activation and antigen uptake. Thus, synthetic bacterial lipids that act as TLR2 agonists have been extensively studied through their attachment to synthesised peptides to serve as potential adjuvants. However, using synthetic peptides allow for recognition of only smaller immunogenic fragments/epitopes which limits their target for whole proteins. We set out to formulate an oral mucosal vaccine using recombinant *C. difficile* antigens expressed with a unique N-terminal cysteine used to conjugate a synthetic lipid with a maleimide head group, to mimic bacterial lipoproteins. These semi-synthetic lipoproteins were presented on liposomes as a

delivery vehicle and to also mimic the native presentation of lipoproteins on the bacterial cell surface. In this study we tested two antigens; colonisation factor CD630_08730 and a fragment of the receptor binding domain of TcdB and compared these administered either as antigen alone or with being presented on liposomes with conjugation to a lipid adjuvant. The formulations were lyophilised and packed into enteric coated capsule. Hamsters immunised with both CD630_08730 formulations showed strong intestinal sIgA and serum IgG responses compared to the naïve group and other control groups, which reduced *C. difficile* adherence to Caco-2 cells *in vitro*. The adherence blocking was further reduced for those hamsters receiving CD630_08730 adjuvanted with the synthetic lipid, presented on liposomes compared to the antigen alone. As hamsters receiving CD630_08730 alone showed good immunological responses, its protective efficacy was tested. Hamsters immunised with CD630_08730 antigen alone were challenged with hypervirulent strain R20291*ermB* and showed an 80% increase in mean time to end point compared to naïve animals. The survival was correlated with bacterial clearance and reduced toxin-mediated damage as determined from histopathology assessment of the caecum. This study highlights the potential of using semi-synthetic lipoproteins presented on liposomes as an oral vaccine platform and also highlights the potential of CD630_08730 as a vaccine candidate against CDI.

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Publications

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Abbreviations

°C	Celsius
µl	Microliter
µg	Microgram
µmol	Micromole
AAL	*Aleuria auranitia lectin*
ABC	ATP-binding Cassette
ADP	Adenosine Diphosphate
AIDS	Acquired Immunodeficiency Syndrome
ALUM	Aluminium Hydroxide
APC	Antigen Presenting Cell
APD	Autoprotease Domain
ATEC	Acetyl Triethyl Citrate
ATP	Adenosine Triphosphate
BALT	Bronchus-associated Lymphoid Tissues
BCA	Bicinchoninic Acid Assay
BHIS	Brain Heart Infusion Supplemented
bp	base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CALT	Conjunctiva-associated lymphoid tissues
CAP	Cellulose Acetate Phthalate
CAT	Cellulose Acetate Trimellitate
CBD	Chitin Binding Domain
CCL25	CC-motif Chemokine Ligand 25
CCR9	CC-motif Chemokine Receptor 9
CDC	Centres for Disease Control and Prevention
CDI	*Clostridioides difficle* Infection
CDT	*Clostridioides difficile* Transferase

CFU	Colony Forming Unit
CIAP	Calf Intestinal Alkaline Phosphatase
CO	Codon Optimised
cP	Centipoise
CROPs	Combined Repetitive Oligopeptides
CST	Cell Signalling Technology
CT	Computed Tomography
CWP	Cell Wall Protein
DBP	Dibutyl Phthalate
DBS	Dibutyl Sebacate
DBT	Dibutyl Tartate
DDA	Dimethyldioctadecylammonium Bromide
DEP	Diethyl Phthalate
dH_2O	Distilled Water
diH_2O	Deionised Water
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOTAP	Dioleoyltrimethylammoniumpropane
DPA	Dipicolinic Acid
DPPC	Dipalmitoylphoshatidylcholine
DPPS	Dipalmitoylphophaitdylserine
DTT	1,4- Dithiothreitol
EDA	Experimental Design Assistant
EDC	1-ethyl-3- (-3Dimethylaminopropyl) Carbodiimide
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European Medicine Agency

FACS	Florescence-activated Cell Sorting
FAE	Follicle-associated Epithelium
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FL	Full-length
FMT	Faecal Microbiota Transplantation
FSC	Forward Scatter
g	g force
GALT	Gut-associated Lymphoid Tissues
GC	Germinal Centre
GFP	Green Fluorescent Protein
GI	Gastrointestinal Tract
GTD	Glucosyltransferase Domain
HF	High-fidelity
HIV	Human Immunodeficiency Virus
HMW	High Molecular Weight
HPMCAS	Hydroxypropylmethylcellulose Acetate Succinate
HPMCP	Hydroxypropylmethylcellulose Phthalate
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
HuMAbs	Humanised Monoclonal Antibodies
IMAC	Immobilized Metal Affinity Chromatography
IMPACT	Intein Mediated Purification with an Affinity Chitin-binding Tag
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVCs	Individually Ventilated Cages
IL	Interleukin
kb	Kilobase
kDa	Kilodalton
kg	Kilogram

LB	Luria-Bertani
Lgt	Prolipoprotein Diacylglycerol Transferase
LMW	Low Molecular Weight
Lnt	Apolipoprotein *N*-acyltransferase
LPS	Lipopolysaccharide
LRR	Leucine-rich Repeat
LspA	Prolipoprotein Signal Peptidase
LT	Heat-labile toxin
LUV	Large Unilamellar Vesicles
MA-EA	Methacrylic Acid Ethacrylate Poly
MAdCAM-1	Mucosal Vascular Addressin Cell Adhesion Molecule
MALT	Mucosa-associated Lymphoid Tissue
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
ml	Millilitre
mM	Millimolar
M	Molar
MLN	Mesenteric Lymph Nodes
MLV	Multilamellar Vesicles
MMA	Methacrylic Acid Methyl Methacrylate poly
MOI	Multiplicity of Infection
MPL	Monophosphoryl Lipid A
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
NALT	Nasopharynx-associated Lymphoid Tissues
nm	Nanometre
NBF	Neutral Buffered Formalin
NEB	New England BioLabs

NF-κB	Nuclear Factor Kappa-light chain
NHS	N-Hydroxysuccinimide
Ni-NTA	Nickel-Nitrilotriacetic Acid
NTCD	Nontoxigenic *Clostridioides difficile*
dNTP	Deoxynucleoside Triphosphate
OD	Optical Density
OPV	Oral Polio Vaccine
OVA	Ovalbumin
PAFR	Platelet-activating Factor Receptor
PBS	Phosphate-buffered Saline
PBST	Phosphate-buffered Saline Tween
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PDI	Polydispersity Index
PEG	Polyethylene Glycol
PG	Peptidoglycan
PPs	Peyer's Patches
PRR	Pattern Recognition Receptors
PS	Phosphatidylserine
PVAP	Polyvinyl Acetate Phalate
PVDF	Polyvinylidene Difluoride
RBD	Receptor Binding Domain
rpm	Revolutions per minute
SASP	Small Acid Soluble Proteins
SC	Secretory Component
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Electrophoresis
Sec	Secretory Pathway
SEC	Size Exclusion Chromatography
SEM	Standard Error of the Mean

SH	Sulfhydryl
SLP	S-layer Proteins
SSC	Side Scatter
SUV	Small Unilamellar Vesicles
TAE	Tris-acetate
Tat	Twin Arginine Translocase pathway
TBC	Tributyl Citrate
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline Tween
TCCFA	Taurocholate-Cefoxitin-Cycloserine-Fructose agar
TCEP	tri-(2-Carboxyethly)Phosphine
TEC	Triethyl Citrate
TEM	Transmission Electron Microscopy
TLR	Toll-like Receptor
TMB	3, 3',5 ,5'-Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor α
UEA	*Ulex europaeus* Agglutinin
UV	Ultraviolet
VALT	Vaginal- associated Lymphoid Tissues
VAPP	Vaccine-associated Paralytic Poliomyelitis
WT	Wild Type

Declaration

I declare all work presented in the thesis titled 'Development of an oral vaccine against *Clostridioides difficile*' is my own unless acknowledged in text or by references. No work here has been submitted for another degree here at Nottingham University or any other institute.

Chapter 1

Introduction

1 Introduction

1.1 *Clostridioides difficile*

Clostridioides difficile infection (CDI) is the leading cause of hospital-acquired infection associated with severe diarrhoea and pseudomembranous colitis, accounting for significant morbidity and mortality worldwide (Walker *et al*., 2012). In 2017, 13, 000 cases were reported in England and Wales, resulting in approximately 1000 deaths and accounting for over £120 million spent by the NHS (National Health Service) (Public Health England, 2019). According to the Centres for Disease Control and Prevention (CDC), in 2017, 223, 900 cases occurred in the United States of which 12, 800 resulted in death. The economic burden of such a high number of cases is estimated to be $4.5 billion each year (CDC, 2017). Various factors have been identified which play a role in increased risk of acquiring CDI. These include age (over 65 years), the use of broad-spectrum antibiotics as well as the duration of administration, prolonged hospital stays, co-existing infections and gastrointestinal (GI) procedures and use of proton pump inhibitors (Bignardi, 1998; Thibault *et al*., 1991). A compromised immune system and depletion of normal gut flora resulting from antibiotic usage permits *C. difficile* to flourish and establish infection (Bartlett *et al*., 1978).

The incidence of CDI has increased dramatically since 2003 with the emergence of hypervirulent strains such as 027/BI/NAPI which have caused outbreaks in health care settings in North America and Europe (Barbut *et al*., 2011). With the rapid spread of hypervirulent strains, the spectrum of affected individuals has broadened with more cases occurring within the community including in healthy children (Goorhuis

et al., 2008). Currently the primary treatment against CDI is antibiotic therapy, mainly metronidazole, vancomycin or fidaxomicin. The major concern associated with CDI is the occurrence of relapses and the difficulty in treating these episodes (McDonald *et al*., 2018; Singh *et al*., 2019). With the increase in antibiotic resistance and the lack of approved prophylactics, the need to develop a vaccine is urgent.

1.2 Life cycle of *C. difficile*

C. difficile is a Gram-positive, spore forming, anaerobic bacillus, belonging to the phylum Firmicutes. The bacterium was first discovered in 1935 by Hall and O'Toole during examination of intestinal flora of new-born infants from stool specimens (Hall and O'Toole, 1935). Initially the organism was given the name *Bacillus difficilis* by virtue of the difficulties in isolating and studying the bacterium and was later re-designated *Clostridium difficile.* The bacterium was re-named again in 2016 as *Clostridioides difficile* (Lawson *et al*., 2016). In 1978, the first case of toxin-producing *C. difficile* responsible for causing antibiotic-associated colitis was reported (Bartlett *et al*., 1978). The life cycle begins with ingestion of spores which endure the acidity of the stomach, germinate in the GI tract and subsequently grow into toxin-producing vegetative cells (Sorg and Sonenshein, 2008). The major factors responsible for the clinical manifestation of CDI are the toxins which disrupt the intestinal epithelial barrier (Nusrat *et al*., 2001). Pro-inflammatory cytokines are released by immune cells such as neutrophils and antigen presenting cells (APCs) in the intestine causing an inflammatory response (colitis) (Sun *et al*., 2010). Moreover, the increased permeability of the intestinal epithelium leads to fluid influx which results in diarrhoea (Napolitano and Edmiston, 2017). Symptoms can range from mild to

moderate diarrhoea, pseudomembranous colitis, and fulminant colitis and in the worst case scenario, death (Napolitano and Edmiston, 2017).

1.2.1 Sporulation

Sporulation is initiated as a mechanism of survival of the strict anaerobe. Although signals that trigger *C. difficile* sporulation have not been identified, based on *Bacillus subtilis* (*B. subtilis*) studies, stimuli such as oxygen exposure, nutrient starvation and other stress factors are speculated to initiate sporulation (Higgins and Dworkin, 2012). These dormant spores disseminate via faecal-oral contamination particularly in health-care settings and are highly persistent as they are insensitive to heat or alcohol-based disinfectants (Kochan *et al*., 2018; McFarland *et al*., 1989; Johnson *et al*., 1990). Therefore, spore formation plays an important role in transmission of the disease. *C. difficile* spores are similar to those of other endospore-forming bacteria such as *B. subtilis* and *B. anthracis*, consisting of several layers and having a low water content core which makes them hardy. In brief, the spore core contains DNA which is protected from damage by small acid soluble proteins (SASP) and a high content of dipicolinic acid-DPA combined with calcium (Ca-DPA) as a substitute for water which provides heat resistance (Donnelly *et al*., 2016). The core is surrounded by a phospholipid inner membrane similar to that of *B. subtilis* spores with minimal permeability (Figure 1.1) (Cowan *et al*., 2004). This inner membrane is enclosed by a germ cell wall which is surrounded by a modified peptidoglycan cortex (Figure 1.1). The peptidoglycan side chains are removed from *N*-acetylmuramic acid forming muramic acid-δ-lactam during cortex hydrolysis (Kochan *et al*., 2018). Surrounding this cortex is an outer membrane followed by a layer of proteinaceous spore coat which provides protection against enzymatic activity from antimicrobial reagents.

Finally, an outermost layer known as the exosporium surrounds the spore coat and is involved in adherence and dormancy (Figure 1.1). The exosporium is the feature that differs the most, not only amongst endospore-forming bacteria but also between *C. difficile* strains and not all strains have one (Bara-Carrasco and Paredes-Sabja, 2014; Czepiel *et al*., 2019; Kochan *et al*., 2018; Paredes-Sabja *et al*., 2014; Zhu *et al.,* 2018).

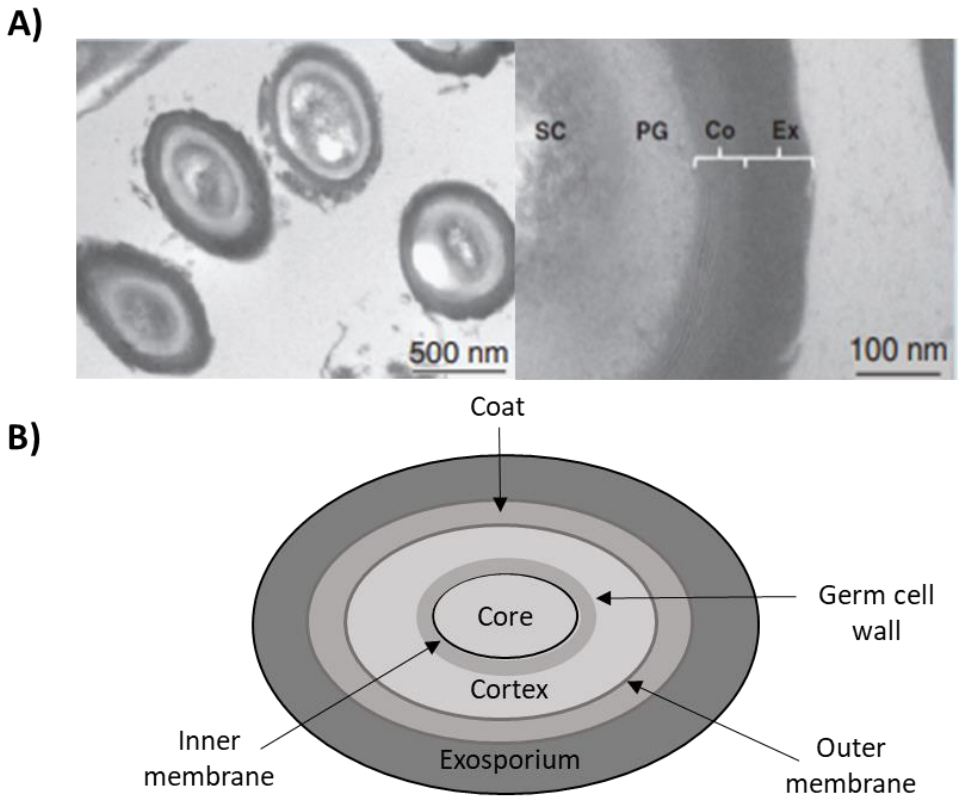

Figure 1.1- Ultrastructure of *C. difficile* spore.
C. difficile strain 630 visualised by A) transmission electron microscopy adapted from Barra-Carrasco and Paredes-Sabja, (2014). SC: spore coat, PG: peptidoglycan germ cell wall, Co: cortex and Ex: exosporium and B) a schematic representation of a typical spore.

1.2.2 Germination

Pathogenesis of *C. difficile* begins with the ingestion of highly resistant spores readily distributed throughout the environment that germinate in the small intestine (Kochan *et al*., 2018). Germination is activated in response to host primary bile salt

germinants such as taurocholate and cholate derivatives and the amino acid co-germinant glycine (Sorg and Sonenshein, 2008). *C. difficile* utilises the subtilisin-like spore germinant receptor, CspC (on the spore coat or outer membrane) for recognition of bile salt germinants, in which loss of this diminishes spore germination (Francis *et al*., 2013). Activation of the cortex lytic enzyme, SleC is required for degradation of the cortex layer, to facilitate spore germination (Figure 1.2) (Burns *et al*., 2010). Expression of the Csp serine protease family operon (*cspBAC*) is responsible for the sequential proteolytic activation of SleC (Figure 1.2). CspBA is present as a fusion protein which undergoes cleavage by the YabG protease leading to the release of CspA and CspB serine proteases (Figure 1.2) (Kevorkian *et al*., 2016). CspB subsequently cleaves pro-SleC resulting in activation of SleC. This causes release of cations and Ca-DPA and degradation of spore peptidoglycan, spore rehydration and hence reactivation of metabolism defined as outgrowth into toxin-producing vegetative cells (Diaz *et al*., 2018; Kochan *et al*., 2018). Recently a novel lipoprotein GerS (CD630_34640) (on the spore coat or outer membrane) has been shown to regulate SleC activity by playing a role in cortex modification which allows SleC recognition and thus cortex hydrolysis (Figure 1.2) (Diaz *et al*., 2018). Although GerS mutant spores expressed SleC at wild type level, cortex hydrolysis was hindered suggesting GerS plays a role in regulation (Figure 1.2). Further to this study, it has also been shown that CwlD (CD630_01060) and PdaA (CD630_14300) enzymes which play a role in cortex modifications in *B. subtilis,* also play a role in *C. difficile* spore germination. GerS, CwlD and PdaA mutants all cause germination defects in *C. difficile* and are required for cortex modification which is recognised by SleC (Diaz *et al*., 2018).

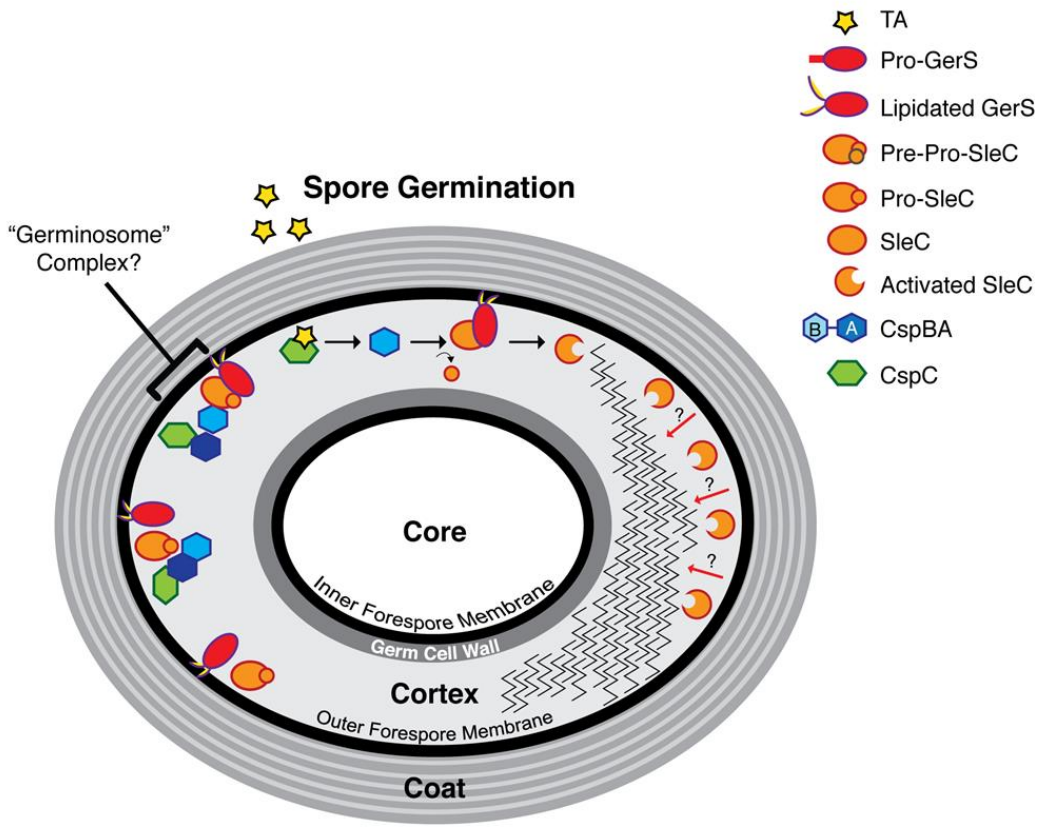

Figure 1.2- Model of *C. difficile* germination regulation and localisation adapted from Fimlaid *et al* (2015).
Germination regulators referred to as germinosome complex are predicted to localise in the cortex and/or outer membrane. Taurocholate-TA (yellow star) binds to CspC receptor (green hexagon) which cleaves CspBA (light blue CspB and dark blue CspA pentagons) for the release of CspB. This then cleaves pro-SleC (double orange circle) into activated SleC (orange circle) which is regulated by GerS (red oval). Red arrows with question marks represent unknown events.

1.2.3 Colonisation

Upon spore germination, metabolically active vegetative cells are capable of colonising the large intestine and producing toxins (Theriot *et al*., 2016). The ability to colonise is greatly facilitated by disruption of the gut flora by antibiotics which lowers the protective colonisation resistance barrier (Theriot *et al*., 2016). Colonisation is a crucial step in pathogenesis and various factors that are thought to be involved in colonisation include the S-layer proteins (SLPs), cell wall proteins

(CWPs), flagella proteins (FliC and FliD), fibronectin-binding protein (FbpA), heat-shock protein (GroEL) and lipoproteins (CD630_08730) (Janoir *et al*., 2016). These will be discussed further. However, the clinical symptoms that present during CDI are attributed mainly to the release of toxins from colonised vegetative cells (Smits *et al*., 2016; Vedantam *et al*., 2012).

1.3 Virulence factors

1.3.1 Toxins

1.3.1.1 TcdA and TcdB

The major virulence determinants produced by *C. difficile* are, toxin A (TcdA) and toxin B (TcdB) (Banno *et al*., 1984). Clinical isolates that manifest disease are toxin producers and conversely isolates which lack toxin production are avirulent (Borriello *et al*., 1990). All virulent strains produce TcdB, however TcdA-negative ($TcdA^{-}TcdB^{+}$) strains exist and have also been shown to be virulent (Vedantum *et al*., 2012). In a study carried out by Lyerly *et al* (1985), purified TcdA alone was shown to cause infection in hamster models whereas TcdB was only toxic when co-administered with small quantities of TcdA suggesting the toxins work synergistically (Lyerly *et al*., 1985). However, in another study, when TcdA and TcdB mutants were tested in a hamster model, it was shown that TcdB was essential in virulence as TcdB mutants (TcdB-/TcdA) did not cause pathology in hamsters but TcdA mutants (TcdA-/TcdB) were able to cause disease in hamsters (Lyras *et al.,* 2009). Kuehne *et al* (2010), then showed that deletion of either of the toxin genes, still caused disease in a hamster model. Furthermore, simultaneous deletion of both toxins led to abolishment of

virulence (Kuehne *et al*., 2010). These studies highlight that although TcdB appears to be the primary virulence determinant, both contribute to CDI.

The genes that encode TcdA and TcdB (*tcdA* and *tcdB,* respectively) are on the 19.6 kb pathogenicity locus (PaLoc) of the chromosome (Barroso *et al*., 1990; Dove *et al*., 1990). Both toxins are large; TcdA is 308 kDa and TcdB is 269.6 kDa and share an overall similarity of 68% amino acid sequence (Pruitt *et al*., 2010; Voth and Ballard, 2005). TcdA and TcdB are monoglucosyltransferases composed of two subunits, A and B (von Eichel-Streiber *et al*., 1996). The A subunit is composed of an N-terminal glucosyltransferase domain (GTD) and the B subunit is responsible for the delivery of GTD to the cytosol of target cells (Pruitt *et al*., 2012). The B-subunit is formed of three domains; an autoprotease domain (APD), a delivery or pore-forming domain and combined repetitive oligopeptides (CROPs) at the C-terminus which forms the receptor binding domain (RBD) (Figure 1.3) (Chandrasekaran and Lacy, 2017; Pruitt *et al*., 2012).

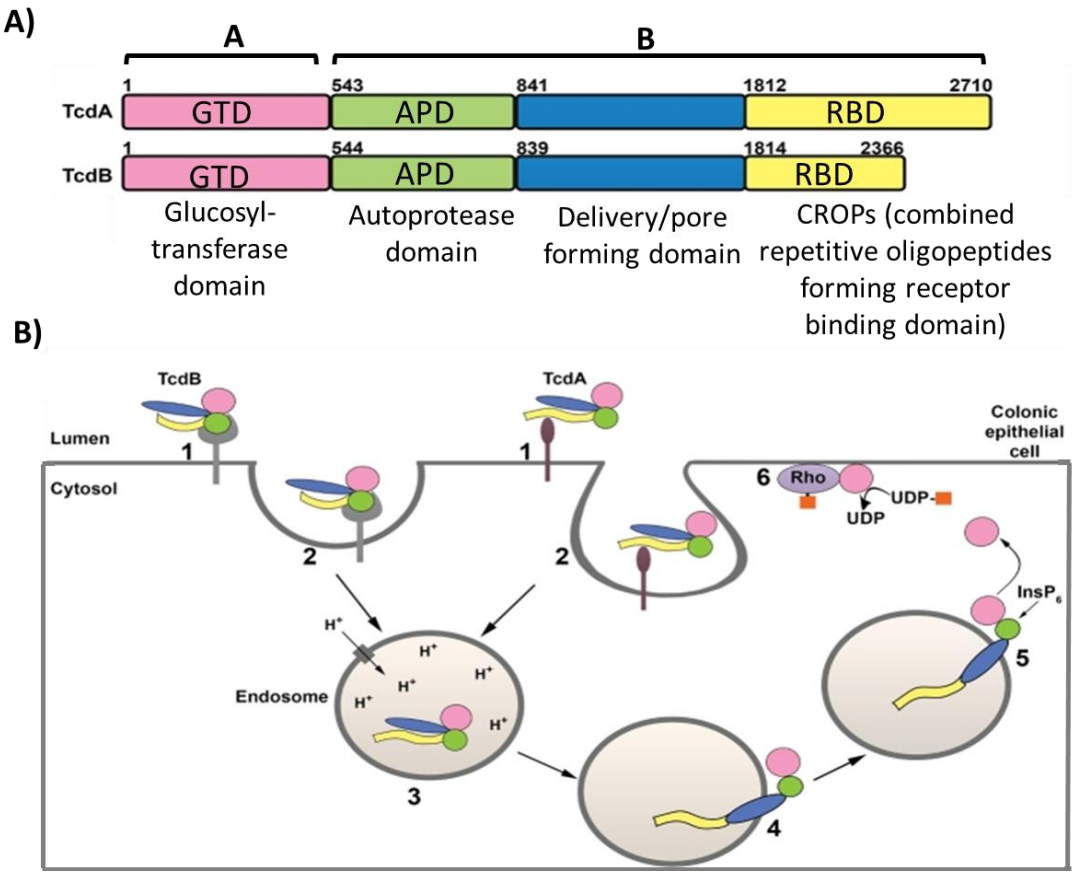

Figure 1.3- Schematic diagram of TcdA and TcdB structure and mechanism of action adapted from Chandrasekaran and Lacy (2017).

A) Four functional domains; glucosyltransferase domain (GTD), the autoprotease domain (APD), the delivery or pore-forming domain and the combined repetitive oligopeptides domain (CROPs). B) Steps involved in binding and intoxication of epithelial cells; 1) binding and internalization by endocytosis, 2) acidic endosome formed, 3) low pH causes conformational change in delivery domain which results in pore formation and 4) translocation of the GTD into cytosol. 5) Inositol hexakisphosphate (InsP6) binds and activates APD, resulting in the cleavage and release of the GTD. 6) Inactivation of Rho by GTD glucosylation and therefore cell rounding and apoptotic cell death.

The RBD is important in binding to receptors on epithelial cells with the toxins subsequently taken up by the cell via endocytosis (Chandrasekaran and Lacy, 2017; von Eichel-Streiber *et al*., 1992). Once internalized, an acidic environment is required for exposure of the delivery/pore forming region (Florin and Thelestam, 1983). This domain is composed of hydrophobic residues which cause insertion into the

membrane forming a pore. This is followed by proteolytic cleavage and the GTD is released into the cytosol (Pfeifer *et al*., 2003). The N-terminal enzymatic domain accounts for the biological activity in the cytosol. The GTD is responsible for the glucosylation and inactivation of Rho-family GTPases (guanosine triphosphate binding enzymes) within the cytosol of target cells which leads to actin cytoskeleton impairment and disruption of epithelial cells causing extensive fluid influx and cell death (Figure 1.3) (Voth and Ballard, 2005). It has recently been shown that a GTD inactivated mutant strain was unable to cause disease in a hamster model compared to wild-type highlighting that the GTD is crucial for disease pathogenesis (Bilverstone *et al*., 2020).

Several studies have focused on different toxin domains to compare their importance in infection and their immunogenicity. It has been shown that truncated fragments of the RBD of both TcdA and TcdB elicit toxin-neutralising antibody production in mice (Belyi *et al*., 2003; Huang *et al.,* 2015; Tian *et al.,* 2017). Vero cells derived from African green monkey kidney (*Ceropithecus aethiops*) epithelial cells are widely used cell lines to study *C. difficile* cytotoxicity/cell rounding due to their sensitivity to the toxins (Yücesoy *et al*., 2002). Huang *et al* (2015) recently investigated three truncated fragments of TcdA-RBD; its N-terminus, the middle portion and the C-terminus. In this study the C-terminal domain was shown to have strong Vero cell binding properties and when used to immunise mice, elicited a protective immune response.

1.3.1.2 Binary Toxin

A third toxin known as the binary toxin or '*C. difficile* transferase' (CDT) is produced by approximately 20% of clinical isolates, specifically hypervirulent strains of ribotype

027, 078 and 023 (Cookson, 2007; Cowardin *et al*., 2016; Gerding *et al*., 2014). CDT is an ADP-ribosyltransferase, distinct from TcdA and TcdB (Popoff *et al.,* 1988). This toxin is composed of two domains, CDTa and CDTb, encoded by two genes *cdtA* and *cdtB,* respectively found within the Cdt locus (CdtLoc) (Perelle *et al.,* 1997). CDTb is involved in binding to target host cells, allowing internalization of the CDTa subunit which is responsible for the enzymatic activity within the cytosol of the cell (Simpson, 1989). CDTa, adenosine diphosphate (ADP)-ribosylates actin which results in inhibition of actin polymerisation and depolymerisation of actin filaments leading to disruption of actin cytoskeleton and the formation of microtubule protrusions on the epithelial cell surface (Gerding *et al.,* 2014).

In the recent years, more studies have unravelled insight into the role CDT plays in pathogenesis. A small number of cases of symptomatic patients infected with CDT-positive but TcdA and TcdB-negative strains have previously been reported (Androga *et al*., 2015; Eckert *et al*., 2015). Studies have shown that patients infected with hypervirulent strains positive for CDT as well as TcdA and TcdB have increased severity and higher rates of recurrence compared to patients infected with strains positive for just TcdA and TcdB (Bacci *et al*., 2011; Barbut *et al*., 2007; Stewart *et al*., 2013). Additionally, the presence of CDT as well as a deletion in the *tcdC* gene, which is a repressor for toxin A and B production, showed the highest rate of recurrence (Stewart *et al*., 2013). However, in one of these studies a similar level of fatality rate was observed in patients infected with either hypervirulent or non-hypervirulent strains that produced all three toxins compared to patients infected with strains that produced only TcdA and TcdB (Bacci *et al*., 2011). These studies suggest that CDT may play a role in pathogenesis and contribute to virulence. Factors identified as

contributors for virulence have been that CDT enhances formation of microtubule protrusions on the epithelial cell surface which increases bacterial adherence and also suppresses protective eosinophilia (Cowardin *et al*., 2016; Schwan *et al*., 2009). Challenge studies in hamsters conducted with strains producing CDT and not TcdA and TcdB showed that CDT alone does not cause disease (Geric *et al*., 2006; Kuehne *et al*., 2014). However, hamsters challenged with a mutant strain producing TcdA and CDT were more virulent when compared to hamsters challenged with a mutant strain producing only TcdA. This study highlights that CDT may act synergistically with TcdA and enhance virulence (Kuehne *et al*., 2014). All together these studies highlight the role CDT plays in pathogenesis and contribution to virulence.

1.3.2 S-layer and cell wall proteins

The S-layer is a crystalline array of proteinaceous molecules coating the entire peptidoglycan cell wall and plays different roles in different organisms (Sara and Sleytr, 2000). The major CWPs forming the S-layer in all *C. difficile* strains are the SLPs. High molecular weight (HMW-SLP) and low molecular weight (LMW-SLP) forms are generated by proteolytic cleavage of the precursor protein SlpA encoded by *slpA* (Awad *et al*., 2014; Bruxelle *et al*., 2016). Both proteins form a heterodimer complex, and are surface-exposed with the LMW-SLP forming the outermost layer (Cerquetti *et al.,* 2000; Eidhin *et al.,* 2006). The HMW-SLP forms the bottom layer and is cell wall anchored and involved in adherence to epithelial cells and normal GI tissue (Calabi *et al.,* 2002). LMW-SLPs and HMW-SLPs are considered immunogenic as studies have shown their ability to elicit pro-inflammatory responses through Toll-like receptor 4 (TLR4) signalling (Ryan *et al.,* 2011). Other cell wall proteins have been identified in

C. difficile strain 630 that are paralogues to HMW-SLP. However, the variation between these SLP between strains renders them unsuitable as vaccine candidates.

The most well characterized CWP is Cwp84. This is a surface-exposed cysteine protease and has an N-terminal domain responsible for the cleavage of SlpA into LMW-SLP and HMW-SLP (Dang *et al.,* 2010; Janoir *et al.,* 2007). Cwp84 is well conserved between *C. difficile* strains and has been shown to be immunogenic as demonstrated by the presence of Cwp84-specific antibodies in patients infected with *C. difficile* (Péchiné *et al.,* 2005; Savariau-Lacomme *et al.,* 2003). It has also been shown to provide 40% protection against CDI in hamster models via oral delivery (Sandolo *et al*., 2011).

Cwp66 is a surface-exposed protein involved in adhesion (Waligora *et al.*, 2001). This cell wall protein is composed of two domains. The N-terminal domain allows cell anchoring and is less variable as opposed to the surface-exposed C-terminal domain. In a study carried out by Péchiné *et al* (2005), the Cwp66 C-terminal domain was found to be highly immunogenic based on ELISA studies using sera of patients with CDI (Péchiné *et al.,* 2005). Despite its immunogenicity, its potential as a vaccine candidate is limited due to its variability between *C. difficile* strains (Waligora *et al*., 2001).

1.3.3 Lipoproteins

Bacterial lipoproteins are proteins that anchor to the cell membrane via their lipid moiety and have been shown to perform various functions including conferring structural integrity to the cell wall, nutrient uptake, cell signalling, sporulation and antibiotic resistance. They play a key role in bacterial virulence such as adhesion and

colonisation and/or elicit the production of protective antibodies and this has attracted interest in their potential as vaccine candidates (Kovacs-Simon *et al.,* 2011; Nguyen and Götz, 2016). An example of a lipoprotein approved as a vaccine is Factor H binding protein (FHbp). FHbp is used to immunise against meningococcal serogroup B isolates in two different licensed vaccines; Bexsero® (GlaxoSmithKline-GSK) and Tumenba® (Pfizer) (Seib *et al*., 2015).

All lipoproteins are synthesised as preprolipoproteins in the cytoplasm and are exported through the cytoplasmic (inner) membrane either via the secretory (Sec) pathway or in some cases by the twin arginine translocase (Tat) pathway (Goosens *et al.,* 2014). This is directed by the N-terminal signal peptide which contains a characteristic lipobox at the C-terminus with the consensus sequence (LVI) (ASTG) (GA) C, ending with a conserved cysteine residue (Hayashi and Wu, 1990). Upon translocation into the periplasm, the preprolipoprotein is processed and lipidated at the N-terminus by a sequential enzymatic process (Figure 1.4).

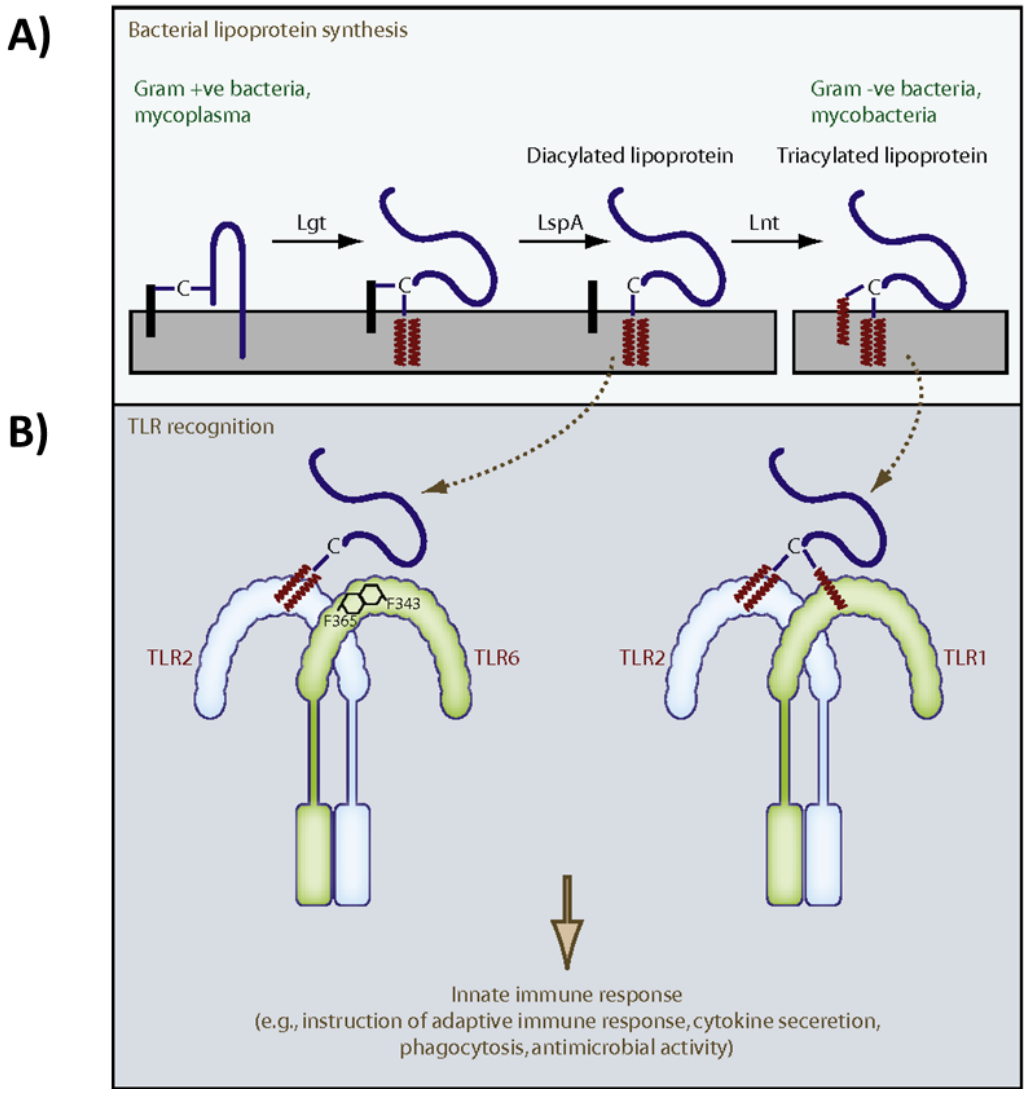

Figure 1.4- Mature lipoprotein synthesis and TLR2 recognition adapted from Schenk *et al* (2009).
Preprolipoprotein translocated across cell membrane and addition of diacylglycerol group (two fatty acids) catalysed by prolipoprotein diacylglycerol transferase (Lgt) (red) and then the signal peptide (black) is cleaved by prolipoprotein signal peptidase (LspA) in Gram-positive bacteria. Addition of third fatty acid in Gram-negative is catalysed by apolipoprotein *N*-acyltransferase (Lnt). B) Recognition of di or triacylated lipoproteins through Toll-like receptor 2 (TLR2) interaction. TLR2 forms a heterodimer with TLR6 or TLR1 which enables binding of diacylated or triacylated lipoproteins respectively.

The first step is the addition of a diacylglycerol group to the conserved cysteine. This is catalysed by prolipoprotein diacylglycerol transferase (Lgt) (Figure 1.4) (Sankaran

and Wu, 1994). These two lipids are attached covalently between the glycerol backbone and the sulfhydryl (SH) group of the N-terminal cysteine. A second enzyme known as the prolipoprotein signal peptidase (LspA) then cleaves the signal peptide at the residue immediate upstream of the conserved cysteine forming a diacylated lipoprotein (Figure 1.4) (Hussain *et al*., 1982). In Gram-negative bacteria and some Gram-positive bacteria with high GC content an additional fatty acid is added to the N-terminal cysteine forming an amide bond via the amino termini. This is catalysed by the enzyme apolipoprotein *N*-acyltransferase (Lnt), generating a triacylated lipoprotein (Figure 1.4, A) (Tokunaga *et al*., 1982). Gram-positive bacteria with low GC-content also referred to as Firmicutes lack Lnt and are therefore unable to produce triacylated lipoproteins with the exception of staphylococci (Kurokawa *et al*., 2012; Machata *et al*., 2008). An *in silico* study conducted by Ruth Griffin (personal communication) identified the Lnt gene (*lnt*) in *C. difficile* strain 630 (CD630_27370). Protein Basic Local Alignment Search Tool (BLASTp®) analysis revealed homologues of the gene in other strains of *C. difficile.* This may indicate that *C. difficile* could potentially produce triacylated lipoproteins.

Bacterial lipoproteins are known to elicit an innate immune response via the activation of TLR2 which results in the establishment of adaptive immunity (Figure 1.4, B). Circulating APCs, such as dendritic cells (DCs), harbour pattern recognition receptors (PRRs) such as TLR2 which act as lipoprotein ligands (Brightbill *et al*., 1999). TLR2 can form a heterodimer with TLR6 or TLR1 which enables a binding pocket for the recognition of diacylated or triacylated lipoproteins respectively (Takeuchi *et al*., 2002). Leucine-rich repeat (LRR) domains found within TLRs contribute to this recognition and form distinct horse-shoe like structures (Kang *et al*., 2009). TLR2 can

bind diacylated lipoproteins by forming hydrogen bonds between the glycerol group (attached to the two lipids) and the protein ligand with its hydrophobic LRR loops. What establishes heterodimer formation with either TLR1 or TLR6 is an additional hydrophobic channel within the TLR1 required for the binding of the third lipid and therefore heterodimer formation between TLR2/TLR1 (Kang *et al*., 2009). However, this channel is blocked in TLR6 by phenylalanine residues (Schenk *et al*., 2009). The TLR2/TLR6 formation also requires the non-occupied amino-terminal cysteine interaction with the hydrophobic residues of TLR2 and TLR6 (Figure 1.4, B). These complexes induce the activation of the transcription factor, nuclear factor kappa-light chain activator of activated B cells (NF-κB) via the MyD88 cascade, which causes the release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), and Interleukins (IL) and therefore, induces phagocytosis (Miggin and O'Neill, 2006). This also leads to upregulation and maturation of DCs and macrophages which is crucial for innate and adaptive immune responses which form antigen specific effector and memory T cells and B cells (Khan *et al*., 2009; Miggin and O'Neill, 2006; Schenk *et al.,* 2009). Due to these TLR2 agonist properties, the use of lipoproteins as vaccine adjuvants has recently attracted great interest (Leng *et al*., 2015; Zaman and Toth, 2013). An example includes work conducted by Moyle *et al* (2014), who compared synthetic dipalmitoyl-*S*-glyceryl-cysteine (Pam2Cys) and tripalmitoyl-*S*-glyceryl-cysteine (Pam3Cys) lipids engineered to contain a cysteine residue conjugated onto recombinant proteins of *Streptococcus pyogenes.* This study demonstrated enhanced serum IgG elicited in mice by lipidated antigens compared to non-lipidated.

The *C. difficile* lipoproteome was studied by Charlton *et al* (2015) in strains 630 and R20291 with bioinformatics analysis followed by experimental confirmation of over 50 predicted lipoproteins. Of these predicted lipoproteins, CD630_08730, an ATP-binding cassette (ABC-type) transporter was identified and previously experimentally shown to be a surface-exposed adhesin (Kovacs-Simon *et al*., 2014). The lipoprotein was designated CD0873 and adherence confirmed by CD0873 mutants showing a significant reduction in adherence to Caco-2 cells (human colorectal epithelial cells) compared to the parent strain, *C. difficile*Δ*erm* (Kovacs-Simon *et al*., 2014). The immunogenicity of this lipoprotein had previously been tested using sera of 6 *C. difficile* patients. Three demonstrated antigen-specific immunoglobulin; IgG, IgA and IgM responses (Wright *et al.,* 2008). Bradshaw *et al* (2019) also showed this lipoprotein to be immunogenic and to provide lower clinical scoring used to immunise mice which were then challenged with *C. difficile*. These studies highlight the importance of CD630_08730 utilisation for colonisation prevention.

CD630_14300 and CD630_27190 (predicted lipoproteins identified by Charlton *et al.,* 2015) are genes that encode *N*-deacetylase PdaA1 and PdaA2 respectively. In *B. subtilis,* PdaA is required for the modification and hydrolysis of the spore cortex which leads to germination (Fukushima *et al*., 2002). Another *C. difficile* lipoprotein identified recently is CD630_34640 (GerS) which has been shown to be involved in the regulation of germination (Fimlaid *et al*., 2015). SleC hydrolase is important for the degradation of the spore cortex which allows *C. difficile* to germinate and transform into toxin-producing, vegetative cells (Fimlaid *et al.,* 2015). In this study, GerS mutants presented with severe germination defects and failed to degrade the cortex despite expressing SleC at wild type levels. From this it is inferred that GerS is

involved in the regulation of germination. In addition to this, a reduction in virulence in hamster models was observed with the loss of GerS (Fimlaid *et al.,* 2015).

1.4 *C. difficile* treatment

1.4.1 Antibiotic therapy

The first line treatment against CDI is antibiotic therapy. Patients presenting with mild to moderate infection are administered with metronidazole and for those with moderate to severe infection, oral vancomycin is preferred (Jarrad *et al*., 2015). Treatment failure after administration of either metronidazole or vancomycin typically results in recurrence of CDI (Vardakas *et al.,* 2012). Both metronidazole and vancomycin are broad-spectrum antibiotics which can also lead to disruption of the normal gut flora and in turn increase susceptibility of the patient to CDI (Dethlefsen and Relman, 2011). In addition to this, with the removal of the gut flora, conversion of primary bile salts into secondary bile salts which inhibits *C. difficile* spore germination is depleted which then favours *C. difficile* germination (Theriot *et al*., 2016). Alternative antibiotics such as rifamycin and fidaxomicin are then usually offered. Fidamoxicin is a narrow-spectrum macrolide antibiotic which causes less disruption to the gut flora and has similar treatment efficacy compared to vancomycin. It has been shown to have less recurrence rates compared to vancomycin (Singh *et al*., 2019). The major issue with antibiotic treatment is the propensity for recurring episodes due to which some cases become impossible to treat and unfortunately lead to death (Jarrad *et al*., 2015).

1.4.2 Alternative therapeutics

1.4.2.1 Faecal microbiota transplantation (FMT)

Faecal microbiota transplantation (FMT) is a treatment used for recurrent and refractory CDI which involves the transfer of faecal matter from an healthy individual via the rectal route as a suspension or oral route in a pill to a patient with CDI aimed at restoring the normal gut flora. The use of this treatment has globally increased and in 2017, the first FMT service in the UK was licenced and launched by the University of Birmingham Microbiome Treatment Centre (McCure *et al*., 2020). This approach is recommended for patients after their third episode of CDI and is aimed to prevent recurrent CDI. It was shown that patients receiving a course of vancomycin who were co-administered with FMT were protected from further episodes of CDI (Fernández-García *et al.,* 2017; van Nood *et al.,* 2013). Several reports of case records have shown success rates between 68-92% in treating recurrent CDI using FMT with no further relapse observed (Lee *et al*., 2014; Nowak *et al*., 2019; Quraishi *et al*., 2017; Tvede *et al*., 2014). This process requires extensive screening of stool and blood samples of healthy donors in order to avoid potential risk of transferring pathogens (Mullish *et al*., 2018). FMT is an accepted and effective treatment for recurrent/refractory CDI. The only concern which remains with this treatment is the uncertainty of long-term side effects and the patient acceptance of using this form of treatment (Kociolek and Gerding, 2016).

1.4.2.2 Spore therapy

Another approach in development is the use of spores that could potentially compete with *C. difficile* and prevent colonisation. Gerding *et al* (2015) assessed the use of spores of a non-toxigenic *C. difficile* strain M3 (NTCD-M3) as a potential therapeutic for prevention of recurrent CDI. After assessing the safety and ability to colonise patients aged over 60, the efficacy for prevention in patients who had received metronidazole and vancomycin treatment with either their first CDI or first recurrence was investigated with comparison to patients receiving placebo. Patients received either 10^4 or 10^7 NTCD-M3 spores for 7 days or 10^7 spores for 14 days. This study showed that patients receiving NTCD-M3 spores had significantly lower recurrence rates with the lowest in patients receiving 10^7 spores for 7 days which showed 5% recurrence compared the placebo group which showed 33% recurrence of CDI. The reduction in recurrence was correlated with NTCD-M3 colonisation. In another study, it was also demonstrated that when a mixture of spores known as ser-109 collected from 50 Firmicutes species were orally administered to patients with recurring CDI, 8 weeks later, 26 out of 30 patients were *C. difficile*-negative (Khanna *et al.,* 2016). However, some adverse side effects such as nausea, mild diarrhoea and abdominal pain were observed. Therefore, the safety of this treatment needs refining.

1.4.2.3 Probiotics

There has been considerable focus on the use of probiotics for both the prevention and treatment of CDI as well as for other gastrointestinal disease. Probiotics are live microorganisms which can block adherence of other pathogens like *C. difficile* by competing for adherence to epithelial cells (Oelschlaeger, 2010). The efficacy of

probiotics for prevention of primary CDI was reviewed by Johnson *et al* (2012). Patients receiving antibiotics were used to compare CDI development with a placebo group also receiving antibiotics with no probiotics. Several studies showed significantly lower CDI rates upon treatment with different probiotic strains including study 1: *Lactobacillus; L. rhamnosus, L. casei, L. bulgaricus* and *Saccharomyces; S. thermophilus* (population n=112) and study 2*:* higher and lower doses of *L. acidophilus* CL1285 and *L. casei* LBC80R (population n=255). It was concluded the risk of CDI is reduced for those patients on long term antibiotics by probiotics, however more studies are required to select the most appropriate strains and to test a larger number of patients (Goldstein *et al.,* 2017).

1.4.3 Passive immunisation

1.4.3.1 Monoclonal antibodies

Immunisations based on delivering polyclonal antibodies against TcdA and TcdB, were first studied as early as 1982 (Giannasca *et al.,* 1999; Libby *et al.,* 1982; Roberts *et al*., 2012). The focus then shifted to monoclonal antibodies to target specific epitopes of these toxins (Bruxelle *et al.,* 2018). Corthier *et al* (1991) were the first to generate mouse monoclonal antibodies against the RBD of TcdA. These were tested in axenic (germ-free) mice models. Given intravenously, they showed protection against *C. difficile* (Corthier *et al*., 1991). However, the concern of an immunogenic reaction in humans given mouse monoclonal antibodies led to the development of humanised monoclonal antibodies (Babcock *et al*., 2006).

The first humanised monoclonal antibodies (HuMAbs) raised against TcdA and TcdB were produced by Babcock *et al* (2006). Toxoid A and toxoid B (inactivated toxins) as

well were used to generate anti-TcdA/TcdB HuMAbs in transgenic mice with human immunoglobulin genes. Following the observation of successful toxin neutralisation in *in vitro* toxin neutralisation assays, CDA1 and MDX1388 HuMAbs directed against the RBD of TcdA and TcdB, respectively were selected for testing in hamsters. Following encouraging results from this study, both HuMAbs progressed to human trials and were out-licenced to Merck (Whitehouse Station, New Jersey, USA) as anti-TcdA, Actoxumab (MK-3415/MDX-066) and anti-TcdB, Bezlotoxumab (MK-6072/MDX-1388/CDB1) in 2009. A two stage (MODIFYI and MODIFYII), phase III clinical trial was conducted assessing prevention of recurrent CDI with groups receiving; Actoxumab or Bezlotoxumab alone, Actoxumab plus Bezlotoxumab and a placebo saline only group. Actoxumab alone was discontinued after MODIFYI proving to have low efficacy and also more serious adverse reactions compared to the placebo saline only group (Wilcox *et al*., 2017). At the end of this trial, it was concluded that Bezlotoxumab alone achieved lower rates of recurrence in patients receiving antibiotics than the placebo saline group and the Actoxumab plus Bezlotoxumab group (Wilcox *et al*., 2017). In 2016, Bezlotoxumab (Zinplava™) was approved by the Food and Drug Administration (FDA) and in 2017 by the European Medicine Agency (EMA). There is some evidence to suggest that this therapy reduces CDI episodes in patients of 18 years of age and older, who are receiving antibiotic therapy against CDI (Péchiné *et al.,* 2017). However, the extortionate expense of this treatment, raises the important question of its availability for all patients.

1.4.4 Active immunisations: vaccines in development

TcdA and TcdB have attracted interest as vaccine candidates over recent years. In one study it was shown that asymptomatic carriers of *C. difficile* and patients who only

encounter a single episode of CDI, have significantly higher levels of circulating anti-TcdA serum IgG compared to patients who develop recurrent CDI (Aboudola *et al.,* 2003). Several studies have shown lower anti-TcdB as well as anti-TcdA IgG titres in patients with recurrent CDI compared to those who have a single episode or compared to asymptomatic carriers (Kyne *et al.,* 2001; Warny *et al*., 1994; Wullt *et al.,* 2012). Thus there appears to be a direct-correlation between the low risk of developing recurrent CDI and the prevalence of protective anti-toxin IgG. As both toxins are known for their major contributions in virulence and both have been associated with eliciting protective TcdA- and TcdB-specific neutralising antibody responses, focus has been directed on testing these toxins as vaccine candidates (Giannasca and Warny, 2004). Human clinical trials using toxoids for safety reasons have been performed and toxin-neutralising serum IgG titres measured as a correlate for protection. This is known as the immunogenicity profile.

The first vaccine tested in humans comprised formaldehyde-inactivated toxoids, TcdA and TcdB developed by Sanofi Pasteur. Both toxoids previously showed protection in hamster models, against *C. difficile* (Libby *et al.,* 1982). This vaccine was then tested in humans and proved safe with a good immunogenicity profile (Aboudola *et al*., 2003; Kotloff *et al.,* 2001). A phase I trial was conducted with intramuscular (i.m.) administration (adjuvanted with aluminium hydroxide-ALUM) to healthy adults (18-55 years) and the elderly (over 65 years) using doses of 2 µg, 10 µg, 50 µg or placebo control at days 0, 28 and 56. No adverse effects were reported. Higher anti-TcdA IgG levels were observed compared with anti-TcdB IgG (Greenberg *et al*., 2012). A two stage phase II clinical trial followed. For the stage I trial, 50 µg or 100 µg of formulation adjuvanted with or without ALUM was administered to

individuals who were at high risk of developing CDI, aged 40-75 years on days 1, 7 and 30. The adjuvanted higher dose showed a superior immunogenicity profile as well as improved anti-TcdB response as this was low compared to anti-TcdA response in phase I and was selected for the stage II trial for administration on days 0, 7, 180 or 0, 30, 180 (de Bruyn *et al.,* 2016). Thus 100 µg of toxoids, adjuvanted with ALUM were administered on days 0, 7 and 30 for phase III clinical trials to test for efficacy against primary symptomatic CDI. However, this trial was terminated after it was shown that the vaccine failed to protect against CDI following patient clinical assessment of loose/watery stool and PCR confirmation of TcdB (NCT01887912) (Riley *et al*., 2019).

In parallel, Pfizer also developed a toxoid vaccine composed of genetically and chemically modified full-length TcdA and TcdB. Briefly, 3 mutations of the GTD and APD portions; D285A/D287A/C700A for TcdA and D286A/D288A/C698A for TcdB were generated and the inactivated toxins treated with formalin. The toxoids given via the i.m. route, showed 60% protection in hamsters against CDI (Donald *et al*., 2013). Some cytotoxicity features remained and therefore, modifications to the chemical treatment using 1-ethyl-3-(-3dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were made and the vaccine taken forward for human trials (Vidunas *et al*., 2016). Phase I clinical trials were conducted in healthy volunteers (50-85 years) using doses of 50 µg, 100 µg, 200 µg or placebo control (with or without ALUM) with i.m. administration at 0, 1, and 6 months (Sheldon *et al*., 2016). The results of this study revealed good immunogenicity profiles 12 months after administration and good tolerance. The toxoid formulation alone gave a better immunogenicity profile with higher toxin-neutralising antibody titres compared to

the adjuvanted vaccine (Sheldon *et al.,* 2016). This successfully progressed on to phase II trials with healthy adults aged 65-85 years receiving doses of 100 µg, 200 µg or placebo control (without ALUM) and two regimens; 0, 1 and 6 months or 1, 8, 30 days. The results showed that the higher dose along with the month regimen generated higher anti-toxin antibody titres which persisted 12 months after the final dose. The vaccine was further confirmed to be safe and well tolerated for progression to a phase III trial, currently on-going (Kitchin *et al*., 2020).

The third vaccine VLA84, produced by Valneva is composed of a fusion protein containing a portion of the RBD of TcdA (amino acids 2273-2710) linked via a 4 amino acid linker to a portion of RBD of TcdB (amino acids 1851-2366). A phase I trial was conducted in two stages using i.m. administration. Stage I was with healthy adults aged 18-65 years receiving 20 µg (adjuvanted with ALUM), 75 µg or 200 µg (adjuvanted with or without ALUM) on days 0, 7 and 21. Stage II was with adults aged over 65 receiving 75 µg or 200 µg (adjuvanted with or without ALUM) on days 0, 7, 28, and 58. Phase I results revealed good immunogenic responses in both groups and good tolerance (Bézay *et al.,* 2016). Individuals who received 75 µg (without ALUM) showed the greatest immunogenicity profile. A phase II trial has been completed to identify the optimal dose with healthy adults over the age of 50, receiving 75 µg (without ALUM), 200 µg (adjuvanted with or without ALUM) on days 0, 7 and 28. VLA84 formulation using 200 µg (without ALUM) gave the best immunogenicity profile for both toxins (Dubischar *et al*., 2017).

Active immunisation potentially results in persistent protection, unlike passive immunisation which is short-lived. The vaccines currently in clinical trials are all

composed of inactivated TcdA and TcdB or toxin fragments and elicit anti-toxin neutralising IgG responses. They are aimed at prevention of infection in individuals at risk. However, since anti-TcdA and anti-TcdB antibodies target secreted toxins, they may not bind to the bacterial cell. It is preferable to use antigens that are surface-associated that could also block colonisation. Given the failure of the Sanofi Pasteur toxoids vaccine to provide protection via the i.m. route, which only targeted the toxins, raises concerns for the protection efficacy of the Pfizer and Valneva vaccines currently undergoing clinical trial, which are also i.m. administrations targeting the toxins. It could be argued that the most concerning feature of these vaccines is the route of delivery and lack of colonisation targeting. Vaccines delivered parenterally are capable of eliciting a systemic immune response, *i.e.*, circulation of IgG in the blood however the gut wall is impermeable to serum antibodies (Czerkinsky and Holmgren, 2015). Prevention from infection by gut pathogens relies mainly, if not exclusively on local secretory IgA (sIgA). *C. difficile* has been considered a non-invasive pathogen until recently where it has been shown *in vivo* that *C. difficile* spores are capable of crossing the ileal and colonic mucosa and entering epithelial cells where they reside which is suggested to contribute to recurrence (Castro-Córdova *et al*., 2021). With the epithelial damage caused by the secretion of toxins by vegetative cells and the observed invasion of the mucosal barrier by spores, mucosal vaccines that are capable of generating local immune responses are urgently required.

1.5 Mucosal immunisation

1.5.1 Mucosal vaccines

Most pathogens infect via openings to nasal, respiratory, gastrointestinal or genital mucosal tracts. Immunising through the natural route of infection aims to mimic natural protection. Mucosal vaccination via the natural portal of entry of the pathogen can activate effector immune cells locally providing potentially protective mucosal responses at the target site as well as systemic immune responses. Conversely parenteral vaccines provoke systemic responses with no or very weak local responses. Other advantages of mucosal administration is that it negates the need for needles, reducing cost, needle-stick injuries and pain, making it easier for mass vaccination (Azegami *et al*., 2014; Lycke, 2012). Mucosal vaccines can be delivered via the oral, intranasal, sublingual, pulmonary (inhalation), genital, rectal or ocular routes (Kim and Jang, 2014). There are currently several licenced mucosal vaccines and most of these are orally delivered (Table 1.1) (Miquel-Clopés *et al*., 2019). Despite their success, mucosal vaccines remain few in number. There are important obstacles to overcome particularly with oral vaccines. Such obstacles include oral tolerance, identifying suitable adjuvants and overcoming the harsh environment of the stomach as discussed later.

Table 1.1- Licenced mucosal vaccines adapted from Miquel-Clopés *et al* (2019).

Pathogen	Trade name	Delivery route	Formulation
Vibrio cholera	Dukoral®	Oral (liquid)	Inactivated (plus recombinant cholera toxin subunit B)
	Schanchol®, Euvichol®	Oral (liquid)	Inactivated
	Vaxchora®	Oral (liquid)	Live attenuated
Influenza type A and B virus	FluMist™	Intranasal (spray)	Live attenuated
Polio virus	Biopolio™ B1/3 and other oral polio vaccine (OPVs)	Oral (liquid)	Live attenuated
Rotavirus	Rotarix® and RotaTeq®	Oral (liquid)	Live attenuated
Salmonella typhimurium	Vivotif®	Oral (capsule)	Live attenuated
Adenovirus	Adenovirus	Oral (Tablets)	Live attenuated

1.5.2 Mucosal immunity

Mucosal immune responses are initiated when antigens from pathogens are encountered in the mucosa-associated lymphoid tissue (MALT). The specialised tissues which make up the different MALTs in the body are gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT), conjunctiva-associated lymphoid tissue (CALT) and the vaginal-associated lymphoid tissue (VALT) (Nizard *et al*., 2014).

The focus here will be on gut mucosal responses comprising the GALT. The most important inductive sites within the GALT are the Peyer's Patches (PPs) and the mesenteric lymph nodes (MLNs) found in the small intestine. The PPs are aggregated

lymphoid follicles surrounded by follicle-associated epithelium (FAE) which are the interface between the gut lumen and GALT (McGhee and Fujihashi, 2012) (Figure 1.5). The mucosal surface of the intestine is composed of a villous epithelium mainly consisting of enterocytes (adsorptive), goblet cells (mucus producing) and paneth cells (anti-microbial peptide producing) in the crypt base. The villous epithelium is covered with mucus which provides a protective barrier against pathogen invasion (Ohno, 2016; Jung *et al*., 2010). The FAE has a reduced number of goblet cells compared to the small intestine epithelium which makes the mucus layer thin and allows interaction with invading pathogens. Another important feature of the FAE is the presence of specialised cells known as M cells (microfold cells) which are involved in antigen or pathogen uptake and are responsible for the initiation of innate and adaptive immune responses (Nakamura *et al*., 2018) (Figure 1.5).

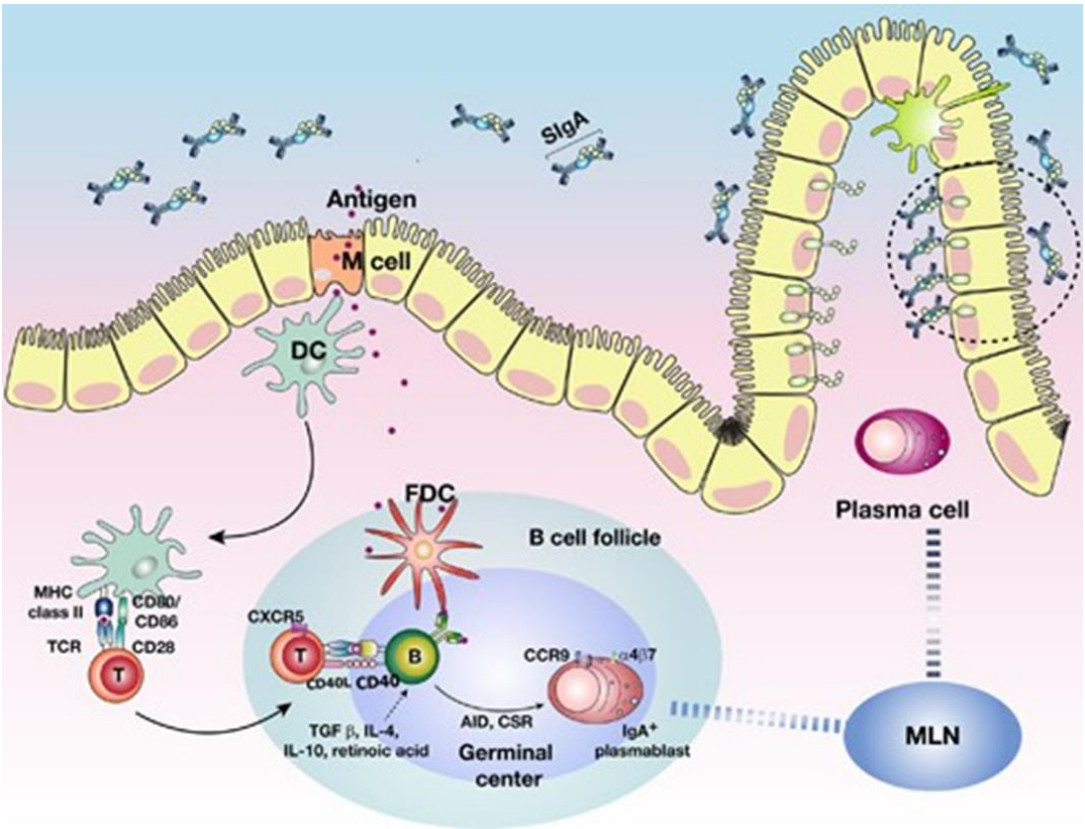

Figure 1.5- Schematic diagram of immune cell induction adapted from Kim and Jang (2014).

Antigens taken up by dendritic cells (DCs) in the Peyer's Patches (PPs) are presented to naïve T cells which become effector $CD4^+$ T cells. $CD4^+$ T cells activate B cells via CD40 ligand binding which allows class switching in B cells. Secretory IgA (sIgA) producing plasma B cells are generated which express receptors α4β7 and CCR9 (gut homing receptors). sIgA producing plasma B cell migrate via the mesenteric lymph nodes (MLNs) to the Lamina Propria.

1.5.2.1 M cells

M cells are distinct from enterocytes found within the FAE in that they lack microvilli and instead have irregular, short-like folds (Figure 1.5) hence the name microfold. In addition to this, M cells also have a pocket-like structure forming the basal plasma membrane which allows interaction with APCs such as DCs, B cells, T cells and macrophages (Kim and Jang, 2017). M cells can take up antigens or pathogens from the lumen via endocytosis, phagocytosis or transcytosis (Azizi *et al.*, 2010). This can

be achieved via receptors present on the apical surface of M cells that bind specific pathogenic ligands (Kim and Jang, 2014) (Table 1.2). Particle size also affects uptake efficiency, with particles 100 nm and above being conducive for uptake by M cells (Miquel-Clopés *et al*., 2019; Williams and Owen, 2015). Once taken up, DCs residing in the pocket of M cells recognise antigens via PRRs such as TLR and process these antigens for presentation by major histocompatibility complex (MHC) class II molecules to naïve $CD4^+$ T cells. DC priming leads to T cell differentiation into T helper (Th) or T regulatory (Treg) cells in the germinal centre (GC) of the PPs. Activated $CD4^+$ T cells subsequently activate and initiate class switching in naïve B cells which results in IgA producing plasma IgA^+ B cells and memory B cells within the GC (Azegami *et al*., 2014; Lamichhane *et al*., 2014). During DC activation of T cells and hence IgA^+ B cells, the expression of gut homing receptors $\alpha_4\beta_7$ integrin (receptor for mucosal vascular addressin cell adhesion molecule-MAdCAM-1) and CCR9 (CC-motif chemokine receptor) (a receptor for CC-motif chemokine ligand CCL25) are enhanced. MAdCAM-1 and CCL25 are expressed on vascular endothelial cells which allows plasma IgA^+ B cells and memory B cells to migrate via the MLNs to the effector site known as the Lamina Propria found beneath the epithelium villous of the intestine (Boyaka and Fujihashi, 2019). In addition, DCs migrate via the MLNs to further activate naïve $CD4^+$ T cells and thus further stimulate IgA^+ B cell production. Secretory IgA (sIgA) produced by plasma cells is translocated across the epithelium and released into the lumen (Figure 1.5) (McGhee and Fujihashi, 2012; Williams and Owen, 2015; Kim and Jang, 2014).

Table 1.2- M cell receptor and binding ligands adapted from Kim and Jang (2014).

Ligand	M cell Receptor
UEA-1	a1,2 Fucose
AAL	a-L-Fucose
Galectin-9	N-glycans/repeated oligosaccharide
Peptide Co1 (SFHQLPARSPLP)	C5aR
Antibody NKM 16-2-4	α1,2 Fucose-containing carbohydrate
Antibody LM112	Sialyl Lewis A
Antibody 3G7-H9	Glycoprotein 2
s1 protein (reovirus)	α2,3 Sialic acid
Invasion (*Yersinia*)	β1 Integrin
Long polar fimbriae (*E. coli*, *Salmonella*)	Unknown
FimH (*E. coli*, *Salmonella*)	Glycoprotein 2/uromodulin
OmpH (*Yersinia*)	C5aR
LPS	TLR4
Lipoteichoic acid	TLR2
Phosphorylcholine moiety of LPS	PAFR
Hsp60 of *Brucella abortus*	Cellular prion protein
Lipid A domain of LPS (Gram-negative bacteria)	AnxA5
Bacterial peptidoglycan	PGLYRP-1
SIgA	Unknown
c-Term domain of enterotoxin (*Clostridium perfringens*)	Claudin 4

Abbreviations 1: AAL, Aleuria auranitia lectin; AnxA5, Annexin A5, LPS: lipopolysaccharide, PAFR: platelet-activating factor receptor, PGLYRP-1: peptidoglycan recognition protein-1, TLR: Toll-like receptor, UEA-1: Ulex europaeus Agglutinin- 1.

1.5.3 sIgA

Mucosal surfaces throughout the human body are highly abundant in sIgA and play an important role in protection from invasion by pathogens. sIgA keeps the gut microbiota healthy by continual surveillance, discriminating between commensal and pathogenic microorganisms and selectively clearing the latter (Corthésy, 2013). Mucosal sIgA exists as a dimeric structure as opposed to serum IgA which is

monomeric (Breedveld and Egmond, 2019). Dimeric IgA produced by plasma B cells in the Lamina Propria are linked together with a J chain and bind to the polymeric immunoglobulin receptor (pIgR) found on the basolateral side of the mucosal epithelium (Figure 1.6).

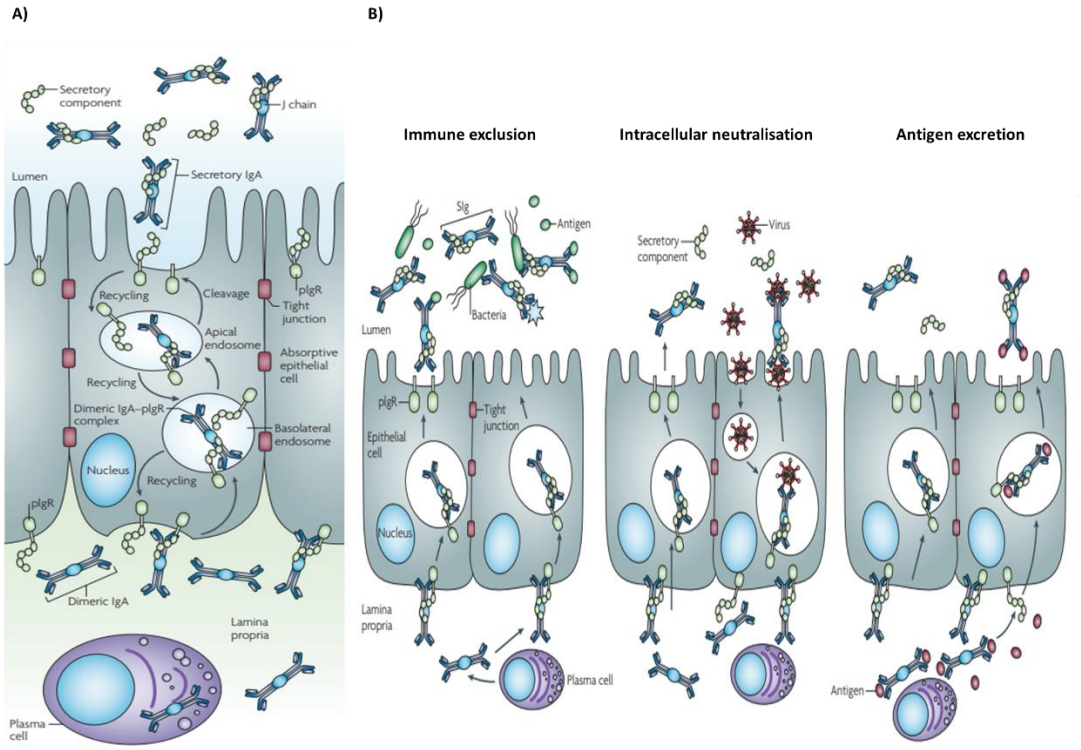

Figure 1.6- Secretion of IgA and function at the mucosal surface within the Lamina Propria adapted from Strugnell and Wijburg, 2010.
A) Secretion of IgA with release of dimeric IgA from plasma cells, linked together via the J chain which specifically binds to the polymeric immunoglobulin receptor (pIgR) on the basolateral side of the endothelial cell. The pIgR-dimeric IgA complex is taken up via endocytosis and the pIgR-IgA complex binds to the apical surface. The pIgR is cleaved such that the secretory component, bound to the dimeric IgA is released via exocytosis into the lumen forming secretory IgA (sIgA). B) Function of sIgA providing protection by immune exclusion where by sIgA binds to the pathogen blocking attachment and therefore invasion, intracellular invasion in which the pIgR-dimeric IgA complex neutralises internalised pathogens and by antigen excretion whereby antigens are removed from the Lamina Propria with the attachment of the pIgR-dimeric IgA complex and excreted back into the lumen.

The pIgR-dimeric IgA complex is taken up by endocytosis and the pIgR attaches to the apical surface of the epithelial cell undergoing cleavage of the portion bound to dimeric IgA known as the secretory component. The secretory component remains bound to the dimeric IgA and ultimately forms sIgA. Thus, sIgA is released into the gut lumen via exocytosis (Figure 1.6, A) (Breedveld and Egmond, 2019; Strugnell and

Wijburg, 2010). sIgA provides mucosal protection by several mechanisms (Figure 1.6, B). One mechanism is immune exclusion in which sIgA transported into the gut lumen blocks pathogen entry, preventing their attachment to epithelial cells and therefore preventing invasion. If the pathogen has become internalised, the endosomal fusion with sIgA destined for secretion to the apical surface can neutralise the pathogen en route (Corthésy, 2013). This is known as intracellular neutralisation. Finally, pathogens that enter the Lamina Propria can be excreted back into the lumen through binding to dimeric IgA which is subsequently transported to the lumen by forming the pIgR-dimeric IgA complex as mentioned above (Figure 1.6, B) (Strugnell and Wijburg, 2010). In addition to the induction of sIgA, it has been shown that activated DCs migrate from the mucosal site to the lymph nodes and spleen for the induction of systemic IgG secretion by plasma cells (Boyaka and Fujihashi, 2019).

A protective sIgA response is expected to occur upon gut mucosal epithelial cells encountering vegetative cells of *C. difficile*. Indeed there is a correlation between increased faecal anti-TcdA sIgA in healthy adults compared to those with recurrent CDI (Warny *et al*., 1994). A higher total sIgA titre in faeces has been shown to correlate with reduced *C. difficile* colonisation in infants (Bridgman *et al*., 2016).

1.5.4 *C. difficile*-directed oral vaccines tested preclinically

Several different mucosal delivery systems carrying different antigens have been tested for their efficacy against *C. difficile*. In 2011, Sandolo *et al*, tested oral administration of Cwp84-encapsulated in pectin beads and showed 40% of hamsters survived in challenge studies. Pectin is a natural plant-based substance and is used to form an outer coating when formulated with zinc and calcium, which is enzymatically

broken down in the colon for targeted release. A similar approach using pectin encapsulation of *C. difficile* was applied to FliC-previously shown to have adjuvant properties (Bruxelle *et al*., 2018). Oral, intrarectal (i.r.) and intraperitoneal (i.p.) deliveries were compared. Oral delivery provided partial protection with 50% of hamsters surviving 18 days post challenge compared to non-immunised control with 17% survival 18 days post challenge (Bruxelle *et al*., 2018). Immunological assays showed greater serum IgG levels for animals immunised via the i.p. route, with no detection of serum IgG in animals vaccinated orally or via the i.r. route. The greater protection seen in hamsters immunised orally is likely due to a local mucosal sIgA response, however, due to the lack of availability of anti-hamster secondary antibodies, detection of sIgA was not possible (Bruxelle *et al*., 2018). Another delivery system tested was *B. subtilis* spores that can out-compete *C. difficile* colonisation. The *B. subtilis* spore expressing TcdA-RBD (TcdA $_{\text{amino acids 2388-2710}}$) fused to *B. subtilis* spore coat proteins on the surface resulted in 75% of hamsters surviving in a challenge study, compared with 0% in the i.m. administration of $TcdA_{2388-2710}$ alone and in the placebo control groups (Hong *et al*., 2017). Similarly, NTCD spores engineered to express a chimeric protein of the RBD of TcdA and the GTD (point mutations in GTD) and APD of TcdB, referred to as NTC_mTcd138 were tested after oral delivery in mice and hamsters (Wang *et al*., 2018). *C. difficile* challenge studies conducted in both models revealed complete protection in mice and about 75% in hamsters with less than 20% survival in both animals that received PBS alone (Wang *et al*., 2018). Another recent approach has been to test engineered attenuated *S. typhimurium* strain YS1646 harbouring a plasmid encoding TcdA_RBD and TcdB_RBD (non-chromosomal integration) (Winter *et al*., 2019). This strategy exploits the ability

of this enteric intracellular organism to secrete heterologous antigens into the host cytoplasm. Immunogenicity studies in mice showed the most effective regimen to be with 1 i.m. injection with RBD of TcdA and TcdB and 3 oral doses of attenuated YS1646 (with plasmid expression of RBD toxins) which resulted in 100% and 82% of animals surviving, with and without the i.m. delivery respectively, and 33% in the PBS only control group (Winter *et al*., 2019).

1.5.5 Challenges with oral vaccination

One of the challenges with oral vaccines is 'oral tolerance'. Antigens are constantly encountered by an individual, from the environment and from the food consumed which results in tolerance to avoid continuous stimulation of the immune system (Mowat *et al*., 2004). Only when there is a strong enough immunogen does an individual elicit an immune response. For this reason, oral vaccines require a potent adjuvant to successfully initiate an immune response and these have been difficult to identify (Lycke, 2012; Pasetti *et al*., 2011). In addition to this, a robust delivery vehicle is essential. The formulation must bypass the harsh conditions associated with the stomach including acidity and enzymatic activity. Often high concentrations of antigen are required to overcome antigen loss encountered in the stomach. Several delivery systems have thus been studied including polymer-based systems, modified liposomes such as bilosomes (virosomes and archeasomes) and transgenic plant based systems expressing target protein (Srivastava *et al*., 2015).

1.6 Liposomes as vaccine delivery systems

Since the discovery of liposomes in the mid-1960 (Bangham *et al*., 1965), they have been exploited as delivery vehicles, particularly for cancer therapy and as

prophylactics with several products now in clinical trials (Bulbake *et al.,* 2017). Approval for use of liposomal delivery was granted in the late 1990's for treatment against ovarian cancer, specifically acquired immunodeficiency syndrome (AIDS)-associated Kaposi's sarcoma (Doxil®) and human immunodeficiency (HIV)-associated Kaposi's sarcoma (DaunoXome®). Other liposomal cancer therapeutics have followed since (Bulbake *et al*., 2017). Advancements in liposomal vaccines against infectious diseases have also been made with the approval of Inflexal®, Epaxal® and Cervarix® against influenza virus, hepatitis A and human papillomavirus (HPV) respectively (Nisini *et al*., 2018). In addition, there are currently several clinical trials underway for liposomal vaccines against malaria, dengue fever, tuberculosis, HIV, influenza and visceral leishmaniasis (Bernasconi *et al*., 2016).

1.6.1 Characteristics of liposomes

Liposomes are spherical vesicles which form a lipid bilayer with an aqueous core. These vesicles are made up of amphiphilic phospholipids composed of a hydrophobic tail (two lipid chains) and a hydrophilic phosphate head group. When dispersed in aqueous solution, the phospholipids self-assemble as a bilayer such that the hydrophilic head groups form the outer and innermost faces in contact with the aqueous environment and the hydrophobic tails face away from the aqueous environment (Figure 1.7). Liposomes are attractive vehicles due to their non-toxic, biodegradable and versatile nature. The most exploited property of liposomes is their ability to encapsulate hydrophilic constituents in their core which provides protection from degradation and therefore safe delivery to the target site. Hydrophilic molecules can also be attached to the liposomal surface via covalent attachment and hydrophobic molecules can be entrapped within the lipid bilayer itself (Figure 1.7)

(Marasini *et al*., 2017). Liposomes can be classified into three types; small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) or multilamellar vesicles (MLV). The choice of phospholipids used in the formulation depends on the delivery route chosen and thus the stability and surface charge required (section 1.6.2). The size of the liposomes is also chosen according to the route of administration and thus target cell.

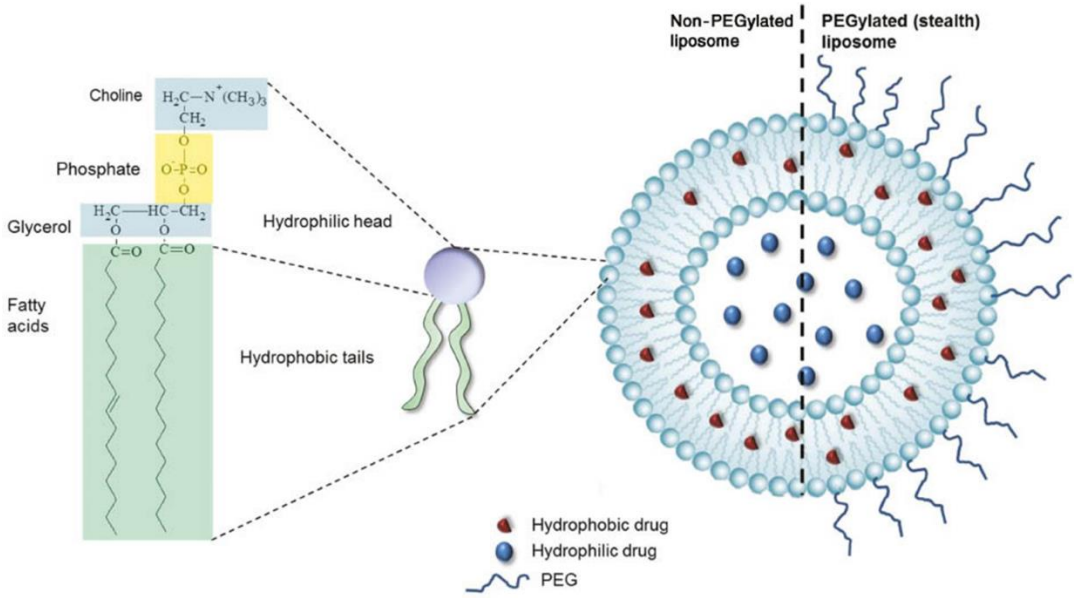

Figure 1.7- Schematic diagram of liposomes and properties exploited adapted from Ahmed *et al* (2016).

Phospholipids form a lipid bilayer with hydrophilic head group (blue) facing towards aqueous environment and hydrophobic tails (green) facing away from aqueous environment. Hydrophilic molecules (dark blue) can be encapsulated in the liposomal core or attached covalently to the liposome surface, for example by using polyethylene glycol (PEG) (blue lines). Hydrophobic compounds can be entrapped in the lipid bilayer (red).

1.6.2 Uptake of liposomes by host cells

Although the mechanism of liposomal uptake is still poorly understood, early studies using transmission electron microscopy (TEM) to analyse the PPs from orally vaccinated rats showed M cell take up of liposomal vesicles (Childers *et al*., 1990). Since then, several studies have focused on ways to target M cells (Kim and Jang, 2014).

Incorporation of specific ligands to not only target M cells but also provide adjuvancy has been explored. Examples include UEU-1 which binds to α-1,2 fucose residues on M cells, TLR agonists such as monophosphoryl lipid A (MPL), *E. coli* heat-labile toxin

(LT) and cytosine-phosphate-guanine (CpG) or even influenza viral proteins such as HINI and also antibodies have been used (Bernasconi *et al.,* 2016; Gupta and Vyas, 2011; Read *et al*., 2005) (Table 1.2). Moreover, each altered factor can have dramatic changes on the liposomal formulation. Fine tuning of liposomal composition is required for mucosal liposomal delivery that could provide stability as well as effective adjuvancy.

Other studies have tested the addition of polysaccharides or polymers to increase the interaction of liposomes with mucosal epithelial cells or compared adjuvants that engage different receptors for enhanced immune responses (He *et al*., 2019). Intranasal delivery of liposomes is preferred compared to oral delivery since it avoids the harsh conditions associated with gut such as the low pH, bile acid and enzymatic digestion (Bernasconi *et al*., 2016). Intranasal delivery is desirable for stimulating an immune response in the NALT, BALT and lower respiratory tract whereas oral delivery initiates immune responses in the gut as well as the NALT (Miquel-Clopés *et al*., 2019). As the mucosal surface is negatively charged, liposomes that are cationic can be favoured, forming electrostatic interactions thereby attaching by charge attraction resisting clearance and increasing the chance of uptake.

1.6.3 Composition of liposomes tailored for stability and immunogenicity

Optimising the composition of lipids of liposomes is vital for achieving stability and adjuvancy. Examples of commonly used lipids include phosphatidylcholine (PC) and phosphatidylserine (PS) as their neutral properties provide biocompatibility (less toxic to host tissues) and cholesterol which has been shown to enhance lipid-bilayer stability (Wang *et al*., 2019). Han *et al* (1997) tested the stability of liposomes *in vitro*

in simulated gut conditions using various ratios of phospholipids including dipalmitoylphophaitdylserine (DPPS), dipalmitoylphoshatidylcholine (DPPC), and diestearoylphosphatidylcholine (DPSC) with or without cholesterol. Liposomes containing DSPC showed good stability as well as liposomes formulated with DPPS, DPPC and cholesterol. When delivered orally in mice, the combination of DPSC, DPPC and cholesterol, and the combination of DPPS, DPPC and, cholesterol showed intestinal sIgA responses against entrapped model antigen ganglioside GM1 compared to other combinations tested. The liposomes containing DPPS, DPPC and cholesterol were also tested orally *in vivo* with the co-entrapment of TLR4 agonist MPL as an adjuvant, which showed enhanced intestinal sIgA responses (Han *et al*., 1997; Watarai *et al*., 1998). Intranasal delivery of cationic liposomes dioleoyltrimethylammoniumpropane (DOTAP) with dimethylaminoethan-carbamoyl (DC)-cholesterol and dimethyldioctadecylammonium bromide (DDA) to entrap ovalbumin (OVA) has also demonstrated mucosal sIgA responses and DC maturation in the nasal mucosa (Corthésy and Bioley, 2018; Tada *et al*., 2015; Yusuf *et al*., 2017).

The inclusion of PEG by covalent attachment to the surface of liposomes is used for parenteral delivery of cancer drugs such as Doxil® and Onvivyde™. PEG is beneficial for shielding of surface-exposed components such as adjuvants, ligands or proteins and also confers stability (Bulbake *et al*., 2017). PEGylation has been exploited in both oral and sublingual formulations and shown to provide resistance to GI degradation. PEGylated liposomes can elicit greater mucosal sIgA responses compared to non-PEGylated (Minato *et al*., 2003). PEGylation has also been shown to have muco-adhesive properties enabling liposomes to be retained more effectively within the small intestine (Iwanaga *et al*., 1999; Minato *et al*., 2003; Oberoi *et al*., 2016). Another

well studied muco-adhesin is chitosan. Chitosan is a positively charged polysaccharide and can be used for coating the surface of liposomes. It has been shown to form electrostatic interactions with mucosal surfaces thereby increasing retention time, and also transiently opening tight junctions within epithelial cells allowing transportation of liposomes (Corthésy and Bioley, 2018; Filipović-Grcić *et al*., 2001; Nguyen *et al*., 2014). The combination of PEGylation and chitosan coating, adjuvanted with CRX-601 (synthetic TLR4 agonist) has been tested by sublingual delivery against influenza virus (Oberoi *et al*., 2016). Higher mucosal sIgA and systemic IgG responses were observed with adjuvanted coated liposomes compared to non-coated liposomes in mice (Oberoi *et al*., 2016).

1.7 Lipoproteins as vaccine candidates

Bacterial lipoproteins have been of interest in recombinant vaccine development owing to their adjuvant properties, specifically as TLR2 agonists. TLRs are abundantly present on DCs and macrophages and play a key role in modulating the innate and adaptive immune responses as mentioned above (Leng *et al*., 2015). The first recombinant lipoprotein vaccine licenced for use was against Lyme disease; the *Borrelia burgdorferi* outer membrane lipoprotein, OspA. Challenge studies in immunised mice showed protection with lipidated OspA unlike its non-lipidated counterpart which failed to protect mice against the disease (Fikrig *et al*., 1990). This vaccine was licenced from 1998 to 2002 then removed from the market due to adverse reactions at the site of injection (Steere *et al*., 1998). Valneva is currently conducting a phase II trial using a multivalent OspA vaccine against several serogroups of *B. burgdorferi* (NCT03769194). The second lipoprotein vaccine licenced

uses the lipoprotein FHbp to target meningococcal serogroup B; Bexsero® (GlaxoSmithKline-GSK) and Tumenba® (Pfizer) (Seib *et al*., 2015). As mentioned earlier, it is known that lipid attachment using analogues with TLR2 agonistic properties could enhance immunogenicity (Chua *et al*., 2012; Moyle *et al*., 2014). Therefore, exploiting *C. difficile* protein antigens with attachment of a suitable synthetic lipid presented on liposomes could provide an attractive delivery platform, mimicking the presentation of lipoprotein antigens found on the bacterial cell wall (Figure 1.8).

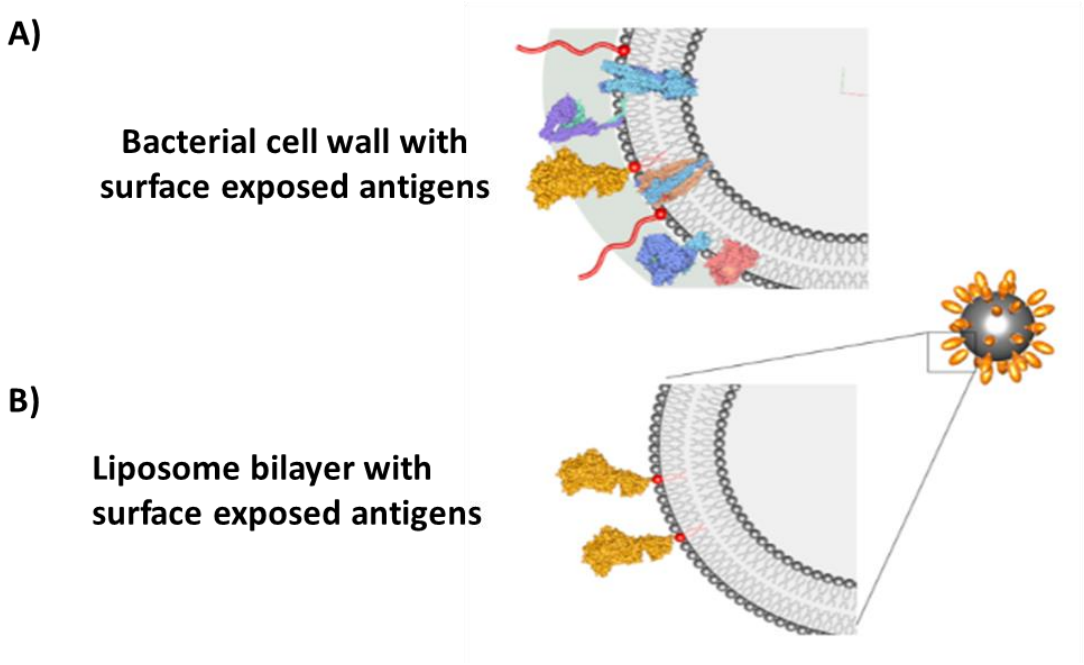

Figure 1.8- Schematic diagram showing presentation of antigens on the bacterial cell wall and surface of liposomes.
Representation of surface exposed protein antigens on A) bacterial cell wall and B) liposomal surface.

1.8 Aims of this project

As previously mentioned, *C. difficile* is the leading cause of hospital acquired infections posing a global threat with an ever increasing number of cases reported for individuals not previously considered to be at risk (Balsells *et al*., 2019; Lessa *et al*., 2012). The major issue associated with CDI is relapse and the difficulties in treating these recurrent cases with available antibiotics. The only way to provide sustained long-term protection against *C. difficile* is to vaccinate. Initial studies testing *C. difficile* patient serum, highlighted the strong association between high anti-TcdA/TcdB IgG titres and clinical recovery with no relapse. On the other hand, patients which failed to mount anti-TcdA/TcdB serum IgG titres, were associated with increased risk of relapse (Aboudola *et al*., 2003; Aronsson *et al*., 1985; Kyne *et al.,* 2001; Leav *et al.,* 2010; Wullt *et al.,* 2012). Various preclinical studies conducted in hamsters or mice have shown the parenteral delivery of toxoids or toxin-fragments to elicit high titres of toxin-neutralising serum IgG and provide varying degrees of protection (Anosova *et al*., 2013; Bruxelle *et al*., 2018; Giannasca and Warny, 2004; Libby *et al.,* 1982).

Hence, parenteral vaccines directed against *C. difficile* have entered clinical trials and are composed of toxoids or toxin-fragments. These have proven to be safe and immunogenic. However, the observed protection in preclinical trials was not observed in human trials as the phase III clinical trial of the Sanofi Pasteur toxoid vaccine was terminated. The failure of the i.m. administration of the Sanofi Pasteur toxoid vaccine to prevent CDI does not bode well for the other parenteral vaccines in trials relying on the same mode of action. The most effective way of immunising

against the gut pathogen, *C. difficile* would be to directly target the small intestine for a local protective sIgA response via the oral route which mimics the natural route of infection.

In this study we set out to develop an oral vaccine against *C. difficile*. Using a panel of vaccine candidates selected from literature involved in different stages of infection, a semi-synthetic lipoprotein approach will be elucidated by attaching recombinant *C. difficile* proteins to a synthetic lipid adjuvant formulated into liposomes as a delivery vehicle. The immunogenicity of both lipidated proteins presented on the liposomal surface and non-lipidated free proteins, encapsulated in enteric coated capsules will be tested for oral delivery *in vivo* using a hamster model. Furthermore, the protective efficacy of vaccine candidates will be tested following a *C. difficile* challenge study in hamsters.

Chapter 2

Material and methods

2 Material and methods

2.1 General microbiological techniques

2.1.1 Bacterial strains and growth conditions

2.1.1.1 Bacterial strains

All bacterial strains used throughout this study are listed in Table 2.1. NEB® 5-alpha competent *E. coli* were used for all cloning procedures and T7 Express competent *E. coli* (High Efficiency), New England BioLabs (NEB, Ipswich, Ma, USA) cells were used for expression of recombinant proteins.

Table 2.1- List of bacterial strains used throughout this study.

Strain	Description	Source
NEB® 5-alpha *E. coli* (High Efficiency)	Cloning host	NEB
T7 Express competent *E. coli* (High Efficiency)	Expression host	NEB
C. difficile strain 630	PCR ribotype 012	Trevor Lawley
C. difficile strain R20291*ermB*	Erythromycin resistant *C. difficile* R20291 (*ermB* gene cloned into genome)	Kelly *et al* (2016)

2.1.1.2 Growth medium and supplements

All media was autoclaved by heating to 121°C at 1 bar pressure for 10 minutes and allowed to cool to 56°C prior to addition of appropriate supplements. All media and supplements used in this study are stated in Table 2.2 and Table 2.3.

Table 2.2- Media for bacterial growth and components used throughout this study.

Media	Components	g/L
Luria-Bertani (LB) broth	Sodium	5
	Tryptone	10
	Yeast extract	10
LB agar	Sodium	5
	Tryptone	10
	Yeast extract	10
	Agar	10
Brain Heart Infusion Supplemented (BHIS) broth	Brain Heart Infusion	37
	Yeast extract	5
	L-cysteine	1
BHIS agar	Brain Heart Infusion	37
	Yeast extract	5
	L-cysteine	1
	Agar	10
Taurocholate-Cefoxitin-Cycloserine-Fructose agar (TCCFA)	*Clostridium difficile* Agar	69
	Yeast extract	5

Table 2.3- Growth medium supplements and antibiotics used throughout this study.

Supplement	Stock concentration mg/ml	Working concentration µg/ml	Solvent
Ampicillin	100	100	dH_2O
Clindamycin	30	-	dH_2O
C. difficile selective supplement:			
D-cycloserine	62.3	250	dH_2O
Cefoxitin	2	8	dH_2O
TCCFA supplement:			
C. difficile selective supplement as above			
Sodium taurocholate	1	10	dH_2O
Amphotericin	0.25	2.5	dH_2O

2.1.1.3 Glycerol stocks

Bacterial cells were stored at -80°C in 15% (v/v) glycerol prepared by mixing 500 µl broth culture with 500 µl of 30% (v/v) sterile glycerol.

2.1.1.4 Aerobic strain growth conditions

E. coli strains were grown overnight at 37°C in LB broth with 200 rpm shaking or on LB agar plates. Where appropriate ampicillin was added into broth cultures or agar plates prior to pouring plates (Table 2.3).

2.1.1.5 Anaerobic growth conditions

C. difficile strains were cultured using reduced BHIS broth or supplemented BHIS agar (Table 2.2 and Table 2.3) at 37°C using an anaerobic workstation (MG1000 Mark II, Don Whitley Scientific Ltd, Bingley, UK) with an atmosphere of CO_2 (10%), H_2 (10%) and N_2 (80%).

2.1.2 *C. difficile* spore preparation

An overnight *C. difficile* culture grown on supplemented BHIS agar, was used to set up a 10 ml inoculum using a 10 µl loop in BHIS broth and incubated anaerobically at 37°C overnight. The culture was diluted 1:5 using BHIS broth and 100 µl were plated onto 5 BHIS plates without supplement. Following a 5 day incubation period, bacterial culture from all 5 plates were scrapped and re-suspended in 1 ml PBS. The suspension was heat shocked at 60°C for 30 minutes for the removal of vegetative cells and then centrifuged at 16, 000 x *g* for 1 minute. The supernatant was discarded and the pellet was washed in 1 ml dH_2O and centrifuged again. This wash step was repeated 3 times. The final pellet was re-suspended in 1 ml dH_2O and stored at -80°C.

2.1.2.1 Quantification of *C. difficile* spores

Frozen stocks of spores were enumerated as colony forming unit per ml (CFU/ml). Spores were serially diluted 1:10 (10^{-1} to 10^{-8}) in PBS and plated onto BHIS or TCCFA plates containing *C. difficile* selective supplement and sodium taurocholate (Table 2.3) to promote germination. Plates were divided into quadrants and 20 µl of each dilution was spotted 3 times in each quadrant and incubated overnight. Dilutions which gave the most separate colonies were used for enumeration.

2.2 Bioinformatics manipulations and tools

2.2.1 Basic Local Alignment Search Tool (BLAST®)

Similarity in nucleotide or protein sequences with available sequenced genomes of *C. difficile* in National Centre for Biotechnology Information (NCBI) was analysed using the BLASTn® or BLASTp® tool; https://blast.ncbi.n.lm.nih.gov/Blast.cgi.

2.2.2 Identifying putative *C. difficile* lipoproteins

Using the refined G+Lppv2 pattern recommended for screening Gram-positive proteomes for lipoproteins, the proteome of *C. difficile* strain 630 from SwissProt was screened in ScanProsite (https://prosite.expasy.org/scanprosite/). From the dataset retrieved, proteins not predicted to be lipoproteins by both DOLOP (https://www.mrc-lmb.cam.ac.uk/genomes/dolop/) and LipoP (http://www.cbs.dtu.dk/services/LipoP/) were discarded as false positives. In order to identify any false negatives, all lipobox consensus sequences from DOLOP were used as query sequences in BLASTp® analysis against the sequenced genome of *C. difficile* strain 630 (section 2.2.1). Matches found near the N-terminus of proteins

were further investigated for the remaining signal peptide. These proteins were validated as above using LipoP and DOLOP (Griffin and Minton, 2017).

2.2.3 Plasmid sequence maps

All plasmid sequence maps were generated using the ApE plasmid editing software or the Benchling online tool available at https://www.benchling.com/.

2.2.4 DNA Oligos

All primers were purchased from Merck (Merck group, Darmstadt, Germany). Primer sequences were manually designed and analysed for secondary structures using the Merck analysis tool available at; https://www.sigmaaldrich.com/pc/ui/tube-home/standard.

2.2.5 Sequence alignments

Sequence alignments were analysed using the Benchling online alignment tool and the Clustal Omega tool for multiple alignments available at https://www.ebi.ac.uk/Tools/msa/clustalo/.

2.3 Genetic manipulations

2.3.1 Gene synthesis

Genes encoding selected *C. difficile* proteins were codon-optimised using ThermoFisher's online tool for optimising expression in *E. coli* available at https://www.thermofisher.com/uk/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis.html. All gene strings were designed and purchased from Invitrogen GeneArt® Gene synthesis (ThermoFisher Scientific, Waltham, MA, USA).

2.3.2 Plasmid DNA extraction

Plasmid DNA extraction was performed using the Monarch® Plasmid Miniprep Kit according to the manufacturer's instructions (NEB) and stored at -20°C.

2.3.3 DNA quantification

Plasmid DNA concentration and purity were measured by NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific).

2.3.4 Gene clean

Gene clean was performed on all polymerase chain reaction (PCR) products, restriction digests and dephosphorylation reactions using the Monarch® PCR & DNA Cleanup Kit (NEB) following the manufacturer's instructions.

2.3.5 Polymerase chain reaction (PCR) and primers

DNA amplification was carried out using Q5® High-Fidelity DNA Polymerase (NEB) for cloning genes and *Taq* DNA Polymerase (NEB) for confirming cloning. The PCR reaction was conducted using the components and conditions shown in Table 2.4 and 2.5 respectively in 50 µl or 25 µl reactions, following the manufacturer's instructions. The reactions were conducted in an Eppendorf® Mastercycler® (Eppendorf, Hamburg, Germany). All primer annealing temperatures were calculated using the NEB Tm calculating tool available at; https://tmcalculator.neb.com/. All primers and annealing temperatures are shown in Table 2.6. PCR products were then purified (section 2.3.4).

Table 2.4- Components of PCR using Q5® High-Fidelity DNA Polymerase and *Taq* DNA Polymerase.

Components	DNA (µl)	No DNA (µl)
Q5® High-fidelity DNA Polymerase		
Q5® HF Reaction Buffer (5X)	10	10
dNTPs (10 mM)	1	1
Forward primer (10 µM)	2.5	2.5
Reverse primer (10 µM)	2.5	2.5
Template DNA (50-100 ng)	1	-
Q5 High-Fidelity DNA polymerase	0.5	0.5
Nuclease free water	**to 50**	**to 50**
***Taq* DNA polymerase**		
10 X standard *Taq* Buffer	2.5	2.5
dNTPs (10 mM)	0.5	0.5
Forward primer (10 µM)	0.5	0.5
Reverse primer (10 µM)	0.5	0.5
Template DNA (50-100 ng)	1	-
Taq DNA polymerase	0.5	0.5
Nuclease-free water	**to 25 µl**	**to 25 µl**

Table 2.5- The conditions used for PCR when using Q5® High-Fidelity DNA Polymerase and *Taq* DNA Polymerase.

	Q5® High-Fidelity DNA Polymerase			***Taq* DNA Polymerase**		
Step	**Temperature (°C)**	**Time**	**Cycle**	**Temperature (°C)**	**Time**	**Cycle**
Initial denaturation	98	30 secs	**1**	95	30 secs	**1**
Denaturation	98	10 secs	**35**	95	30 secs	**35**
Annealing	variable	30 secs		variable	60 secs	
Extension	72	20-30 secs / kb		68	1 min / kb	
Final extension	72	2 min	**1**	68	5 min	**1**

Table 2.6- Primers used to assemble pTWIN1.His constructs with restriction sites underlined.

Primer	Sequence (5'-3')	Restriction Site	Annealing temp. (°C)	Product size (bp)
pET52b For	GGTGGTGGATCCGCTGGTGCCACGCGGT	*Bam*HI	66	71
pET52b Rev	GGTGGTGCTCAGCTTAGTGGTGGTGATGGTG	*Blp*I		
08730 For	GGTGGTTGCTCTTCCAACTGTAGCCAAGGTGGTGATAG	*Sap*I	67	971
08730 Rev	GGTGGTCTGCAGCTCTTGCTTGGTTTTCACGT	*Pst*I		
08760 For	GGTGGTTGCTCTTCCAACTGTTCTCAAAATGATGGCTCCAA	*Sap*I	61	982
08760 Rev	GGTGGTCTGCAGTTTTGCAGATTTTGCATTTT	*Pst*I		
27190 For	GGTGGTTGCTCTTCCAACTGCAGCAACAGCCAGAAT	*Sap*I	54	872
27190 Rev	GGTGGTCTGCAGATATTCCAGCAGCTCAAATTC	*Pst*I		
34640 For	GGTGGTTGCTCTTCCAACTGTCAGAAACGTCAGAGCAC	*Sap*I	64	545
34640 Rev	GGTGGTGGATCCTTGCGATACTCAAAATCTTT	*Bam*HI		
TcdA-RBD For	GGTGGTTGCTCTTCCAACTGTAAAGCAGTTACCGGTTGGCAGACC	*Sap*I	66	1194
TcdA-RBD Rev	GGTGGTGGATCCCCATAAATACCCGGTGCTTTCAC	*Bam*HI		
TcdB-RBD For	GGTGGTTGCTCTTCCAACTGTATTACCGGT	*Sap*I	48	1565
TcdB-RBD Rev	GGTGGTGGATCCTCGCTAATAACCAG	*Bam*HI		
Cwp84 For	GGTGGTTGCTCTTCCAACTGCGAAAATCATAAAACC	*Sap*I	64	2300
Cwp84 Rev	GATCTTGGATCCGTGGTGCTGCCTTTACC	*Bam*HI		
GFP For	GTGGTTGCTCTTCCAACTGC	*Sap*I	60	750
GFP Rev	GGTGGTCTGCAGCTTGTACA	*Pst*I		

2.3.6 Colony PCR

Colony PCR was conducted as a method for quick screening of multiple colonies where necessary without the need for genomic extraction. Briefly, individual colonies were picked using a sterile 1 µl loop and suspended gently in 30 µl of nuclease-free water. The suspension was heated at 95°C for 5 minutes and 1 µl of this used as template DNA to conduct PCR amplification as described in section 2.3.5 using the *Taq* DNA polymerase (NEB) (Table 2.4 and 2.5).

2.3.7 Agarose gel electrophoresis

The size of DNA fragments were verified by electrophoresis using 1% (w/v) agarose gel stained with 0.01% (v/v) SYBR-Safe DNA gel stain (Invitrogen™) in 1X Tris-acetate-EDTA buffer (TAE) (ThermoFisher Scientific). The X-Cell SureLock (Bio-Rad) electrophoresis chamber was used and electrophoresis was conducted at 100 volts for 50 minutes. 1kb Plus DNA ladder (Invitrogen™) and GeneRuler 1 kb Plus DNA ladder (Invitrogen™) were used as a size marker for agarose gels. Verification was performed by loading each gel with a total volume of 6 µl containing 1 µl 6X gel loading dye (NEB) and 5 µl PCR product. DNA loading for gel extractions was carried out in a total volume of 50 µl containing 8.3 µl 6X gel loading dye (NEB) and 50 µl PCR product. All agarose gels were imaged using the Gel Doc™ XR+ with the Image Lab software (BioRad, California, USA).

2.3.8 Restriction digest

Restriction digests of PCR products and plasmid DNA were performed in a total volume of 50 µl containing the components listed in Table 2.7. All enzymes were purchased from NEB. Compatible reaction buffers were selected for each double

digest according to manufacturer's recommendations. All reactions were carried out at 37°C for 2 hours followed by gene clean or gel extraction for plasmid digest as described in section 2.3.4 and 2.3.10.

Table 2.7- Components of a typical restriction digest of DNA.

Components	Volume (µl)
DNA (1 µg)	Variable
NEBuffer (10 X)	5
Restriction enzyme 1	1
Restriction enzyme 2	1
Nuclease-free water	**to 50 µl**

2.3.9 Dephosphorylation of plasmid vector

Plasmid DNA was dephosphorylated following digestion using Calf Intestinal Alkaline Phosphatase (CIAP) (Invitrogen™). The reaction was performed by adding 1 µl CIAP enzyme into the restriction digest reaction and incubating for an additional 5 minutes at 37°C followed by either gene clean or gel extraction (section 2.3.4 and section 2.3.10).

2.3.10 Gel extraction

DNA purification from agarose gel was performed following excision of the appropriate band using the Monarch® DNA Gel Extraction Kit according to the manufacturer's instructions (NEB).

2.3.11 Ligation reactions

In order to determine the concentration of insert required for ligation reactions, the following formula was applied;

$$\text{ng of insert required} = \left(\frac{\text{ng of vector} \times \text{base pair of insert}}{\text{base pair of vector}}\right) \times \text{molar ratio}\left(\frac{\text{insert}}{\text{vector}}\right)$$

All ligation reactions were performed with a constant vector concentration of 25 ng and a molar ratio of 1:6, vector to insert. Each reaction contained the required quantity of insert DNA and plasmid vector, 1 µl T4 DNA ligase (NEB), 1 µl T4 DNA ligase buffer (10X) (NEB) and nuclease-free water to a total volume of 10 µl. Ligations were incubated overnight at 4°C. A vector-only reaction was included alongside all ligation reaction as a negative control.

2.4 Transforming *E. coli*

2.4.1 Making competent *E. coli* cells

Using an overnight *E. coli* culture, 500 µl were used to inoculate 50 ml LB broth containing ampicillin (Table 2.3) in a 250 ml flask and incubated at 37°C with 200 rpm shaking until an optical density at 600 nm wavelength (OD_{600}) 0.2-0.4 was reached. Cells were then transferred to a 50 ml falcon tube and incubated on ice for 30 minutes followed by centrifugation at 1, 600 x *g* for 10 minutes at 4°C. The pellet was gently re-suspended in 20 ml sterile 0.1 M $MgCl_2$ and incubated on ice for 30 minutes followed by further centrifugation. The pellet obtained was re-suspended in 12.5 ml sterile 0.1 M $CaCl_2$ and incubated on ice for 30 minutes followed by centrifugation. The final pellet was re-suspended in 2 ml 0.1 M $CaCl_2$ and stored in 15% (v/v) sterile glycerol in 50 µl volumes at -80°C.

2.4.2 Transforming competent *E. coli*

All ligations were first transformed into NEB® 5-alpha *E. coli* (High Efficiency) (NEB) and once verified, these constructs were transformed into T7 Express competent *E.*

coli (High Efficiency) (NEB) cells for expression. The heat shock method was used for all transformations (Froger and Hall, 2007). In brief, 50 µl of competent cells were thawed on ice followed by the addition of 1-5 µl of ligation reaction and mixed gently. Cells were incubated on ice for 30 minutes followed by heat shock for 30 seconds at 42°C then transferred onto ice immediately. All contents were transferred into a 15 ml falcon tube with 950 µl of LB broth and were left shaking horizontally at 200 rpm for 1 hour at 37°C. Following this, 200 µl of the cell suspension were plated onto LB agar containing ampicillin (Table 2.3). The remaining cells were transferred to a 1.5 ml Eppendorf tube and centrifuged for 3 minutes at 3, 400 x *g*. Most of the supernatant was removed and the remainder used to re-suspend the pellet for plating onto LB agar containing ampicillin (Table 2.3). Plates were incubated overnight at 37°C.

2.4.3 Screening *E. coli* transformants

The transformation plates were assessed for the presence of colonies subject to confirmation of zero growth on the vector-only negative control plates. Single colonies were picked and used to inoculate 10 ml LB broth containing ampicillin. Following overnight incubation at 37°C with 200 rpm shaking, plasmid DNA was extracted as described (section 2.3.2) and quantified (section 2.3.3).

2.4.4 Verifying *E. coli* transformants

Two transformants were selected from each transformation and the expected size base pair (bp) for the insert was verified by PCR amplification (section 2.3.5). pTWIN1.His constructs were verified using *Ssp* DnaB intein forward and His reverse primers (Table 2.8). The *Ssp* DnaB intein primer anneals from 107 bp upstream of the

*Sap*I restriction site and the His reverse primer anneals from 141 bp downstream of the *Bam*HI site. Plasmids were further confirmed by sequencing (DeepSeq, Nottingham University) using the same primers stated in Table 2.8. Internal primers listed in Table 2.8 were used for constructs where full-length sequencing reads were not obtained. All constructs produced are listed in Table 2.9.

Table 2.8- Primers used to verify pTWIN1.His constructs.

Primer name	Sequence (5'-3')	Annealing temp. (°C)
Ssp DnaB intein For	ACTGGGACTCCATCGTTTCT	58
His check seq Rev	ATAGTTCCTCCTTTCAGC	
Internal primers used for sequencing		
Cwp84 CO internal For	CGATAGCGTTGGTGCCAA	57
Cwp84 CO internal Rev	TTGGTCGGAACATTGATA	

Table 2.9- Plasmids constructs used throughout this study.

Plasmid	Description	Source
pET-52b(+)	expression vector /pBR322/T7*lac* promotor/10X His tag	Novagen
pTWIN1	expression vector/pBR322/ T7 promotor/*Ssp* DnaB intein tag	NEB
pTWIN1.His	His tag gene cloned into PTWIN1 vector	This study
pTWIN1-CD630_08760.His	*C. difficile* gene CD630_08760 cloned in pTWIN1.His plasmid	This study
pTWIN1-CD630_08730.His	*C. difficile* gene CD630_08730 cloned in pTWIN1.His plasmid	This study
pTWIN1-CD630_27190.His	*C. difficile* gene CD630_27190 cloned in pTWIN1.His plasmid	This study
pTWIN1-CD630_34640.His	*C. difficile* gene CD630_34640 cloned in pTWIN1.His plasmid	This study
pTWIN1-CD630_TcdA-RBD.His	*C. difficile* gene CD630_TcdA-RBD cloned in pTWIN1.His plasmid	This study
pTWIN1-CD630_TcdB-RBD.His	*C. difficile* gene CD630_TcdB-RBD cloned in pTWIN1.His plasmid	This study
pTWIN1-CD630_cwp84.His	*C. difficile* gene CD630_cwp84 cloned in pTWIN1.His plasmid	This study
pTWIN1-GFP.His	GFP gene cloned in pTWIN1.His plasmid	This study

2.5 Protein expression and purification of double tagged protein

2.5.1 Protein expression

Expression of double affinity tagged proteins was conducted using the T7 express competent *E. coli* cells (NEB). Using a single colony following an overnight plate incubation (section 2.1.1.4), 10 ml LB broth containing ampicillin was inoculated (Table 2.3) and incubated with 200 rpm shaking at 37°C overnight. The following day, 10 ml of overnight culture was used to inoculate 1 L LB broth containing ampicillin in a sterile 2.5 L flask incubated with shaking at 200 rpm at 37°C until an OD_{600} 0.6-0.7

was reached. The cultures were then induced for protein expression using 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) with an overnight incubation at room temperature with shaking at 200 rpm. Non-induced controls were included in this experiment to distinguish expression of the target protein from native proteins. The cultures were then transferred to 500 ml Nalgene® centrifuge bottles and centrifuged at 5, 000 x *g* for 10 minutes at 4°C.

2.5.2 Protein extraction

Proteins were extracted using a binding buffer composed of 20 mM Tris-HCl, 1 M NaCl and 40 mM imidazole at pH 7.4, which formed the basis for all buffers used throughout the purification process. The proceeding steps were conducted on ice and the bacterial cells were lysed in the following manner. The cell pellets were re-suspended in 100 ml refrigerated binding buffer and sonicated in 40 ml volumes with 10 seconds on and 30 seconds off pulse for 18-20 minutes at 40% amplitude, on ice, using the Fisherbrand™ Q500 sonicator-500 W, 20kHz (ThermoFisher Scientific) with a 13 mm diameter probe tip. Lysed cells were then centrifuged at 19, 000 *x g* for 30 minutes at 4°C. The supernatant containing proteins in the cytosol (soluble fraction) was harvested. In order to check if the protein of interest had formed inclusion bodies or aggregated, the pellet (insoluble fraction) was further re-suspended in 40 ml of binding buffer. A sample of both the soluble fraction and insoluble fraction were analysed by sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) (section 2.5.3).

2.5.3 Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE)

All cell lysates were fractionated using 10% (w/v) Tris-glycine SDS-PAGE gels. The lysate samples were prepared by using 20 µl of 2X SDS sample buffer containing 100 mM Tris-HCl, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 200 mM 1, 4- Dithiothreitol (DTT), mixed with 20 µl cell lysate followed by incubation at 95°C for 5 minutes. Each lysate including the non-induced negative control was loaded in a 10 µl volume along with 3 µl Colour Pre-stained Protein standard (NEB) on each gel. Gels were run using 1X Tris-glycine buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS at pH 8.3 in the X-Cell SureLock (Bio-Rad) tank at 120 volts for 1 hour and 30 minutes. The protein gels were then Coomassie blue stained using 1% (w/v) Coomassie brilliant blue, 50% (v/v) methanol and 40% (v/v) diH_2O with incubation at room temperature for 20 minutes and gently shaking. This was followed by incubation with a destain solution containing 50% (v/v) methanol, 10% (v/v) glacial acetic acid and 40% (v/v) diH_2O for 1 hour with further removal of Coomassie blue stain by incubating the gels overnight in diH_2O. Gels were imaged using the Gel Doc™ XR+ with the Image Lab software (Bio-Rad).

2.5.4 Immobilized metal affinity chromatography (IMAC) for purification of soluble recombinant His-tagged proteins

Following verification of expression, crude cell lysates were passed through a Ni^{2+}-charged affinity column using the nickel-nitrilotriacetic acid HisPur™ Ni-NTA Resin (Thermo Scientific, Rockford, USA) and all steps performed at room temperature. Binding buffer was used for all wash steps. The target protein was eluted using increasing concentrations of imidazole elution buffers containing 0.1 M Tris-HCl and 2.5 M NaCl with 50 mM, 100 mM, 250 mM and 500 mM imidazole. Purification steps

were as follows. In a PD-10 column (GE Healthcare Life Sciences), 3 ml of resin slurry was loaded obtaining a 1.5 ml bed volume of resin. The resin was washed with 5X bed volume of dH_2O followed by the same amount of binding buffer. Using a 10 ml volume, lysates were loaded onto the resin and the flow-through collected. The resin was then washed with 20X bed volume with binding buffer for the removal of non-bound proteins and the flow-through collected. The protein was then eluted using increasing concentrations of imidazole; 50 mM, 100 mM, 250 mM and 500 mM. All eluates were collected and fractionated by SDS-PAGE (section 2.5.3). All elutions containing protein were combined and dialysed in PBS wit gently stir overnight for downstream applications using the Biodesign™ Cellulose Dialysis Tubing strip with a 14 kDa molecular weight cut off (MWCO) (Fischer Scientific, Leicestershire, UK).

2.5.5 On column solubilisation and purification of recombinant His-tagged proteins using IMAC

On column solubilisation was performed for insoluble proteins found within cell lysates pellets (section 2.5.2.). The binding buffer containing 20 mM Tris-HCl, 1 M NaCl and 40 mM imidazole, pH 7.4 was used throughout with decreasing concentrations of urea. The insoluble pellet was re-suspended in breaking buffer containing binding buffer with 6 M guanidine-HCl and incubated by stirring at 100 rpm for 1 hour at 4°C. This was then centrifuged at 19, 000 x *g* for 30 minutes at 4°C. The supernatant was loaded onto nickel-nitrilotriacetic acid HisPur™ Ni-NTA Resin (Thermo Scientific) columns prepared, washed and equilibrated in breaking buffer as described in section 2.5.4. The column was flushed through with 20X bed volume washes of binding buffer containing 6 M, 4 M, 2 M urea and 1 mM reduced and 0.1 mM oxidized glutathione respectively. The protein was then eluted in increasing

concentrations of imidazole and visualised using 10% (w/v) Tris-glycine SDS-PAGE gels (section 2.5.3 and 2.5.4).

2.5.6 Protein concentration determination using Bicinchoninic acid assay (BCA)

Protein concentration was determined using the Pierce™ BCA protein assay kit (ThermoFisher Scientific), following manufacturer's instructions. Purified bovine serum albumin (BSA) standards ranging from 200-2000 µg/ml were prepared in PBS. Protein was serially diluted 2-fold (1:2-1:32) in PBS to ensure detection. A total of 25 µl of each sample, standards and PBS control were dispensed in duplicates into a 96-well plate Costar™ (ThermoFisher Scientific). BCA reagents A and B were pre-mixed using a ratio of 50:1 and added to each well in a total volume of 200 µl. The plate was briefly mixed using the plate shake setting and incubated at 37°C for 30 minutes. Absorbance values were measured at 562 nm wavelength using the CLARIOstar^{Plus} (BMG LABTECH, Aylesbury, UK) plate reader.

2.5.7 Western immunoblotting and whole cell Immuno-dot blotting

Tris-glycine SDS-PAGE gel (section 2.5.3) was transferred to the pre-cut blotting transfer pack (Trans-Blot® Turbo™ Mini PVDF Transfer Packs) (Bio-Rad) and positioned directly above the polyvinylidene fluoride (PVDF) membrane in between two stacks of wet filter paper. The transfer was conducted using the Trans-Blot® Turbo™ Transfer system (Bio-Rad) at 25 volts for 7 minutes. All following incubations and wash steps were conducted with gentle shaking at 50 rpm. Once, transferred the membrane was blocked for 1 hour at room temperature in 5% (w/v) dry-milk dissolved in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBST). The membrane was then incubated overnight at 4°C with the primary antibody diluted in

1% (w/v) dry- milk in TBST. The membrane was then washed for 2 hours at room temperature in TBST with solution changes every 20 minutes. This was followed by a 2 hour incubation at room temperature with HRP-conjugated secondary antibody diluted in 1% (w/v) dry-milk in TBST. The membrane was then washed in TBST for 1 hour with 15 minute interval solution changes at room temperature. Protein bands were detected using 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) and visualised using the Gel Doc™ XR+ with the Image Lab software (Bio-Rad). Whole cell Immuno-dot blot was performed in a similar manner with 5 µl of whole cell suspensions spotted onto Nitrocellulose membrane then Western immunoblotting process from the blocking stage conducted as described above.

2.5.8 Microarray Enzyme-linked immunosorbent assay (ELISA)

Recombinant proteins were tested for their binding to anti-human IgG antibody and anti-human IgA antibody using pooled *C. difficile* patient serum (n=20). Microarray ELISA was performed with the assistance of Patrick Tighe (University of Nottingham, Life Sciences department). Antigens were diluted to a concentration of 50 µg/ml in 2-3 butainediol-betaine (1 M) printing buffer in a 384-well plate. All antigens were printed onto an epoxy silane-coated glass slide in replicates (n=6) using the BioRobotics MicroGrid II arrayer (MicroGrid 610, Digilab, Malborough, MA, USA). All wash steps were performed at room temperature using the Precision™ XS Microplate Sample Processor (Biotex, Vermont, USA) in which samples were prepared in a 96-well plate and processed as stated in Table 2.10. Slides were then scanned using the Odyssey Imaging system (LI-COR, USA) using the Odyssey application software.

Table 2.10- ELISA wash steps performed on microarray printed slides.

	Steps	Substrate	Volume	Time
1	Blocking	The Blocking Solution (CλNDOR)	240 µl	1 hour
2	Washes	PBST (PBS containing 0.05% Tween-20)	240 µl	5 X 30 seconds
3	1º Antibody	Serum diluted in low cross buffer (CλNDOR) (1:100)	240 µl	1 hour
4	Washes	PBST (PBS containing 0.05% Tween-20)	280 µl	5 X 30 seconds
5	2º Antibody	Goat anti-human IgG IRDye® (LI-COR) (1:10 000) and Biotinylated goat anti-human IgA (Invitrogen™) (1:1000) diluted in low cross buffer (CλNDOR)	240 µl	1 hour
6	Washes	PBST (PBS containing 0.05% Tween-20)	240 µl	5 X 30 seconds
7	Streptavidin labelled antibody	IRDye® 800CW Streptavidin (1:20 000) diluted in low cross buffer (CλNDOR)	240 µl	1 hour
8	Washes	PBST (PBS containing 0.05% Tween-20)	240 µl	5 X 30 seconds
9	Biotinylated antibody	Biotinylated Goat Anti-Streptavidin (Vector Laboratories) (1:1000) diluted in low cross buffer (CλNDOR)	240 µl	1 hour
10	Washes	PBST (PBS containing 0.05% Tween-20)	240 µl	5 X 30 seconds
11	Repeat from step 7 and leave slides to dry overnight			

2.6 Liposomal preparations

2.6.1 Preparation of maleimide lipid containing liposomes as a delivery vehicle

Liposome preparation and conjugation of antigens were performed by our collaborators Nicholas Mitchell and Panayiota Palazi (University of Nottingham, Chemistry department). The liposomes were formulated with 25%

dipalmitoylglycerophosphoserine (DPPS) (2.5 µmol), 25% dipalmitoylphosphatidylcholine (DPPC) (2.5 µmol), 40% cholesterol (4 µmol) and 10% of synthetic lipid with a maleimide head group (*N*-(2, 3-bis(hexadecyloxy)propyl)-3-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) propanamide) (Mal lipid) (1 µmol) at a final concentration of 1 mM. The lipids DPPS and DPPC were purchased from Avanti® Polar Lipids (Alabama, US). In brief each lyophilised lipid was dissolved as follow; DPPS=2.5 ml (1:1, chloroform: methanol), DPPC=2.5 ml (chloroform), cholesterol=4 ml (chloroform) and Mal lipid=1 ml (chloroform). All lipids were combined forming a total volume of 10 ml and mixed well using a 25 ml round bottom flask. The solvent was then evaporated off using the Rotavapour® R-114 (Büchi, Suffolk, UK) with the flask immersed midway in the Waterbath B480 (Büchi) at 40°C. The resulting film layer of lipids was further dried in a fume hood, under high vacuum for 4 hours using a liquid nitrogen stream. In order to disperse the lipids and form liposomal particles, the 1 mM film layer was re-suspended in 10 ml PBS and sonicated on ice using a 6 mm tip probe at 30% amplitude with 10 seconds on and 30 seconds off pulse for a total of 30 minutes with the Fisherbrand™ Q500 sonicator -500 W, 20kHz (ThermoFisher Scientific).

2.6.2 Dynamic Light Scattering (DLS)

The size of liposomal particles and their polydispersity were measured using the Zeitasizer Nano-ZS (Malvern Panalytics, Malvern, UK). Four measurements were taken for each sample using 1 ml of liposomal suspension in a glass cuvette with square aperture at 25°C. The liposomal refractive index was set at 1.45 with absorbance at 0. 100 and PBS viscosity at 1.330 cP. Particle size range of 100-300 nm was deemed acceptable for use *in vivo*.

2.6.3 Protein attachment to Mal lipid containing liposomes

The protein of interest was attached to the surface of liposomes via conjugation of the N-terminal cysteine to exposed Mal lipid formulated with the liposomal lipids. To minimise the volume and force conjugation, both the liposomal suspension and protein were concentrated down to approximately 1.5 ml using the Vivaspin® 20; 50 kDa and 10 kDa (MWCO) columns (GE Healthcare Life Sciences) respectively. In a 15 ml falcon tube, conjugation was conducted by incubating the liposomal suspension and protein using a 2:7.6 or 2:13 molar ratio of protein to Mal lipid in the presence of tri-(2-carboxyethly)phosphine (TCEP) (pH 7.5) using a molar ratio of 1:2 protein to TCEP overnight with gentle shaking at 50 rpm.

2.6.4 Size exclusion chromatography (SEC)

In order to remove any unbound protein which had not attached to the liposomal surface and purify liposomes to which protein had successfully conjugated, SEC was performed using the ÄKTA pure device with the Superdex™ 200 Increase 10/30 Prepacked Tricorn™ Column (GE Healthcare Life Sciences). Using a 500 µl injection loop, a flow rate of 0.75 ml/minute was used for equilibration and elution in degassed PBS analysed by the UNICORN software. Elution fractions were further analysed by 10% (w/v) Tris-glycine SDS-PAGE and the protein concentration determined using the Pierce™ BCA protein assay kit (ThermoFisher Scientific) (section 2.5.3 and 2.5.6).

2.6.5 Liposome analysis with Florescence-activated cell sorting (FACS)

Liposomal preparations were analysed using FACS performed with the assistance of David Onion and Nicola Croxall using the Astrios EQ Cell sorter (Beckman Coulter-Life Sciences, Indianapolis, USA) within the Flow Cytometry Facility (University of

Nottingham). A 488 nm laser was used for forward (FSC) and side scatter (SSC) measurements. A total of 400 µl of sample was used and data analysis was performed using the Kaluza Analysis 2.1 software (Beckman Coulter-Life Sciences).

2.6.6 Lyophilisation

All formulations prepared for *in vivo* oral delivery were lyophilised following snap freezing in liquid nitrogen using the FreeZone® Benchtop Freeze dryer 2.5 L 84°C (Labconco, Kansas City, MO, USA) with 0.113 mbar pressure.

2.7 Enteric capsule coating

2.7.1 Capsule packing and dip coating

Gelatin capsules of size 9 (8.4 mm length and 2.7 mm eternal diameter) (Torpac®, Fairfield, USA) were packed using the appropriate funnel, tamper and stand provided by the manufacturer. These capsules were dip coated using the capsule holder provided by the manufacture. Capsules were inserted into the designed holes, holding 6 capsule at any one time. The capsule holder was inverted and dipped into coating material approximately 2/3 of a way and then held upright to air dry for 45 minutes. Once dry, the capsules were inverted to dip the remainder 1/3 in the same manner.

2.7.2 *In vitro* testing of enteric coated capsule dissolution in varying pH

To achieve coating and delivery of the vaccine components *in vivo*, gelatin capsules of size 9 (Torpac®) were selected. In order to obtain release of content in the intestines and prevent complete disruption of the capsules in the stomach, optimal capsule enteric coating was tested. 1 or 2 coats of Eudragit® L100 (Evonik, Essen, Germany) enteric coat variations dissolved in isopropanol combined with or without

triethyl citrate (TEC) and dH_2O, were assessed *in vitro* for dissolution in simulated gastric fluid pH 1.2 (J.T.Baker, Aventor, Allentown, PA, USA) and simulated intestinal fluid (PBS at pH 6.7). Firstly, the capsules were packed with 22 mg of bromophenol blue and glucose powder mixture (1:2.5) to be able to measure the release of content in absorbance. Each capsule was then coated in 1 or 2 coats of each formulation and allowed to dry overnight (section 2.7.1). In duplicates, the enterically coated capsules were placed in 5 ml of first gastric fluid for 5 hours and absorbance measurements were taken at 450 nm wavelength using the CLARIOstar^{Plus} (BMG LABTECH) plate reader with the removal of 50 µl every 30 minutes. At the end of 5 hours the capsules were placed in intestinal fluid and the above was repeated at 590 nm wavelength.

2.7.3 *In vivo* testing of enteric coated capsule dissolution in hamsters

The enteric coat formulation, 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O, which revealed the most delayed release in gastric fluid and release in intestinal fluid *in vitro* was also studied *in vivo*. Female Golden Syrian hamsters (n=4), 12-14 weeks old, purchased from Janvier Labs (Le Genest-Saint-Isle, France) were administered with a capsule packed with barium sulphate ($BaSO_4$) and coated with the above formulation. This was used to visualise the movement and the release of contents using Computed Tomography (CT) imaging over a time period of 5 hours. The CT imagining was performed by Jeni Luckett and Michelle Kelly using the BioScan SPECT/CT. Two Luer lock dosing applicators, devise 9 and 9hEC syringes, were tested to ensure gavaging pass the oesophagus to avoid disruption. In brief, 1.5 hours following administration of the capsule, the hamsters were anaesthetised by inhalation using 1.5% (v/v) Isoflurane in 100% oxygen (AB) and the first CT scan was performed. The hamsters were allowed to recover from the anaesthetic using oxygen

and were re-anaesthetised after 1.5 hours to ensure complete recovery and the second CT scan was performed (3 hours post administration). This was repeated in the same manner for the third CT scan (5 hours post administration)

2.7.4 Enteric coating for *in vivo* immunogenicity

The optimal enteric coating, 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O + isopropanol was used for coating capsules containing the vaccine formulations. Each capsule was coated with a single coat using the dipping process mentioned in section 2.7.1.

2.8 *In vivo* immunogenicity and challenge study using hamster

2.8.1 *In vivo* immunisation and sample collection

Female Golden Syrian hamsters (12-14 weeks old) weighing approximately 150 g were purchased from Janvier Labs and used for the *in vivo* immunisation, all housed individually using individually ventilated cages (IVCs). All animal experiments were conducted with the assistance of Michelle Kelly. Each hamster received a total of 3 immunisations, orally gavaged (using the Luer lock dosing applicator, device 9) on day 1, 15 and 30. Hamsters were weighed prior to each immunisation. Hamsters were euthanised using exposure to CO_2 in a gas chamber (3 ml/L) followed by cervical dislocation 14 days post final immunisation. Blood samples were collected via cardiac puncture and faecal pellets were extracted from the large intestines and stored at -80°C. A small portion (~ 2 cm) of small intestine (ileum), large intestine and caecum were removed and fixed in 10 ml of 10 % (v/v) neutral buffered formalin (NBF) (Sigma-Aldrich). The remainder of the small intestine was placed in 5 ml PBS containing 1X SIMGA*FAST*™ protease inhibitor tablet (Sigma-Aldrich).

2.8.1.1 Serum and intestinal fluid preparations

Biological fluids were prepared as described below and stored at -80°C for subsequent use for *in vitro* assays.

Serum:

Blood withdrawn at the experimental end point were collected in 2 ml Eppendorf tubes and allowed to clot overnight at 4°C. The tubes were then centrifuged at 2, 000 x *g* for 10 minutes at 4°C and the supernatant collected and stored at -80°C.

Small intestine:

The small intestine was placed in 5 ml PBS containing 1X SIMGA*FAST*™ protease inhibitor cocktail (Sigma-Aldrich), vortexed for 20 seconds and flushed through twice with 1 ml of the suspension in a 50 ml falcon tube. The suspension was then centrifuged at 2, 500 x *g* for 30 minutes at 4°C. The supernatant was collected and stored at -80°C.

2.8.1.2 ELISA

Anti-IgG levels in serum were detected using indirect ELISA. All incubation steps were performed with gentle shaking at 50 rpm followed by gentle removal of contents and 5 washes with 200 µl PBST (PBS containing 0.01% (v/v) Tween-20). In brief, 96-well Nunc MaxiSorp™ plates (ThermoFischer Scientific) were coated with 100 µl purified recombinant proteins at a concentration of 2.5 µg/ml in 0.2 M sodium bicarbonate, pH 9.4 and incubated overnight at 4°C. Contents were removed and gently tap dried on tissue. Wells were blocked with 200 µl of 5% (w/v) dry-milk in PBST for 2 hours at room temperature. Wells were then incubated overnight at 4°C with 100 µl serum at

1:10 dilution in PBST followed by removal of contents and a wash step. Wells were then incubated for 2 hours at room temperature with 100 µl goat anti-hamster IgG (H +L) highly cross adsorbed-Biotin antibody (Sigma-Aldrich) at a 1: 20 000 dilution in PBST followed by a wash step. This was followed by a 2 hour incubation at room temperature with Strepavidin-HRP (RD Systems-Fisher Scientific) at 1: 200 dilution in PBST and proceeded by a wash step. Absorbance measurements were taken following incubation with 100 µl of TMB (Sigma-Aldrich) for 15 minutes at room temperature at 650 nm wavelength using the CLARIOstar^{Plus} (BMD LABTECH) plate reader. Detection of anti-IgA levels in the faecal and intestinal fluid samples was not possible due to the lack of availability of anti-hamster IgA secondary antibody.

2.8.1.3 Toxin neutralisation assay using Vero cells

Vero cells were maintained in a T25 cell culture flask (Corning®) using 15 ml Dulbecco's Modified Eagles Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) penicillin/streptomycin (Gibco™-ThermoFisher Scientific) at 37°C with 5% CO_2. Cells were passaged 2-3 times weekly and reseeded at a ratio of 1:5. Vero cell counts were conducted by first removing the medium and adding 200 µl 1X trypsin-EDTA (Sigma-Aldrich) to the monolayer of cells to detach the cells from the bottom of the well. Detached cells were harvested and the wells washed with 300 µl PBS and added to the 200 µl of detached cells. Cells counts were determined using a haemocytometer under the Motic AE2000 light microscope and seeded onto a 96-well plate (Corning®) at a density of 1 x 10^5/ml in a volume of 100 µl per well 24 hours prior to the assay. All cell lines were maintained and assays assisted by Jaime Hughes.

The serum and intestinal fluid were filter sterilised using the Costar Spin-X centrifuge tubes (0.22 µm) (Corning®) at 14, 000 x *g*. All dilutions were conducted in DMEM containing 1% (w/v) penicillin/streptomycin. Serum and intestinal fluid were added to a separate 96-well plate in triplicates in a volume of 25 µl with a series of 2-fold dilution in non-supplemented DMEM. Full-length toxin A and toxin B *C. difficile* VPI 1043 strain (FL TcdA and FL TcdB) (Public Health England) was added to each well in a volume of 25 µl to a final concentration of 50 ng/ml and 0.25 ng/ml respectively. The plate was incubated at 37°C with 5% CO_2 for 1 hour. Following removal of DMEM from Vero cells, each serum/intestinal fluid + toxin suspension was added to these cells and incubated for 20 hours at 37°C with 5% CO_2. As controls, cells only and FL TcdA and FL TcdB added to cells were also included. A colorimetric assay was used to detect cell death/cell rounded with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich) added to each well in 50 µl volume at 0.5 mg/ml and incubated for 4 hours 37°C with 5% CO_2 . MTT solution was removed and wells incubated with 75 µl of dimethyl sulfoxide (DMSO) for 10 minutes at room temperature. Absorbance measurements were taken at 570 nm wavelength using the CLARIOstar^{Plus} (BMD LABTECH) plate reader.

2.8.1.4 Adherence blocking assay using Caco-2 cells

Caco-2 cells were maintained and passaged as stated in section 2.8.1.3 using DMEM supplemented with 10% (v/v) FBS and 1% (w/v) penicillin/streptomycin (Gibco™-ThermoFisher Scientific). For the adherence assay, Caco-2 cells were seeded at a density of 1 x 10^5 /ml in a total volume of 500 µl per well in a 24 well plate (Corning®). Cells were left to grow for 14 days and the media changed every 2-3 days. The

medium was also changed 24 hours prior to conducting the assay. Caco-2 cells counts were conducted as mentioned in section 2.8.1.3.

The entirety of the assay was performed under anaerobic conditions at 37°C in the following manner. A 10 ml overnight grown *C. difficile* strain 630 culture was centrifuged at 5, 000 x *g* for 6 minutes and washed once in PBS followed by a repeat centrifugation step. The pellet was re-suspended in serum-free DMEM. For retrospective multiplicity of infection (MOI) determination, 100 µl of the cell suspension was serially diluted 1 in 10 in PBS (10^{-1} to 10^{-7}) and 100 µl of each dilution was plated onto BHIS plates containing *C. difficile* selective supplement (Table 2.3). Fifty µl of serum (1:5) /intestinal fluid (1:2) were added to 50 µl of bacterial cells of the different dilutions and incubated for 1 hour. This mixture was then added to Caco-2 cells following removal of medium and incubated for 2 hours. This suspension was then removed and the Caco-2 cells washed 3 times with 500 µl PBS and then incubated with 200 µl 1X trypsin-EDTA (Sigma-Aldrich). The detached cells were removed and re-suspended in 300 µl DMEM containing 10% (v/v) FBS. This cell suspension was then serially diluted (10^{-1} to 10^{-3}) in PBS and 100 µl plated on BHIS plates supplemented with *C. difficile* selective supplement (Table 2.3) and incubated overnight for CFU/ml counts.

2.8.2 *In vivo* challenge study using *C. difficile* R20291*ermB* strain spores and sample collection

Female Golden Syrian hamsters were immunized as described in section 2.8.1. 14 days post the final immunisation, hamsters were then orally gavaged with clindamycin (30 mg/kg) on day 0, using a feeding needle. On day 5, hamsters were challenged with 100 µl of 1 x 10^3 *C. difficile* strain R20291*ermB* spores prepared as

describe in section 2.1.1 and diluted in PBS (Kelly *et al*., 2016). The disease progression was monitored closely using an approved scoring system (Figure A1) with changes being scored from 1-3 (1-mild and 3-severe). Briefly disease symptoms in the following parameters were monitored: weight loss, observation of wet tail, loose faeces, hunched posture response to stimuli and activity. Animals that reached the humane end point with total scoring of 15 were euthanised and samples including caecum contents were collected as described in section 2.8.1. Daily weight measurements and faecal collections were taken throughout the study. Animals that survived until the experimental endpoint *i.e.*, 14 days post infection were euthanised. All tissue samples collected for histological preparations were processed as stated in section 2.8.1.1 and 2.8.2.4.

2.8.2.1 Faecal and caecum content processing

Faecal pellets and caecal content was processed in order to confirm presence of *C. difficile* infection.

Faecal:

For every 100 mg of faecal pellet, 1 ml PBS was added to 2 ml Precellys® Lysing Kit CK28 tubes and homogenised using the Precellys® 24 tissue homogenizer (Bertin instruments, Montigny-le-Bretonneux, France) for 1 minute and 30 seconds at 3, 400 x *g*. Homogenates were then centrifuged at 7, 000 x *g* for 15 minutes at 4°C. The supernatant was collected and stored at -80°C.

Caecum content:

Caecum was weighed as wet weight and homogenised in the same manner as faecal pellets.

2.8.2.2 Faecal bacterial load

In order to confirm that challenged hamsters were colonised with *C. difficile* strain R20291*ermB* and determine the bacterial load, processed faecal pellets were enumerated as described in section 2.1.2.1 using TCCFA plates (Table 2.2 and 2.3).

2.8.2.3 Confirmation of faecal and cecum content spores

C. difficile colonies detected following spotting of processed faecal pellets onto TCCFA (Table 2.2 and 2.3) plates were confirmed by PCR amplification using the primers specific to the *C. difficile* strain R20291 harbouring the *erm*B Kelly *et al* (2016) (section 2.3.5 and 2.3.6). Where colonies were not detected using faecal homogenates, caecum content homogenates were plated onto TCCFA plates and colonies used for confirmation instead. Colonies were also then confirmed by 16S sequencing via BLASTn® analysis (section 2.2.1) using the following primers stated in Table 2.11.

Table 2.11- Primers used for *C. difficile* strain confirmation.

Primer name	Confirmation	Sequence (5'-3')	Annealing temp. (°C)
cdi-630-pyrD-sF1	*C. difficile* R20291*erm*B	TAGAGAAGGAATAAAAAGTTTAGACGAAATAAGAGG	58
ermB-HindII-R		AAAAAAAAGCTTTTATTTCCTCCCGTTAAATAATAGATAACTATTAAAAATAG	
Univ-0027-F	16S sequencing	GCGAGAGTTTGATCCTGGCTCAG	55
Univ-1492-R		CGCGGTTACCTTGTTACGACTT	

2.8.2.4 Histopathological processing and assessment

All tissue samples were fixed in 10 ml 10% (v/v) NBF overnight at room temperature and then processed by Nottingham University Hospital Trust, Translational Research, Histopathology Department and assessed by Philip Kaye. In brief, samples were processed overnight on a 14 hour programme in a TP1020 Automatic Benchtop Tissue processor (Leica Biosystems, Nussloch, Germany). Samples were then paraffin embedded in the Histostar embedding Workstation (ThermoFischer Scientific) and sectioned at 5 µm, mounted on glass slides and Hematoxylin and Eosin (H&E) stained (Leica ST 5020 Multistainer). Slides were imaged using the Nanozoomer (Hamamatsu Photonics, Japan). Blinded analysis was conducted by an experienced pathologist using an established scoring system (Pawlowski *et al*., 2010). Sections were assessed for oedema (0-3), neutrophil infiltration (0-3) and tissue damage (0-3).

2.8.2.5 Ethics statement

Animal experiments were proceeded using the Experimental Design Assistant (EDA) online tool and the Golden Syrian hamster model was utilised correspondingly with the UK Home Office Inspectorate under the Animals (Scientific Procedures) Act 1986. Approval for experimental procedure was granted by the University of Nottingham Animal Welfare and Ethical Review Body and the UK Home Office under Michelle Kelly's project licence- PPL14712E8BB. Animals were euthanised with CO_2 followed by cervical dislocation.

2.8.2.6 Statistical analysis

All statistical analysis was conducted using the GraphPad Prism 7 software. All data was tested for normality using the D'Agostino & Pearson and the Shapiro-Wilko normality test. A non-parametric Kruskal-Wallis test was performed followed by Dunn's multiple comparison test to compare means of control groups with experimental groups for ELISA and adherence blocking assays. A log-rank Mantel-Cox test was performed for survival percentage analysis and a Mann-Whitney U test was performed for time to end point, percentage weight loss, bacterial spore counts and histopathological scoring.

Chapter 3

Purification of recombinant *C. difficile* vaccine candidates and sero-reactivity screening

3 Purification of recombinant *C. difficile* vaccine candidates and sero-reactivity screening

3.1 Introduction

Live attenuated or inactivated vaccines which relies on using viable whole pathogens with reduced virulence or inactivation are the traditional forms of vaccine technologies (Zaman and Toth, 2013; Kim and Jang, 2017). Live attenuated vaccines can provide a strong immunogenic response with long lasting immunity. However, the unpredictability of host susceptibility to infection or reversibility of virulence is of concern (Lauring *et al*., 2010). Inactivated vaccines reduce the possibility of reverting back to a virulent form as the pathogen is not viable, however this strategy provides a shorter length of immune protection and therefore, may require boosters (Nascimento and Leita, 2012). Subunit vaccines classified as non-live vaccines have emerged as an alternative to use known immunogenic antigens that will not cause the disease such as proteins/recombinant proteins/peptides/polysaccharides involved in virulence using various expression systems such as *E. coli*, yeast, insect cells and mammalian cells (Nascimento and Leita, 2012). This strategy reduces the risks encountered with traditional vaccines, particularly with live attenuated vaccines. Nevertheless, this strategy requires the use of adjuvants to enhance the immune response to provide protection (Lee and Nguyen, 2015). The purpose of adjuvants are to act as a danger signal and trigger an innate immune response hence, amplifying the antigen specific adaptive responses. Examples of different subunit vaccine technologies include recombinant protein vaccines against hepatitis B, meningococcal serogroup B and HPV; conjugate vaccines against influenza,

meningococcal serogroup ACWY/C, pneumococcal; toxoid vaccines against diphtheria, pertussis and tetanus; virus-like particles against hepatitis B, and HPV and outer membrane vesicles against meningococcal serogroup B (Pollard and Bijker, 2021). The crucial step in subunit development is the identification of vaccine candidates that serve as targets. This typically involves selection of immunogenic antigens that play a role in infection, are surface-exposed or secreted by the pathogen and can provide broad coverage of protection against infecting strains.

3.1.1 Identification of candidate vaccine antigens

Inclusion of vaccine candidates involved germination and colonisation, in addition to the major virulence factors TcdA and TcdB could potential enhance vaccine efficacy by preventing early stages of CDI. The *C. difficile* antigens selected as vaccine candidates for this project were chosen based on their role in pathogenesis, putative surface-exposure, and conservation between strains.

1) Colonisation lipoproteins; CD630_08730 has been shown to be a surface-exposed lipoprotein adhesin involved in colonisation (Charlton *et al*., 2015; Karyal *et al*., 2021; Kovacs-Simon *et al*., 2014). It has also been shown to be immunogenic with detection of anti-CD630_08730 IgG, IgA and IgM in *C. difficile* patient serum (Wright *et al.,* 2008). Immunisation via the i.p. route with this lipoprotein was shown to generate a degree of protection from CDI in the mouse model (Bradshaw *et al*., 2019). Therefore, these properties have rendered this lipoprotein as an attractive vaccine candidate. Following *in silico* screening of the *C. difficile* strain 630 proteome, using DOLOP (https://www.mrc-lmb.cam.ac.uk/genomes/dolop/) and LipoP (http://www.cbs.dtu.dk/services/LipoP/) (section 2.2.2), CD630_08760 was

identified as a putative lipoprotein. Given its high sequence identity with CD630_08730 of 70% amino acid identity across the entire protein, it was chosen for this study as it could potentially be immunogenic.

2) Germination lipoproteins; GerS (CD630_34640) involved in germination regulation (Fimlaid *et al*., 2015) and PdaA (CD630_27190), a lipoprotein *N*-deacetylase involved in spore modification. The exact localisation of these lipoproteins are yet to be identified, however they have been selected as vaccine candidates to target the germination stage (Diaz *et al*., 2018; Charlton *et al*., 2015).

3) Toxins; TcdA and TcdB. TcdA and TcdB play a major role in pathogenesis and are secreted making them exposed (Di Bella *et al*., 2016; Voth and Ballard, 2005). Specifically the receptor binding domains (C-terminal domain) were chosen due to their known immunogenicity, TcdA-RBD$_{(\text{amino acids } 1934\text{-}2710)}$ and TcdB-RBD$_{(\text{amino acids } 1852\text{-}2366)}$ (Huang *et al*., 2015; Liu *et al*., 2017; Luo *et al*., 2019).

4) CWP Cwp84; a surface protein, known to be immunogenic and eliciting 40% protection in hamster models was chosen for these properties as a vaccine candidate (Banno *et al.,* 1984; Péchiné *et al*., 2005; Sandolo *et al*., 2013; Savariau-Lacomme *et al*., 2003).

3.1.2 Intein-mediated recombinant protein ligation

In means of using recombinant or full-length proteins as vaccine candidates, attachment of an adjuvant can be achieved via the N- or C-terminal cysteine of a protein. This can be achieved with the use of self-cleavage protein domains known as inteins involved in protein splicing. This process is a 4 step mechanism with an intein situated between N- and C-terminal exteins in protein splicing whereby 1) an acyl

shift at the intein N-terminal cysteine forming a thioester, 2) transesterification by the nucleophilic attack on the thioester by the cysteine of the C-terminal extein, 3) release of the intein by succinimide formation of the C-terminal intein asparagine residue and finally 4) formation of an amide bond between the exteins by an acyl shift (Shi *et al*., 2003; Tarasava and Freisinger, 2014). The commercially available IMPACT™-TWIN (NEB) system allows expression of recombinant proteins with either an N-terminal cysteine and/or a C-terminal reactive thioester. This can be achieved by using the pTWIN1 vector in which the target protein can be cloned in frame with either, an N-terminal intein derived from *Synechocystis sp dnaB* gene (*Ssp* DnaB intein) which can be cleaved upon pH and temperature shift or the C-terminal intein derived from *Mycobacterium xenopi gyrA* gene (*Mxe* GyrA intein) cleaved with the addition of thiols. For the purpose of downstream attachment of a synthetic lipid adjuvant to an N-terminal cysteine of recombinant proteins the pTWIN1 vector was selected for use in this study. The pTWIN1 vector harbouring the N-terminal *Ssp* DnaB intein tag contains a chitin binding domain (CBD) which allows purification of the target protein using chitin beads. The intein tag is cleaved during purification after a shift in temperature (from 4°C to room temperature) and pH (from pH 8 to pH 7), which allows the release of the target protein with an N-terminal cysteine. Once, a pH and temperature shift is induced, the peptide bond between the intein tag and target protein is cleaved. This is caused by the side chain cyclisation of the asparagine residue on the end of the intein forming a succinimide group (IMPACT™-TWIN, NEB) (Figure 3.1).

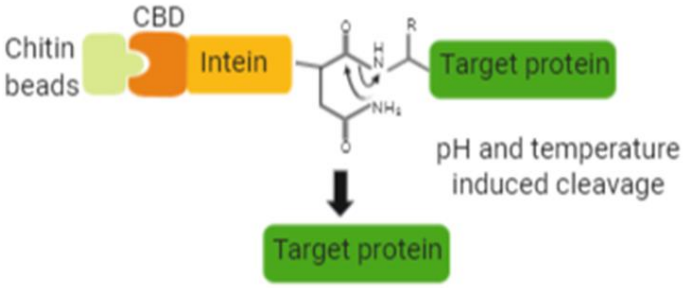

Figure 3.1- Release of target protein from the intein tag with the target protein now bearing an N-terminal cysteine residue.
Intein tag cleavage following a shift in pH and temperature during purification. Cleavage of peptide bond caused by C-terminal asparagine residue side chain cyclisation forming a succinimide group. This causes release the release of the target protein.

3.1.3 Aims of this study

Given that the primary targets of current vaccines in clinical trial against *C. difficile* are the two secreted major virulence factors TcdA and TcdB, inclusion of additional target antigens against germination and colonisation could provide enhanced protection by not only preventing symptomatic disease but also preventing colonisation. As vaccine delivery of recombinant proteins alone may not be sufficient, producing recombinant proteins with an N-terminal cysteine to enable attachment of an adjuvant could enhance the immunogenicity of vaccine candidates as a semi-synthetic lipoprotein approach. The aim of this study was to express and purify *C. difficile* vaccine candidate antigens harbouring an N-terminal cysteine for downstream synthetic lipid adjuvant attachment and test their sero-reactivity to enable selection for *in vivo* testing as oral vaccine candidates. For this, the pTWIN1 vector will be exploited to enable expression of recombinant proteins with a unique N-terminal cysteine and the sero-reactivity of successfully purified recombinant

proteins will be tested using *C. difficile* patient serum and by assessing their binding to anti-human IgG and IgA.

3.2 Analysis of conservation of selected antigens of *C. difficile* strain 630 using BLASTp®

The protein sequence of all antigens selected from the *C. difficile* strain 630 for this study were blasted against sequenced strains of *C. difficile* in the NCBI using BLASTp® (https://blast.ncbi.n.lm.nih.gov/Blast.cgi) (section 2.2.1). The ranges of percent identity and percentage cover was recorded for the top 100 matches for each query sequence with the exception of CD630_34640 which gave 33 matches in total (Table 3.1).

Table 3.1- BLASTp® of selected antigens from *C. difficile* strain 630 against sequenced strains of *C. difficile* strains in the National Centre for Biotechnology Information (NCBI) database. Analysis performed on 15.06.2021.

	Percent identity range with No. of matched sequences shown in brackets (%)						
Query cover (%)	**CD630 08760**	**CD630 08730**	**CD630 27190**	**CD630 34640**	**CD630 TcdA$_{(1934-2710)}$**	**CD630 TcdB$_{(1852-2366}$**	**CD630 Cwp84**
100	100 (36)	90-100 (33)	85-100 (51)	96-100 (42)	99-100 (100)	99-100 (100)	99-100 (100)
90-99	63-72 (48)	67-100 (51)	96-100 (8)	99-100 (24)	-	-	-
80-89	71-100 (5)	70-100 (5)	-	100 (3)	-	-	-
70-79	72-100 (5)	58-100 (5)	49-100 (32)	99-100 (9)	-	-	-
60-69	70-100 (3)	70-100 (4)	-	100 (6)	-	-	
50-59	70-100 (2)	78 (1)	95-98 (3)	98 (3)	-	-	-
40-49	95 (1)	99 (1)	94-100 (5)	98 (3)	-	-	-
30-39	-	-	100 (1)	100 (3)	-	-	-
20-29	-	-	-	98-100 (6)	-	-	-

All selected antigens for this study showed high protein sequence similarity amongst diverse *C. difficile* strains. Importantly all gave hits for clinically relevant ribotypes in which examples include ribotypes 001 (strains; DSM 29745, BI9), 012 (strain DSM 27639), 017 (strain CF5) 027 (strains; E7, R20291, 2007855, DSM 27638/27640, BI1, CD 196, UK1), 078 (strains; TW11, CD21062, M120), 087 (strain VPI 10463) and 106 (DH/NAPI/106/ST-42, BR81) (Groß *et al*., 2018; Zhu *et al*., 2018; Kociolek *et al*., 2018; Spigaglia *et al*., 2015; Stabler *et al*., 2009). This is important to potentially enable

broad protection across diverse strains. As expected CD630_TcdA-RBD, CD630_TcdB-RBD and CD630_cwp84 showed the highest protein sequence conservation. The remainder of the antigens, 60-80% of the sequences that matched, showed between 60-100% identity/similarity over a coverage between 90-100%.

3.3 Adaptation of pTWIN1 plasmid vector

Gene strings encoding all candidate vaccine antigens were cloned into the pTWIN1 vector and expression and purification attempted. However, natural self-cleavage of the intein tag *in vivo* during expression prior to purification resulted in the loss of the CBD within the intein tag and therefore loss of the target protein as the protein could no longer be trapped by chitin beads (Figure A2). To prevent this, a 10X histidine (His) tag (from the pET-52b(+) vector) (Novagen, Merck Group, Darmstadt, Germany) was cloned into the pTWIN1 vector for C-terminal fusion to the target protein. This would allow proteins that had undergone premature cleavage of the intein tag to now be trapped by a Ni^{2+} column (Figure 3.2).

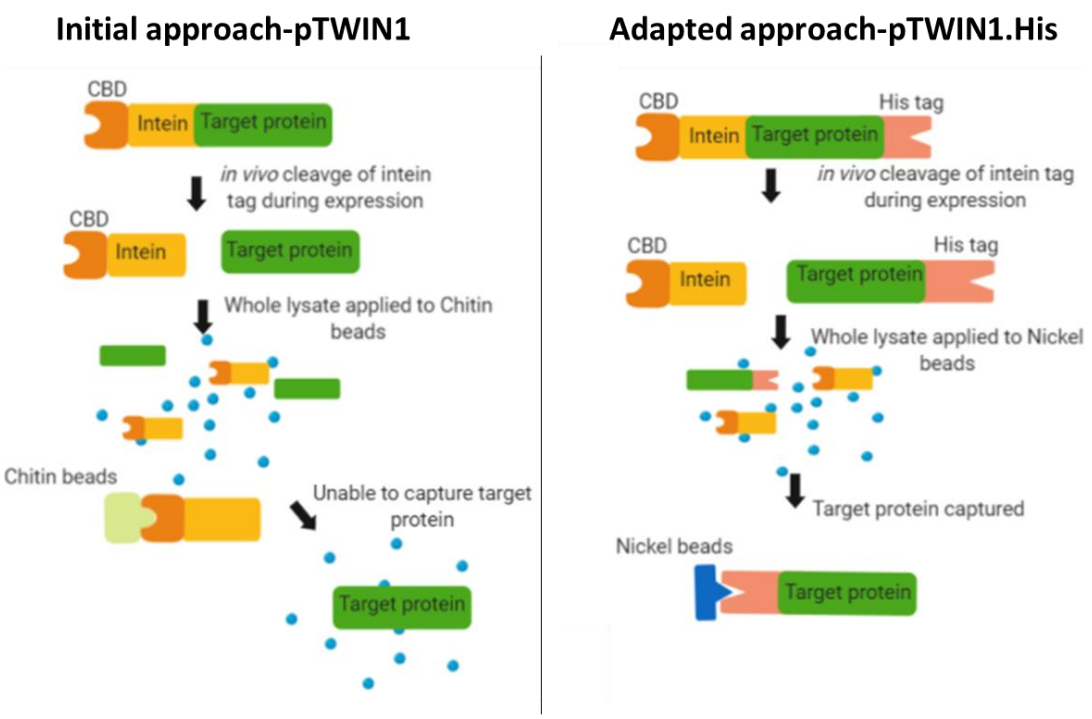

Figure 3.2- Adaptation of the pTWIN1 system.
Left hand-side, the initial approach resulted in the loss of the target proteins during purification due to the natural *in vivo* cleavage of the intein tag. Right hand-side, the adapted approach includes a His tag at the C-terminus of the target protein for successful capture of the target protein.

3.3.1 Adaptation of pTWIN1 to incorporate a His tag

The pET-52b(+) plasmid DNA was used as a template in PCR amplification of the region incorporating the His tag and thrombin cleavage site (section 2.3.5). The forward primer incorporated the *Bam*HI site (GGATCC) and the reverse primer incorporated the *Blp*I (GCTCAGC) followed by a stop codon (Table 2.6). The expected band size of 82 bp was confirmed by agarose gel electrophoresis (section 2.3.7) (Figure 3.3). The PCR product was gel extracted and digested with *Bam*HI and *Blp*I (section 2.3.10 and 2.3.8) and ligated into the *Bam*HI-*Blp*I sites of the pTWIN1 vector (section 2.3.11) (Figure 3.3).

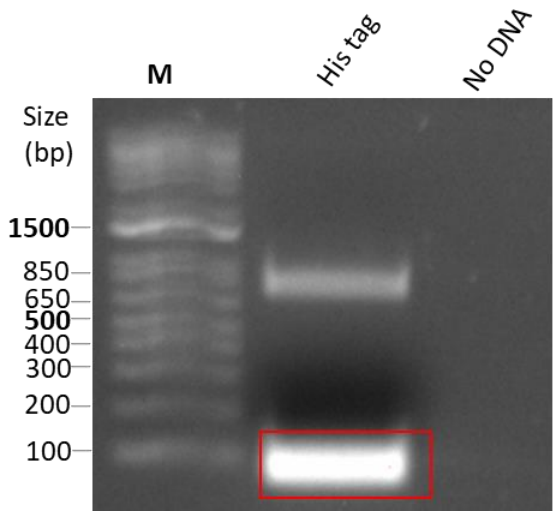

Figure 3.3- PCR amplification of His tag.
The expected band size 82 bp was observed for the His tag PCR amplified from pET52b(+) (highlighted with red border). M: DNA marker 1 kb Plus DNA ladder (Invitrogen™).

Following transformation into NEB® 5-alpha cloning strain (section 2.4.2.), plasmid extraction was performed (section 2.3.2) and clones were verified by DNA sequencing (section 2.4.4) using the primers listed in Table 2.8.

3.3.2 Amplification of genes to be cloned into the pTWIN1.His plasmid vector

Cysteine-free GFP (Suzuki *et al*., 2012) was selected as a prototype protein to optimise the ratio of protein to lipid for liposomal preparations (chapter 4). The DNA encoding each protein was codon-optimised and chemically synthesised with any single cysteine replaced by alanine with the exception of CD630_cwp84 which contained 2 cysteines therefore, both cysteines were retained in order not to prevent protein folding (ThermoFisher Scientific). These gene strings included; CD630_08760, CD630_08730, CD630_27190, CD630_34640, CD630_TcdA-RBD, CD630_TcdB-RBD, CD630_cwp84 and GFP. Each gene string was PCR amplified using the appropriate

forward primer incorporating the *Sap*I site ($GCTCTTCN_1$) followed by a cysteine residue and the reverse primer incorporating either the *Bam*HI (GGATCC) or *Pst*I (CTGCAG) sites as stated in Table 2.6 (section 2.3.5). PCR products were visualised by agarose gel electrophoresis (section 2.3.7), the band of expected size was excised from the gel and gene cleaned or directly gene cleaned (section 2.3.10 and 2.3.4) (Figure 3.4).

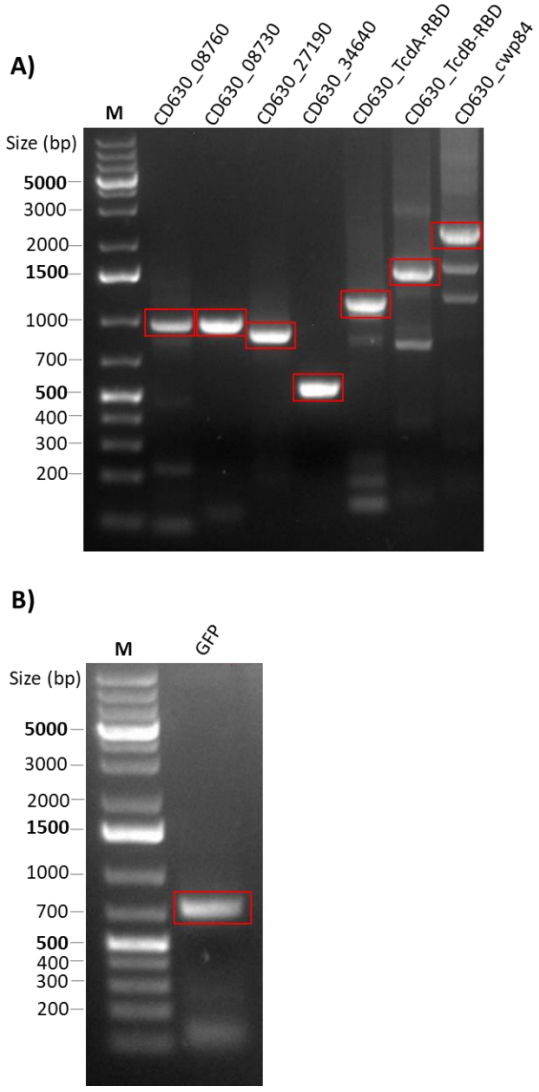

Figure 3.4- PCR amplification of antigen-encoding genes or GFP gene.
PCR product of gene strings A) CD630_08760 (971 bp), CD630_08730 (957 bp), CD630_27190 (879 bp), CD630_34640 (531 bp), CD630_TcdA-RBD (1173 bp), CD630_TcdB-RBD (2286 bp), CD630_cwp84 (2340 bp) and B) GFP (726 bp). M: GeneRuler 1 kb Plus DNA ladder (Invitrogen™). Gel extraction highlighted in red with the expected band observed.

The expected band size for all selected genes was observed by agarose gel electrophoresis and digested as described above for cloning into the pTWIN1.His vector.

3.3.3 Cloning of genes into the pTWIN1.His vector

The PCR products generated were cloned into the pTWIN1.His vector which would enable the gene of interest to harbour an N-terminal *Ssp* DnaB intein tag and a C-terminal His tag. The pTWIN1.His vector was digested with either *Sap*I and *Bam*HI or *Sap*I and *Pst*I (section 2.3.8) followed by dephosphorylation, gel extraction and gene clean (section 2.3.9 and 2.3.10). The gene cleaned PCR products were digested with *Sap*I and *Bam*HI or *Sap*I and *Pst*I (section 2.3.8) and ligated into the digested pTWIN1.His plasmid (section 2.3.11). These constructs (Table 2.9) were transformed into NEB® 5-alpha cells with selection on ampicillin and plasmid extraction was performed on transformants (section 2.3.2 and 2.4.2). An example of a plasmid map of the gene CD630_08730 cloned into pTWIN1.His is shown below (section 2.2.3) (Figure 3.5).

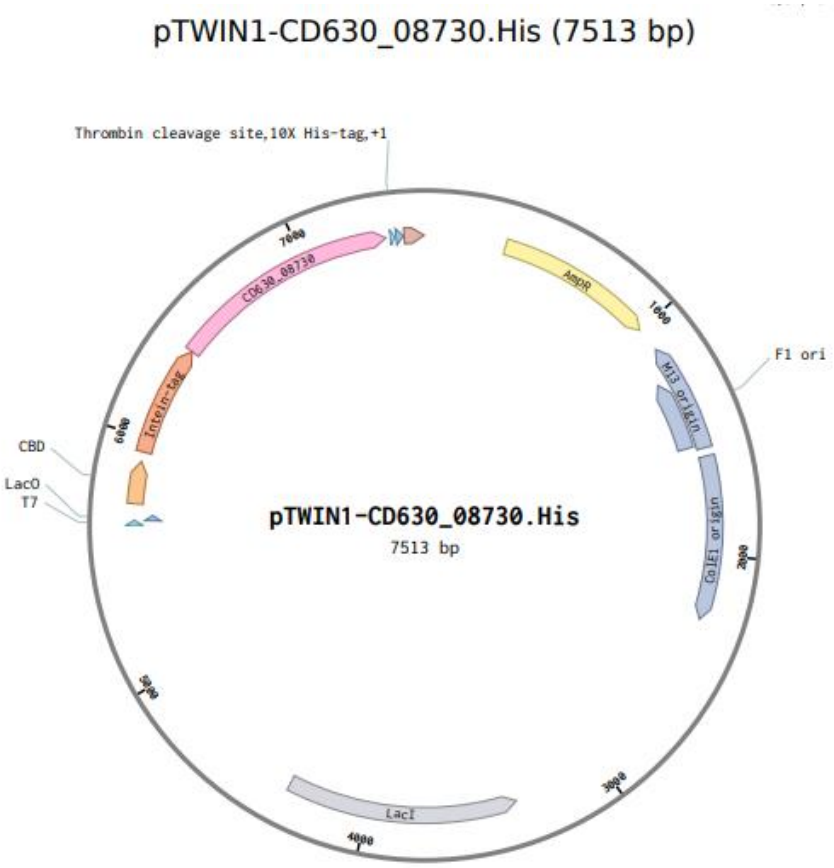

Figure 3.5- Plasmid map showing the pTWIN1-CD630_08730.His construct.
pTWIN1-CD630_08730.His plasmid constructed with the insertion of thrombin cleavage site with a 10X His tag gene followed by a stop codon into the pTWIN1 vector at the *Bam*HI and *Blp*I restriction sites. CD630_08730 gene (with no start codon) was inserted into the pTWIN1.His vector via the *Sap*I and *Bam*HI restriction sites downstream of the intein tag and upstream of the thrombin cleavage site. Gene expression was under the control of T7 promotor and the resistance marker was AmpR.

3.3.4 Verification of cloned double affinity tagged pTWIN1.His constructs

PCR amplification of the constructs was performed using the *Ssp* DnaB intein forward and His reverse primers (Table 2.8). PCR products were visualised by agarose gel electrophoresis (section 2.3.7) and the expected band sizes for each clone were observed (Figure 3.6, A). Each clone was further verified by DNA sequencing (section 2.4.4). Verified constructs were transformed into T7 Express competent cells (NEB)

and further confirmed by PCR amplification using the above primers (section 2.4.2 and 2.3.7) (Figure 3.6, B). All constructs generated are listed in Table 2.9 (Figure 3.6).

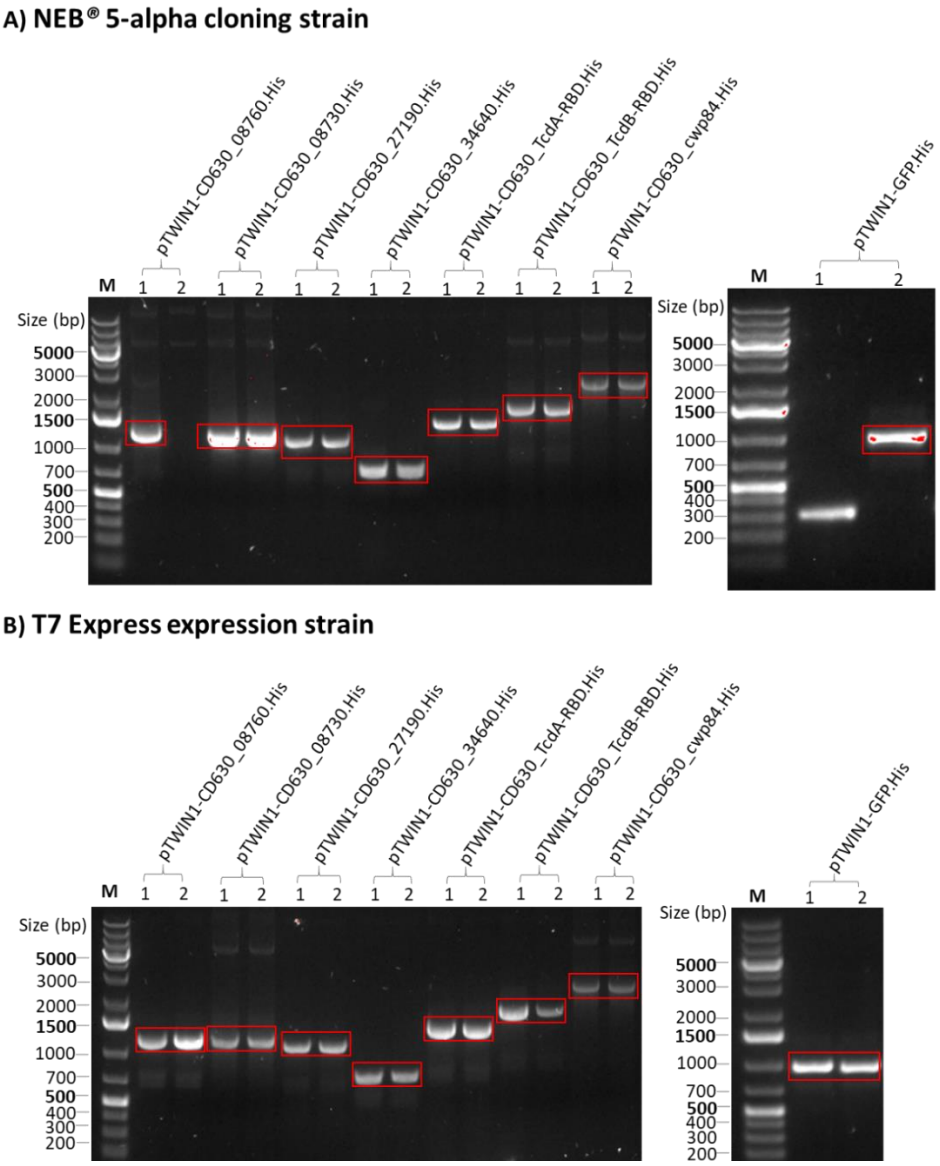

Figure 3.6- Verification pTWIN1.His constructs by PCR amplification.
pTWIN1.His constructs were verified using plasmid DNA of *E. coli* transformants A) NEB® 5-alpha and B) T7 Express cells (NEB). M: GeneRuler 1 kb Plus DNA ladder (Invitrogen™). Expected band size are highlighted in red and as follows; pTWIN1-CD630_08760.His (1224 bp), pTWIN1-CD630_08730.His (1210 bp), pTWIN1-CD630_27190.His (1132 bp), pTWIN1-CD630_34640.His (771 bp), pTWIN1-CD630_TcdA-RBD.His (1414 bp), pTWIN1-CD630_TcdB-RBD.His (1791 bp), pTWIN1-CD630_cwp84 (2580 bp) and pTWIN1-GFP.His (979 bp). Non-highlighted bands or empty wells represent colonies of either self-ligated plasmid vector or unsuccessful PCR amplification respectively.

All transformants were verified by PCR amplification following transformation into the NEB® 5-alpha cloning strain with the selection of two colonies (Figure 3.6, A). The expected band size was observed with at least 1 colony from each transformation. Following transformation of pTWIN1.His and GFP ligations, some colonies contained self-ligated plasmid vector as observed by the lower band (not highlighted in red) (Figure 3.6, A). Transformants that showed the correct expected band size were further confirmed by DNA sequencing with sequence alignments performed using Clustal Omega multiple alignment tool (section 2.2.5) (Appendix 8.2.1). Upon confirmation of successful insertion of each of the above genes into the pTWIN1.His vector, these constructs were transformed into T7 Express cells (NEB) and further confirmed by PCR amplification (Figure 3.6, B).

3.4 Expression and purification of double affinity tagged recombinant proteins

Cell lysates of induced and non-induced T7 Express cells (section 2.5.1 and 2.5.2) harbouring the plasmid of interest were fractionated by 10% (w/v) Tris-glycine SDS-PAGE gels and visualised by Coomassie blue staining (section 2.5.3) (Figure 3.7). The predicted size for recombinant proteins solely bearing a His tag (and not the intein tag) were the sizes observed except for CD630_34640. Specifically, the expected molecular weights (MW) without the intein tag were as follows; CD630_08760 **(37 kDa)**, CD630_08730 **(36 kDa)**, CD630_27190 **(36 kDa)**, CD630_TcdB-RBD **(61 kDa)** and GFP **(29 kDa)** with the exception of CD630_34640 which retained both the intein and His tag **(48 kDa)** (Figure 3.7).

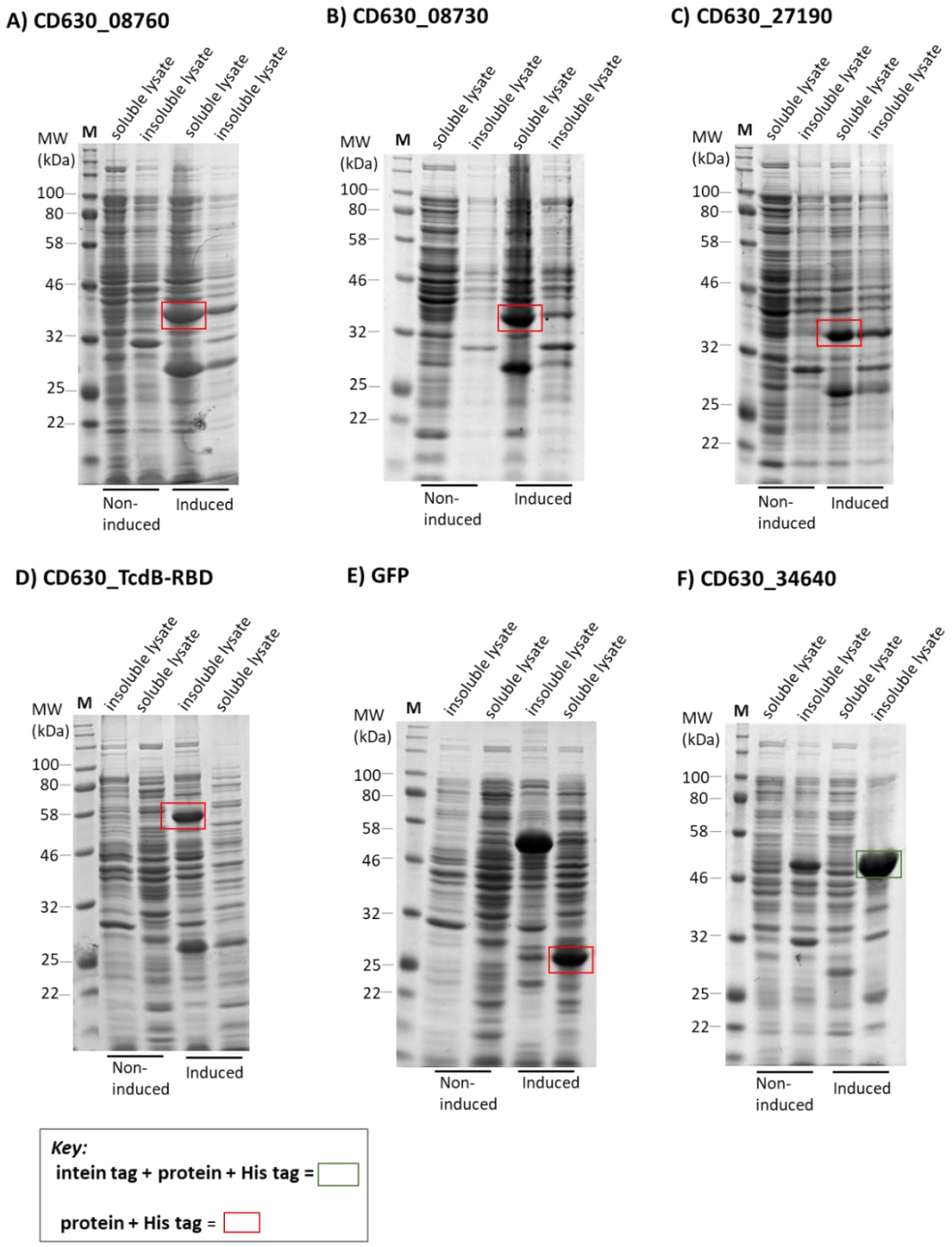

Figure 3.7- Expression of recombinant proteins.

All recombinant proteins were expressed in T7 Express cells (NEB) and induced at room temperature using 0.3 mM IPTG. Non-induced controls were included. Soluble and insoluble fractions were visualised on 10% (w/v) Tris-glycine SDS-PAGE gels stained with Coomassie blue. M: Colour Pre-stained Protein standard (NEB), recombinant proteins bearing the His tag only shown in red box were A) CD630_08760-soluble (37 kDa), B) CD630_08730-soluble (36 kDa), C) CD630_27190-soluble (36 kDa), D) CD630_TcdB-RBD-insoluble (61 kDa), E) GFP-soluble (29 kDa) and F) CD630_34640-insoluble (48 kDa) carrying both the intein and His tag shown in green box.

As seen in Figure 3.7 (A-E), the expression of recombinant proteins CD630_08760, CD60_08730, CD60_27190, CD630_TcdB-RBD and GFP revealed the band size expected for intein-cleaved proteins. For CD630_34640, Figure 3.7 (F), the full-length protein only, containing both the intein and the His tag, was detected in the insoluble fraction.

The pTWIN1-CD630_TcdA-RBD.His and PTWIN1-CD630_cwp84.His constructs were transformed into several *E. coli* expression strains including SHuffle® T7 Express (NEB), NiCo21 (DE3) (NEB) and OverExpress™ C43 (DE3) (Lucigen, Middleton, USA). However, expression of these proteins were unsuccessful or very low in titre in the case of CD630_cwp84.

3.4.1 Protein purification of His tagged recombinant proteins by affinity chromatography

Purification of recombinant proteins CD630_08760, CD630_08730, CD630_27190, and GFP was performed by passing soluble cell lysates from induced cultures through a Ni^{2+} column in binding buffer (20 mM Tris-HCl, 500 mM NaCl and 30 mM imidazole, pH 8) (section 2.5.4). CD630_TcdB-RBD was present in the insoluble fraction and was solubilised in urea after application to the Ni^{2+} column (section 2.5.5). Proteins were eluted in either 250 mM or 500 mM imidazole. Eluates were visualised by 10% (w/v) Tris-glycine SDS-PAGE (section 2.5.3) (Figure 3.8).

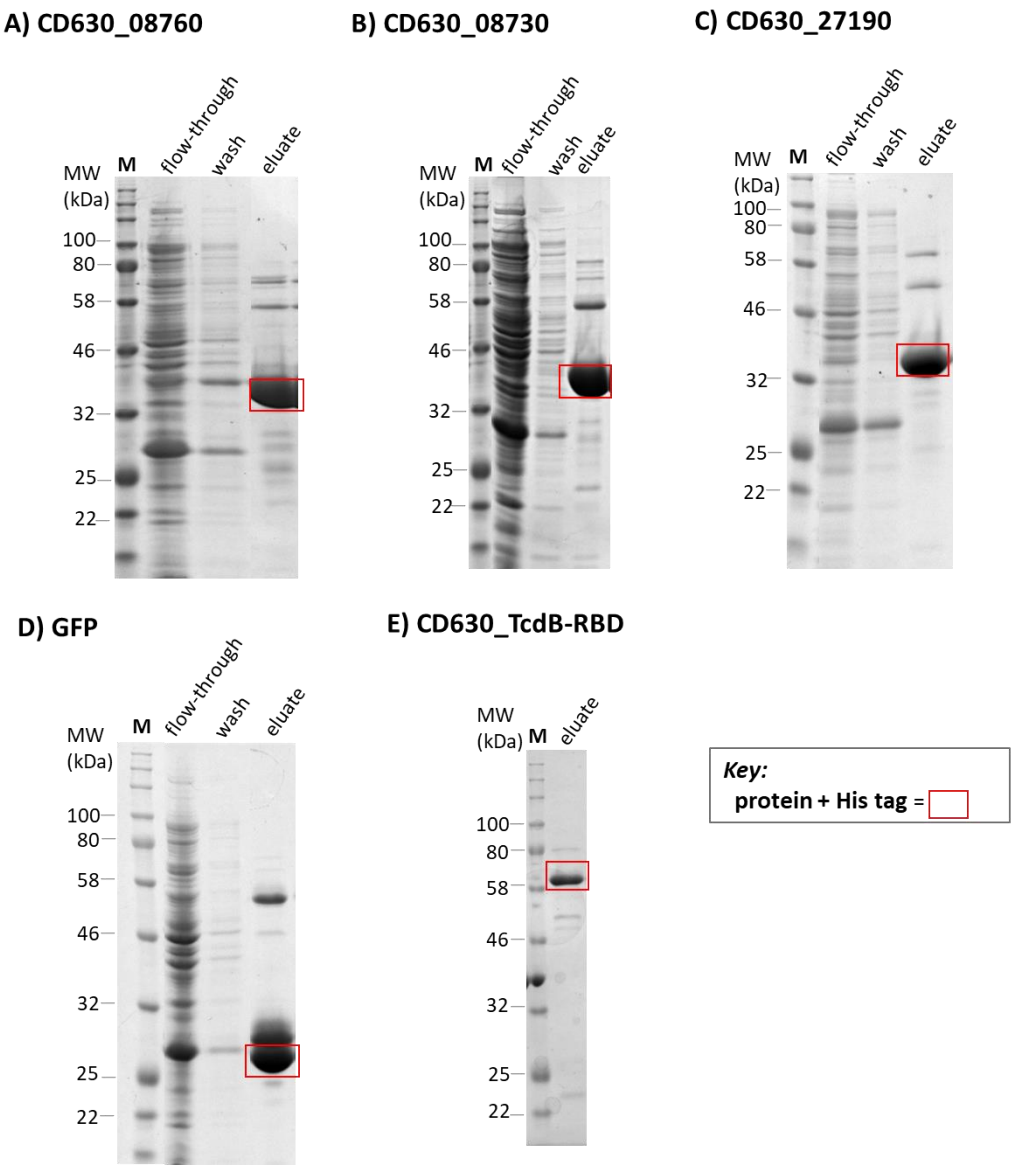

Figure 3.8- Affinity purified recombinant His tagged proteins.
Recombinant proteins were purified using a Ni^{2+} column with HisPur™ Ni-NTA Resin (Thermo Scientific) and eluates visualised by 10% (w/v) Tris-glycine SDS-PAGE with staining by Coomassie blue. M: Colour Pre-stained Protein standard (NEB), purified soluble recombinant proteins with the His tag shown in red box A) CD630_08760, B) CD630_08730, C) CD630_27190, D) GFP and E) on-column solubilised CD630_TcdB-RBD.

The expected sized band for each target protein was observed following affinity purification (Figure 3.8, A-E). Many attempts were made to cleave the intein tag from CD630_3460 without success so further work with this protein was abandoned. The

GFP purification also revealed an additional band directly above the expected band (29 kDa), however this was also observed by Suzuki *et al* (2012). Attempts were made to remove this band via size exclusion chromatography (SEC), however their close sizes made the separation of these proteins difficult.

3.4.2 Sero-reactivity of proteins to *C. difficile* patient serum detected using Microarray ELISA

To test the immunogenicity of the panel of antigens that were successfully purified Microarray ELISA was performed using serum, in collaboration with Patrick Tighe. Full-length (FL) toxins from *C. difficile* strain VPI 10463 (high toxin-producer); FL TcdA and FL TcdB (Public Health England), were included as positive controls and GFP as a negative control (Merrigan *et al*., 2010) (section 2.5.8). The sero-reactivity or binding of the proteins to antigen-specific serum IgG and IgA were detected with goat anti-human IgG IRDye® (LI-COR) used at 1:10 000 dilution and Biotinylated goat anti-human IgA (Invitrogen™) used at 1:1000 dilution respectively. As *C. difficile* antigen specific serum IgG and IgA are observed during CDI infection, (Phillips, 2001; Warny *et al*., 1994), the sero-reactivity of candidate antigens to both was assessed. Protein binding to serum IgG is shown in Figure 3.9 and serum IgA in Figure A3. Six technical replicates were performed for each protein.

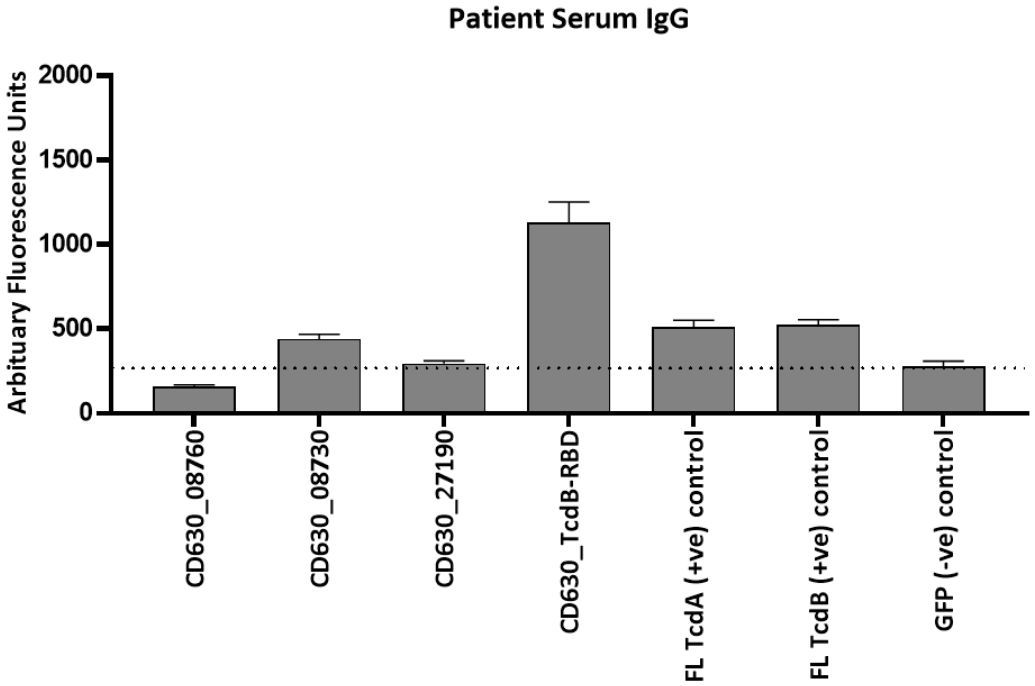

Figure 3.9- Sero-reactivity of purified recombinant protein to pooled *C. difficile* patient serum IgG using Microarray ELISA.
Microarray printing of antigens in replicates of 6 at 50 µg/ml. CD630_08760, CD630_08730, CD630_27190, CD630_TcdB-RBD, full-length *C. difficile* VPI 1043 strain, TcdA and TcdB (FL TcdA and FL TcdB) (positive controls) (Public Health England) and GFP (negative control) were incubated with pooled serum (n=20) from *C. difficile* patients and tested for binding with goat anti-human IgG IRDye® (1:10 000) (LI-COR). Error bars indicating standard error of the mean (SEM). The dotted line represents the baseline detection using GFP as a negative control.

The negative control GFP which is not immunogenic was used to establish the baseline/background level of binding to anti-human IgG. Antigens CD630_08730 and CD630_TcdB-RBD showed higher levels of binding to IgG than GFP. CD630_TcdB-RBD showed the highest level of binding, in particular higher than that of the positive controls (Figure 3.9). This was expected as it has been previously shown that immunisation with the TcdB-RBD can elicit high titres of antibodies (Liu *et al.*, 2017).

Similar results were seen when testing for binding to IgA (Figure A3). Based on the higher binding levels of antigens CD630_08730 and CD630_TcdB-RBD to human IgG by ELISA, these 2 antigens were further analysed by Western immunoblotting.

3.4.3 Confirmation of proteins using Western immunoblotting

Western immunoblotting was performed with the same antigen preparations as those used for Microarray ELISA. CD630_TcdB-RBD and CD630_08730 were loaded onto 10% (w/v) Tris-glycine SDS-PAGE at 50 µg/ml concentration and transferred as described in section 2.5.7. Transferred membranes were first incubated with either rabbit anti-His tag antibody (1:1000) (Cell Signalling Technology®-CST), or rabbit anti-08730 antibody (1:5000) provided by Stephen Michell for CD630_08730 or mouse anti-toxin B antibody (1:1000) (The Native Antigen Company, Oxford, UK) for CD630_TcdB-RBD. This was followed by secondary antibody incubations with anti-rabbit IgG HRP (1:1000) (CST) or anti-mouse IgG HRP (1:1000) (CST) accordingly (Figure 3.10).

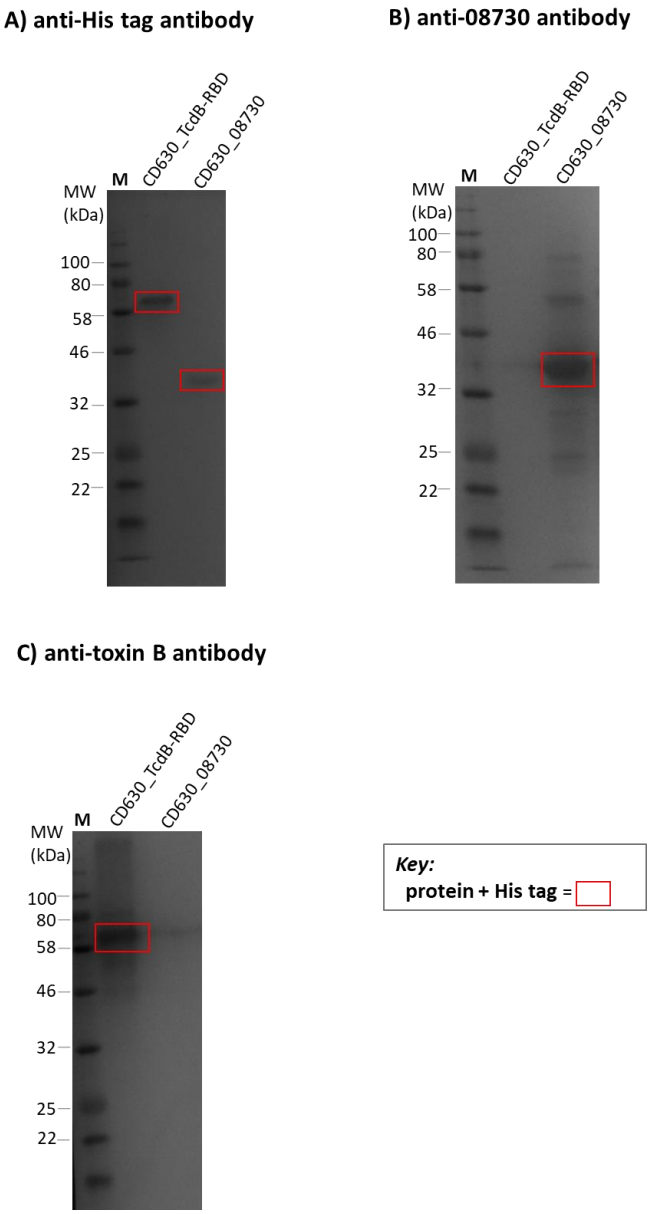

Figure 3.10- Analysis of recombinant proteins CD630_08730 and CD630_TcdB-RBD by Western immunoblotting.

Western immunoblot performed with recombinant proteins CD630_08730 and CD630_TcdB-RBD fractionated by 10% (w/v) Tris-glycine SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. M: Colour Pre-stained Protein standard (NEB), membranes probed with A) rabbit anti-His tag antibody (1:1000) (CST), B) rabbit anti-08730 antibody (1:5000) (Stephen Michell) and C) mouse anti-toxin B antibody (1:1000) (The Native Antigen Company) antibodies. The membranes were then probed with corresponding anti-rabbit IgG HRP (1:1000) (CST) and anti-mouse IgG HRP (1:1000) (CST) antibodies and visualised using 3, 3', 5, 5'-Tetramethylbenzidine (TMB). Recombinant proteins with the His tag shown in red box.

As seen in Figure 3.10 (A-C) bands of the expected MW were observed for each protein probed with the above antibodies.

3.4.4 Increasing the purity of CD630_08730 for larger scale purification for animal studies

One of the impurities previously observed with recombinant CD630_08730 (Figure 3.8, B) was around 60 kDa and likely to be the result of incomplete cleavage of the intein tag. The predicted MW of the full fusion protein (intein tag + CD630_08730 + His tag) is 61 kDa. This impurity was low in abundance. A more prominent impurity was a protein of around 50 kDa. This was suspected to be loss of the CBD from the intein tag as removal of this protein contaminant was not possible when passed through the chitin column. Attempt to minimise the impurities were made by using SEC but this led to a decrease in yield of the target protein. Several attempts were made to increase the intein cleavage and therefore eliminate this higher MW band by adjusting the binding buffer used for the purification as described in section 2.5.2 and 2.5.4. Increasing the concentration of column washes with 1 M NaCl (from 500 mM), 40 mM imidazole (from 30 mM), increasing the volume of washes and adjusting the pH from 8 to 7.4 resulted in improved purity. Since the intein cleavage is promoted by a shift in temperature, purification steps were performed at room temperature following lysis which was performed at 4°C. The purity of the protein was verified by visualising the protein on a 10% (w/v) Tris-glycine SDS-PAGE gel (Figure 3.11).

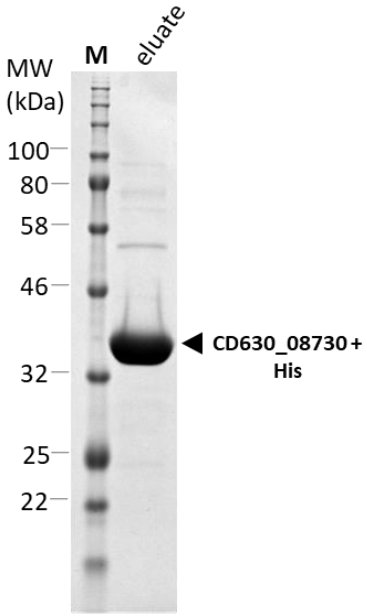

Figure 3.11- Recombinant CD630_08730 from large scale cultures with additional purification.
Impurities were minimised using increased volume of washes with binding buffer containing 20 mM Tris-HCl, 1 M NaCl and 40 mM imidazole at pH 7.4. This was visualised by 10% (w/v) Tris-glycine SDS-PAGE and staining with Coomassie Blue. M: Colour Pre-stained Protein standard (NEB).

Recombinant CD630_TcdB purifications gave sufficient yield and purity and therefore required no further optimisation. Large scale purifications of recombinant proteins CD630_08730 and CD630_TcdB were performed for *in vivo* studies and stored at -20°C.

3.5 Discussion

The pTWIN1 expression system was exploited to produce recombinant protein antigens bearing an N-terminal cysteine. In order to strictly allow downstream attachment of a synthetic lipid adjuvant only to the N-terminal cysteine, all codon-optimised *C. difficile* protein sequences with a single cysteine present in the coding region other than the N-terminal residue, was replaced with alanine. Alanine and

cysteine share a similar small size therefore substitution will have less of an impact on the protein structure or function (Betts and Russell, 2017). However, in the case of CD630_cwp84, the presence of two cysteine residues meant substitution of both with an alanine was not possible in order to not disrupt protein folding. Optimisation of the pTWIN1 vector system was required as initial expression of selected *C. difficile* proteins resulted in premature *in vivo* cleavage of the intein tag (Figure A2). The loss of the intein tag prior to purification meant purification of the target protein using chitin beads was not possible. Although this may have been expected to a small degree, the extent of cleavage was of surprise as the *Ssp* DnaB intein present in the pTWIN1 vector is engineered to lack the N-terminal cysteine to prevent auto-cleavage by a nucleophilic attack and rather be dependent on a shift in pH and temperature with the C-terminal asparagine residue present (Mathys *et al*., 1999). The observed premature *in vivo* cleavage has previously been reported and remains as a limitation of intein mediated purification systems (Cui *et al*., 2006; Du and Rehm, 2017; Qi *et al*., 2019). As the intein tag cleavage is dependent on pH and temperature, attempts were made to minimise this by trying a range of lower and higher induction temperatures with various lengths of duration and by adjusting the pH of the growth medium. In addition to this, various expression strains where also tested using these conditions. Despite all attempts, either complete loss of expression or lower expression titres of the fusion protein harbouring the intein tag was observed. It has been shown that when using the pTXB1 and pTXB2 vectors (IMPACT™) containing the *Mxe* GyrA intein in which cleavage is induced with the presence of thiol reagents, *in vivo* cleavage was influenced by the amino acid residues of the target protein closest to the intein and this may have been the case for this study (Southworth *et al*., 1999).

Furthermore, 20 amino acids were tested and it was observed that none led to *in vivo* cleavage when induced at 37°C although, when induced at 19°C, different levels of *in vivo* cleavage was observed. However, in this study testing induction at 37°C led to no expression at all of the selected *C. difficile* target proteins. It was observed by Southworth *et al* (1990) that serine and glycine residues closest to the N-terminus fused *Mxe* GyrA intein caused *in vivo* cleavage at 19°C. Out of the proteins which presented with *in vivo* cleavage in this study, CD630_08760, CD630_08730, CD630_27190 contain a serine residue proceeding the N-terminal cysteine which might explain the observed *in vivo* cleavage. However, CD630_TcdB-RBD in which the cysteine is proceeded by a threonine also presented with *in vivo* cleavage. Specifically for the *Ssp* DnaB intein, Evans *et al* (1999) observed that a glycine residue present next to the N-terminal cysteine caused *in vivo* cleavage and arginine was found to have prevent this. The only condition that provided high expression titres in this study was with an overnight induction at room temperature which ultimately resulted in *in vivo* cleavage of the intein tag. Hence, the pTWIN1 system was adapted for inclusion of a C-terminal His tag in order to capture and successfully purify the target protein (Figure 3.2). The inclusion of the thrombin cleavage sequence upstream of the His tag allowed for removal of the tag if required. Most importantly, it allowed for purification of recombinant proteins of very high yields. As oral delivery may require high antigen concentrations, this expression system allowed for sufficient yields by minimising the time required for large quantities of protein.

From the panel of antigens chosen for this study the proteins successfully expressed purified were CD630_08760, CD630_08730, CD630_27190, CD630_TcdB-RBD and GFP (Figure 3.8). Unlike the premature *in vivo* intein cleavage encountered, in the

case of CD630_34640, inducing cleavage of the intein tag during purification was unsuccessful. As this protein was found in the insoluble fraction, either insufficient folding following solubilisation or the amino acids closest to the intein may have led to incomplete cleavage of the intein tag (Nanda *et al*., 2020). In the case of CD630_TcdA-RBD and CD630_cwp84, no expression was observed. We speculate that the expression of these proteins are detrimental to *E. coli* and it is likely mutations are selected for that, prevent its expression. However, this was not confirmed. This was unexpected as both have been previously been expressed successfully in *E. coli* (Haung *et al*., 2015; Sandolo *et al*., 2011). This could also be that *in vivo* cleavage of the intein tag is happening very early on in translation which may have led to no expression of these proteins.

Following a microarray ELISA immunogenicity screen of these antigens with pooled *C. difficile* patient serum, the antigens, CD630_08730 and CD630_TcdB-RBD were selected for further study since they showed higher binding levels to IgG compared to the other antigens (Figure 3.9). Similar results were obtained with an ELISA detecting the binding to IgA (Figure A3). The sero-reactivity for CD630_08760 and CD630_27190 showed either lower or similar levels of binding respectively as observed for the negative control GFP. This shows their lack of potential as vaccine candidates indicating the absence of antibodies generated my patients towards these proteins during infection. Although from the ELISA results, CD630_08730 appears to be less immunogenic than CD630_TcdB-RDB, it is an attractive vaccine candidate given its role in colonisation (Kovacs-Simon *et al*., 2014). Generating mucosal antibodies to colonisation factors will potentially block the ability of *C. difficile* to adhere to the gut epithelium allowing the targeting of an early stage of

pathogenesis. As several studies have shown that the RBD of TcdB is immunogenic, combining CD630_08730 with CD630_TcdB-RBD, targets more than one stage of infection (Gardiner *et al*., 2009; Huang *et al*., 2015; Liu *et al*., 2017; Luo *et al*., 2019). Given the large size of the RBD, the C terminal portion was chosen since this was shown to be highly immunogenic (Lui *et al*., 2017).

Following selection of antigens CD630_08730 and CD630_TcdB-RBD for *in vivo* studies, larger scale preparations were made followed by additional purification steps to eliminate impurities. The next goal was to enhance the immunogenicity of CD630_08730 and CD630_TcdB-RBD by exploiting their N-terminal cysteine residue for the attachment of a lipid moiety with known adjuvant activity.

Chapter 4

Protein conjugation to the surface of liposomes using a synthetic lipid adjuvant

4 Protein conjugation to the surface of liposomes using a synthetic lipid adjuvant

4.1 Introduction

Recombinant proteins utilised alone as oral vaccine candidates are typically not sufficiently immunogenic (Fikrig *et al*., 1990). The lipid moieties found on bacterial lipoproteins, Pam2Cys or Pam3Cys are potent TLR2 agonists. Attaching these lipid moieties to recombinant protein antigens endows them with self-adjuvanting properties thus increasing their immunogenicity (Leng *et al*., 2015). Lipidation of heterologous antigens can be achieved recombinantly. For example Chen *et al* (2009) created a fusion of an envelope protein (E3) of the dengue virus to the signal peptide of a *Neisseria meningitis* lipoprotein (Ag473) and expressed this in *E. coli* to permit lipidation *in vivo*. Immunogenicity studies in mice revealed that the lipidated protein generated a stronger IgG response compared with the non-lipidated protein. Using the same genetic engineering approach, the Ag473 signal peptide was fused to the RBD of TcdA and resulted in enhanced immune responses in mice compared to that in mice receiving non-lipidated TcdA-RBD (Huang *et al*., 2015).

Lipidation of heterologous proteins or peptides can also be achieved by a synthetic approach (Huang *et al*., 2015; Jackson *et al*., 2004; Khan *et al*., 2009). Synthetic Pam2Cys and Pam3Cys as covalently attached adjuvants to peptides of Influenza A, Hepatitis C virus and group A *Streptococcus* have proven to enhance their immunogenicity (Chua *et al*., 2012; Day *et al*., 2007; Moyle *et al*., 2014). The common strategy for synthetic lipid attachment to target antigens is to exploit cysteine residues for attachment of the lipid via a thioether linkage (Kowalczyk *et al*., 2017).

Since synthetic peptides have a limited number of epitopes as opposed to whole proteins (Kent, 2008) we took the recombinant approach to produce whole proteins and mimic the native form to include all immunological epitopes, or in the case of the RBD of TcdB, a sizeable portion of this. The antigens were produced with an N-terminal cysteine for conjugation to a synthetic lipid. Synthetic lipid adjuvants; Pam2Cys and Pam3Cys linked to a maleimide group, which readily react with thiol groups within cysteine, have previously been covalently attached to recombinant proteins either using truncated proteins with a native C-terminal cysteine or C-terminal cysteine introduced by site-directed mutagenesis (Bader *et al*., 2000; Moyle *et al*., 2013).

We set out to test whether semi-synthetic lipoproteins could be presented by liposomes as a novel oral vaccine approach. Liposomes of 200-500 nm in diameter were previously shown to be readily taken up by M cells in the gut (Aramaki *et al*., 1993; Cortsév and Bioley, 2018). During uptake by DCs, the lipid should activate TLR2 resulting in enhanced MHC class II presentation of the attached protein. In this study, we utilised a modified version of Pam2Cys such that the ester-bonds were replaced with an ether-bond attached to a glycerol moiety with a maleimide head group, Mal lipid (*N*-(2, 3-bis (hexadecyloxy) propyl)-3-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) propanamide) (Figure 4.1). The ether-bond is considered to be more stable in the gut (Hussein *et al*., 2016; Marasini *et al*., 2017).

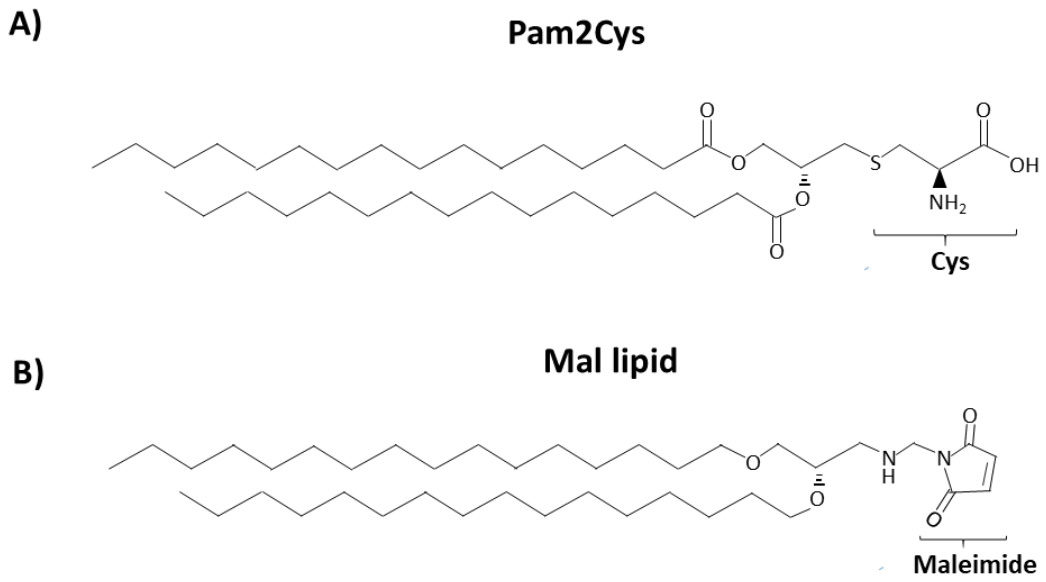

Figure 4.1- Schematic structure of Pam2Cys and Mal lipid.
Schematic diagram of A) typical Pam2Cys harbouring a cysteine (Cys) residue, linked to a glycerol group with ester-linked dipalmitoyl chains and B) synthesised Mal lipid (*N*-(2, 3-bis (hexadecyloxy) propyl)-3-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) propanamide) containing a maleimide group linked to a glycerol group with ether-linked dipalmitoyl chains.

4.1.1 Liposomes containing Mal lipid as a delivery vehicle

Liposomes can serve as versatile delivery vehicles. Their lipid composition can be tailored to suit different needs for example cholesterol can be included to enhance their stability, and their size can be selected depending on the target host cell (Schmidt *et al*., 2016). Some phospholipids are chosen for their inherent adjuvant properties (Schmidt *et al*., 2016). For our vaccine delivery platform, the aim was to formulate liposomes with the synthetic Mal lipid. Recombinant protein could then be conjugated via covalent attachment of the N-terminal cysteine residue with the protruding Mal head group. Specifically the covalent attachment to the maleimide would occur via the SH group present in the N-terminal cysteine forming a thioether linkage (Figure 4.2) (Ravasco *et al*., 2019). The result would be a simplistic mimic of a

bacterial cell with lipoproteins presented at the cell surface via anchorage of their lipid domain into the phospholipid bilayer, resembling the bacterial membrane.

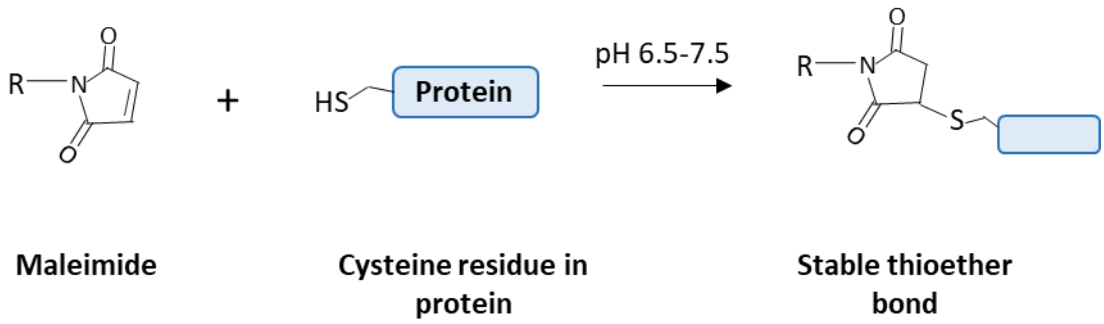

Figure 4.2- Schematic diagram of maleimide reaction with sulfhydryl group of cysteine residue forming a stable thioether bond.
Maleimide group reacts with the sulfhydryl group (SH) present in cysteine residue of the protein in the presence of pH 6.5-7.5. In the basic solution the SH group also known as a thiol, is deprotonated which results in a thiolate anion that attacks the maleimide bond. This results in a stable thioether linked conjugate. R-represents reactive group. Blue rectangle represents protein.

The liposomal lipids were selected based on work conducted by Han *et al* (1997). This group used DPPS, DPPC and cholesterol in a 1:1:2 molar ratio respectively to formulate liposomes to target the gut. Liposomes composed of these phospholipids proved stable in simulated gastric fluid and were found to be capable of eliciting mucosal sIgA against encapsulated antigen (*i.e.*, antigen contained in the inner core of the liposomes) when delivered orally in mice (Han *et al*., 1997; Watarai *et al*., 1998).

In this study, liposomes composed of Mal lipid, DPPS, DPPC and cholesterol in a 0.5:1.25:1.25:2 molar ratio respectively were formulated (Figure 4.3). The nanoparticle size selected was 100-200 nm since it was previously shown that liposomes of up to 500 nm are preferential for M cell uptake (Aramaki *et al*., 1993:

Cortsév and Bioley, 2018; Miquel-Clopés *et al*., 2019; Williams and Owen, 2015). It was anticipated the liposomes would become slightly larger upon conjugation of the protein and therefore sit within the preferred size range for M cell uptake. GFP was selected as our prototype-recombinant protein to assess the conjugation of protein to liposomes for analysis via FACS. The recombinant *C. difficile* antigens could then be conjugated to liposomes followed by lyophilisation and encapsulation. Lyophilisation is commonly used for liposomal dehydration, however this can also lead to liposomal aggregation or damage (Hua *et al*., 2003). Sugars such as trehalose can be used as a cryoprotectant during this process which helps maintain liposomal size and prevent aggregation. Trehalose can form hydrogens bonds between polar head groups of the phospholipids, therefore replacing the bonds formed with water (Madden *et al*., 1985). Therefore, trehalose was selected as a cryoprotectant for lyophilisation of liposomes. Oral delivery requires sufficient antigen to overcome the loss encountered due to degradation in the stomach or during transit through the long intestinal gut (Srivastava *et al*., 2015). We therefore decided to use a total of 1 mg of antigen presented on liposomes for each dose.

The Mal lipid utilised for this study was made by Rhys Griffiths (University of Nottingham, Chemistry department). All liposomal formulations including optimisation of conjugations, purification of liposomal formulations using SEC and analysis via DLS was conducted by Panayiota Palazi with the assistance of Nicholas Mitchell (University of Nottingham, Chemistry department). My involvement in this work was determining protein concentration following liposomal conjugations, data analysis, and analysis of GFP-liposomal conjugates via FACS and assisting in final *in vivo* sample preparations.

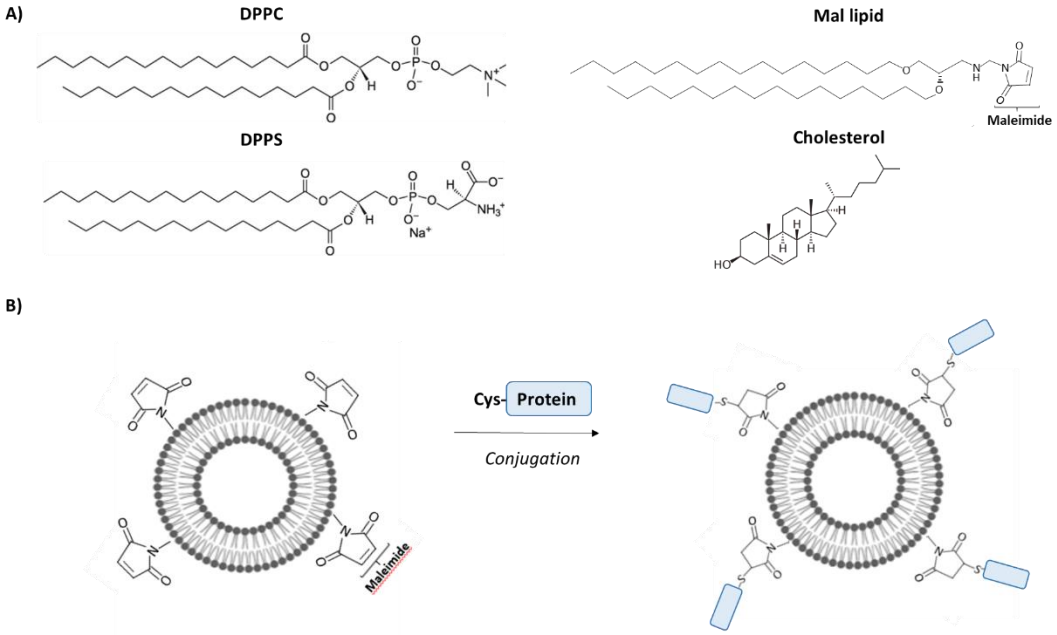

Figure 4.3- Schematic diagram of lipids used for liposomal formulation and Mal lipid attachment to protein.
A) Lipids used to formulate liposomes; dipalmitoylphophaitdylserine (DPPS), dipalmitoylphoshatidylcholine (DPPC), maleimide lipid (Mal lipid) and cholesterol. B) Mal lipid containing liposomes with surface attachment of antigen. This attachment occurs via the maleimide group within the Mal lipid allowing attachment to the sulfhydryl group (SH) present in the N-terminal cysteine residue of recombinant proteins forming a thioether linkage.

4.1.2 Aims of this study

To summarise, the aim of this chapter was to formulate liposomes with synthetic Mal lipid, DPPS, DPPC and cholesterol and optimise the conjugation of protein to the liposomal surface. In order to confirm protein attachment and the optimise ratio of protein to lipid required for maximal presentation of the selected antigens on the liposomal surface, GFP will be used as a test protein to enable direct assessment by FACS analysis. This will be conducted using liposomes with and without Mal lipid and mixed with GFP for confirmation of attachment. In addition to this, in order to determine maximal presentation of protein on the liposomal surface, different ratios

of GFP to Mal lipid will be tested and analysed by FACS. The data could then be used when conjugating the proteins CD630_08730 and CD630_TcdB-RBD with liposomes for maximal presentation of these antigens for testing *in vivo*.

4.2 Liposomal preparations

4.2.1 Determination of liposomal size by DLS

All liposomal preparations were conducted as stated in section 2.6.1 in a total of 10 ml PBS suspension at a 1 mM concentration. Liposomes were prepared using 25% (w/v) DPPS (2.5 µmol), 25% (w/v) DPPC (2.5 µmol), 10% (w/v) Mal lipid (1 µmol) and 40% (w/v) (4 µmol) cholesterol referred to as MalLipo. In order to confirm protein conjugation to liposomes is achieved by the Mal lipid, a non-Mal lipid liposomal control (-MalLipo), was prepared as a negative control and in the same manner using 30% (w/v) DPPS (3 µmol), 30% (w/v) DPPC (3 µmol) and 40% (w/v) (4 µmol) cholesterol. The average size of liposomes and the polydispersity index (PDI) was measured using DLS with 4 replicates (section 2.6.3) (Figure 4.4).

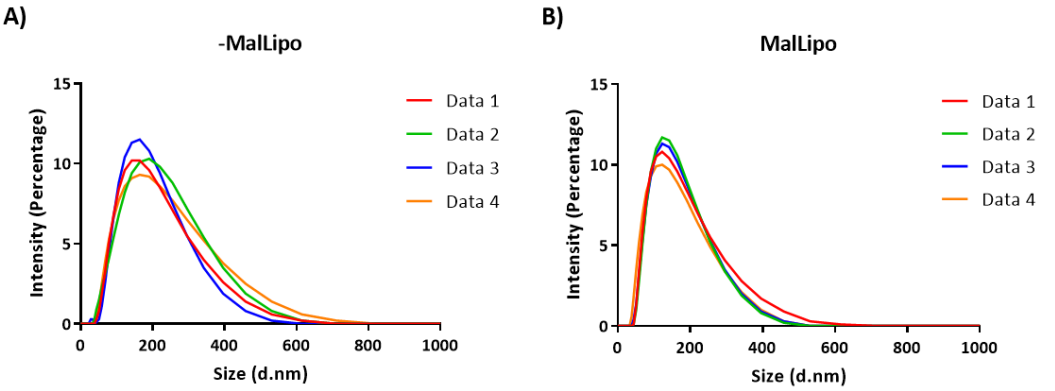

Figure 4.4- Size determination of formulated liposomes; -MalLipo and MalLipo using DLS.

Non-Mal lipid liposomal control A) -MalLipo, using 30% (w/v) DPPS (3 µmol), 30% (w/v) DPPC (3 µmol) and 40% (w/v) (4 µmol) cholesterol with an average size of 124 diameter (d.nm) and a polydispersity index (PDI) of 0.204. Mal lipid containing liposomes B) MalLipo, using 25% (w/v) DPPS (2.5 µmol), 25% (w/v) DPPC (2.5 µmol), 10% (w/v) Mal lipid (1 µmol) and 40% (w/v) (4 µmol) cholesterol with an average size of 113.3 d.nm and a PDI of 0.219.

Liposomes with particle size between 100-200 nm were obtained. -MalLipo gave a mean diameter of 124 nm and MalLipo, 113.3 nm. The PDI represents the uniformity of the population of liposomes with values below 0.1 considered monodisperse and values of 0.1 to 0.4, moderately polydisperse. It is preferential to use a monodisperse population of liposomes to aid accuracy when optimising protein conjugation ratios. Both -MalLipo and MalLipo gave a PDI of 0.204 and 0.219 respectively. This shows that not all particles were of same size however this PDI was acceptable being closer to 0.1 than 0.4. The liposomes were then concentrated down to a volume of 1-2 ml in Vivaspin® 20; 50 kDa MWCO columns (GE Healthcare Life Sciences) and incubated with GFP, using several molar ratios.

4.2.2 Purifying GFP conjugated liposomes using SEC

Purified GFP in PBS was incubated with -MalLipo and MalLipo suspensions as stated in section 2.6.3 using different molar ratios of GFP to Mal lipid and a 1:2 molar ratio of GFP to TCEP. TCEP was used to reduce disulphide bonds that could form between the N-terminal cysteine of GFP resulting in aggregates produced from protein-protein interactions (Cline *et al*., 2004). For -MalLipo incubations with GFP, the amount of GFP used was equivalent to that used for the different molar ratios of GFP to Mal lipid. In order to separate GFP conjugated onto liposomes from non-conjugated GFP *i.e.*, non-bound, free GFP, SEC was performed (section 2.6.4). First MalLipo and GFP samples were analysed by SEC individually to establish the elution volume *i.e.*, the volume at which each sample is eluted from the column, in order to successfully purify these samples. Following the incubation of GFP to MalLipo and -MalLipo in TCEP, SEC was performed to separately purify attached GFP to MalLipo/-MalLipo and non-conjugated GFP. The amount of GFP conjugated to liposomes with and without Mal lipid was subsequently confirmed by performing a BCA assay on the different elution samples collected (section 2.5.6).

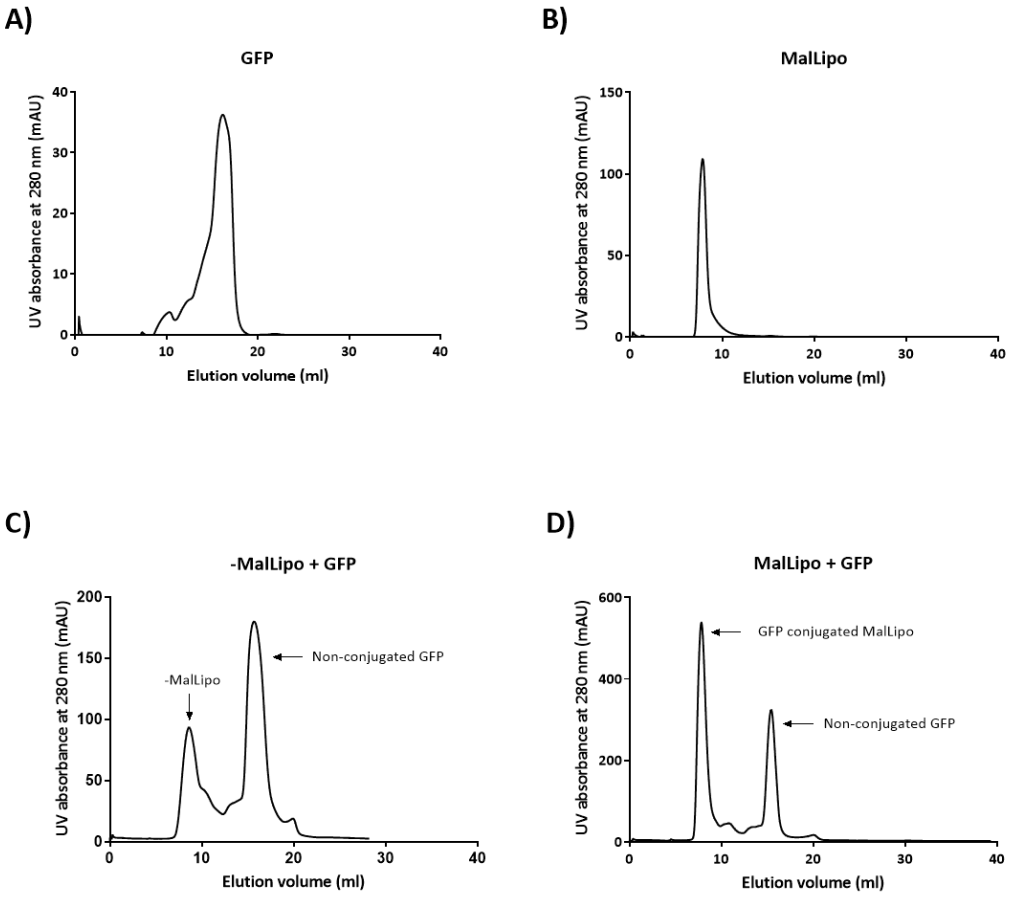

Figure 4.5- Representative SEC chromatogram showing purification of MalLipo and -MalLipo conjugations with GFP.
Using a 500 µl injection loop with a flow rate of 0.75 ml/min the elution volume (horizontal axis) and ultraviolet (UV) absorbance measurement of sample at 280 nm wavelength (vertical axis) of A) GFP and B) MalLipo fractions were determined. This subsequently allowed purification of C) -MalLipo + GFP and D) MalLipo + GFP and the separation of non-conjugated GFP.

The elution volume of GFP and MalLipo purified by SEC was approximately 15 and 8 ml respectively (Figure 4.5, A-B). These elution volumes were used to determine elution fractions of non-conjugated GFP following SEC of -MalLipo + GFP and MalLipo + GFP incubations (Figure 4.5 C-D). As the -MalLipo control should not permit GFP conjugation, the absorbance reading for non-conjugated GFP peak from the -MalLipo + GFP incubation (Figure 4.5, C) is expected to be higher than that of the GFP peak

from the MalLipo + GFP incubation (Figure 4.5, D). The higher peak absorbance is therefore indicative of protein conjugated onto to liposomes. In addition to this, the -MalLipo peak absorbance is expected to be lower compared to the MalLipo absorbance peak following successful conjugation of GFP to the MalLipo. By measuring the GFP concentration from the elutions from both the -MalLipo/MalLipo and GFP, the amount of GFP is determined from the starting concentrations.

4.2.3 Confirming GFP conjugation to MalLipo using FACS

Following SEC of -MalLipo + GFP and MalLipo + GFP incubations, the elutions in PBS were analysed by FACS for two different molar ratios used; 2:1 and 5:1, GFP:Mal lipid *i.e.*, 150 µl of 1 mM liposomal formulation with 15 nmol Mal lipid incubated with 30 nmol and 75 nmol GFP respectively. For -MalLipo incubations with GFP, the amount of GFP equivalent to a 2:1 molar ratio was used *i.e.*, 30 nmol GFP. The amount of GFP used was expected to be in excess in order to saturate all protruding Mal lipid. Since the liposomes are expected to be multilamellar due to their size, about 90% of the Mal lipid was expected to be buried with only around 10% facing outwards (Nick Mitchell personal communication). FSC (forward scatter) and SSC (side scatter) density-plots allowed identification of liposomal populations gating against MalLipo and -MalLipo. Histograms representing the fluorescence intensity signal and number of events were analysed. The negative fluorescence threshold gating was determined using the negative controls; MalLipo and -MalLipo. GFP alone was utilised as a negative control as the protein alone would be too small to be detected using the gating for liposomes. FACS analysis allowed determination of the median fluorescence intensity (MFI) of each sample using the Kaluza Analysis 2.1 software

(Beckman Coulter-Life Sciences). The value obtained was then used to determine the median fluorescence intensity (rMFI) by the equation below.

$$\text{rMFI} = \frac{\text{MFI of all gated liposomal population}}{\text{MFI of gated liposomal population in negative control}}$$

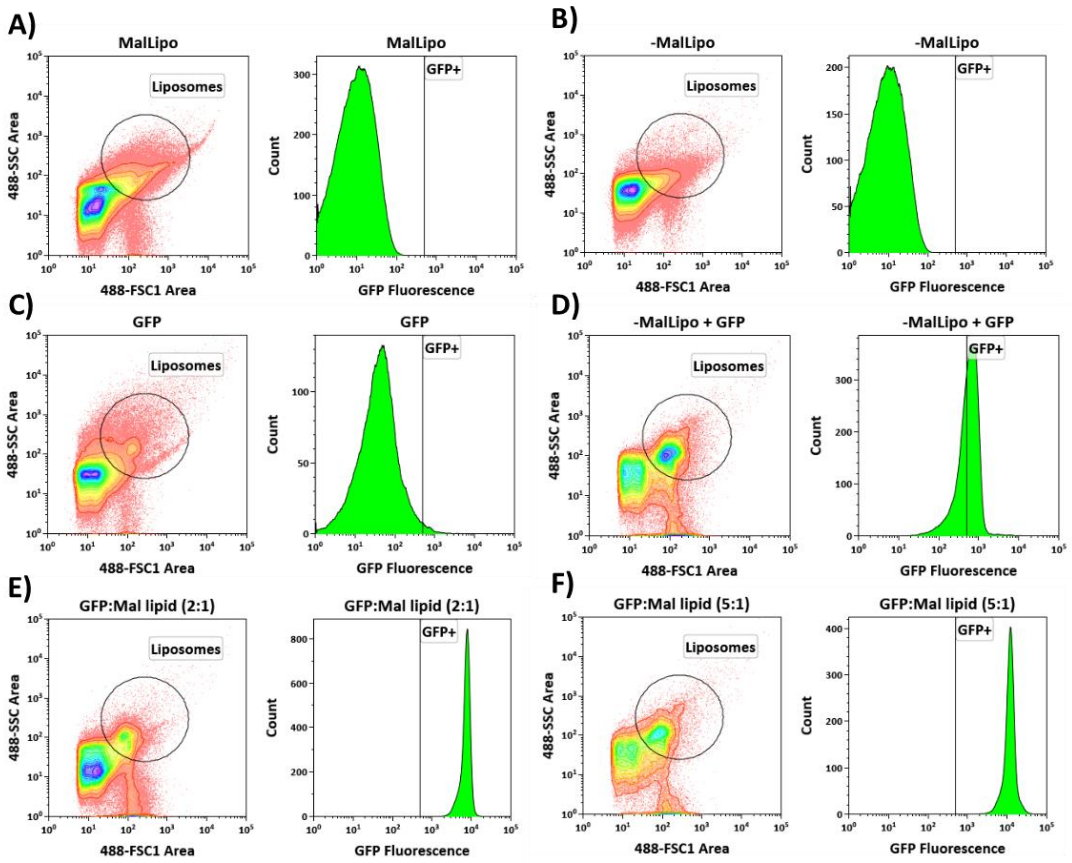

Figure 4.6- Representative FACS density-plot and histogram of liposomal conjugates with GFP.

Representative FACS density-plots and histograms to assess the conjugation of GFP to liposomes. For density-plots, the horizontal axis displays the forward scatter (FSC) and the vertical axis, the side scatter (SSC). For histograms, the horizontal axis represents the GFP fluorescence intensity and the vertical axis, the number of events. Gating for population of liposomes is represented by a circle in the density-plots and negative gating for GFP fluorescence was conducted by analysis of control samples; liposomes alone with the Mal lipid A) MalLipo, and without B) -MalLipo and is shown by the vertical line with GFP+ fluorescence represented to the right of this line. As a negative control C) GFP as purified protein was analysed. The association of GFP with liposomes merely by electrostatic interactions or by specific conjugation was compared by analysis of GFP incubated with liposomes formulated without Mal lipid D) -MalLipo + GFP or with the Mal lipid at molar ratio of E) GFP:Mal lipid (2:1) and F) GFP:Mal lipid (5:1). The histograms were used to determine the median florescence intensity (MFI) using the X-median values determined by the Kaluza Analysis 2.1 software.

The MFI was used to calculate the rMFI for each sample. The florescence signal detection was gated using the negative controls, MalLipo (rMFI= 1) (Figure 4.6, A) and -MalLipo (rMFI= 1) (Figure 4.6, B) which contained no GFP. GFP only control, which may not be detected due the gating used for liposomal sizes, gave a similar signal (rMFI= 1) (Figure 4.6, C). Fluorescence signals were also detected with the negative control liposomes used for conjugations, -MalLipo + GFP (rMFI= 1.7) (Figure 4.6, D), indicating the presence of GFP with a 1.7-fold increase in rMFI relative to the negative controls. However, conjugations conducted with liposomes containing Mal lipid which would aid in covalent attachment of GFP using the molar ratio; GFP:Mal lipid (2:1) (rMFI= 21.5) (Figure 4.6, E) and GFP:Mal lipid (5:1) (rMFI= 32.5) (Figure 4.6, F), revealed a 13-fold and 19-fold increase in florescence signal respectively relative to the negative control -MalLipo + GFP (rMFI= 1.7) (Figure 4.5, D). As both ratios of GFP to Mal gave a similar shift in fluorescence intensity, it was concluded that with the amount of Mal lipid that was used, Mal lipid was indeed saturated using the 2:1 molar equivalents of protein to Mal lipid.

4.3 Preparation of samples for use *in vivo*

In order to perform an *in vivo* study comparing the immunogenicity of an oral vaccine using CD630_08730 and CD630_TcdB-RBD either alone or conjugated onto MalLipo the following was conducted. Samples for the following groups were prepared; MalLipo harbouring 1 mg of each protein or 1 mg purified protein alone as well as control groups, -MalLipo and trehalose only.

4.3.1 Preparation of -MalLipo and MalLipo

-MalLipo and MalLipo were prepared as stated in section 2.6.1 with a total volume of 21.6 ml at 1 mM for each liposomal group. The MalLipo suspensions were concentrated down individually for separate conjugations to each protein to be performed. The -MalLipo suspensions were combined prior to concentrating. The average size of liposomal suspensions were determined by DLS. The mean size and PDI of MalLipo for conjugation to CD630_08730 was 113.3 nm, PDI=0.259 and for conjugations to CD630_TcdB-RBD, 115.1 nm, PDI=0.259. The -MalLipo also gave a similar mean size at 118.2 nm, PDI=0.347. The DLS measurements confirmed that all the liposomal preparations were of similar size with relatively small PDI as desired.

4.3.2 Preparation of *C. difficile* antigen conjugation to MalLipo

In order to maximise the amount of total protein conjugated onto MalLipo, increasing the concentration of MalLipo, thus increasing the overall amount of Mal lipid available for conjugation, should lead to more protein conjugated. To this end, 20 mg of each protein, CD630_08730 and CD630_TcdB-RBD in PBS were mixed with MalLipo suspensions using a molar equivalent of 2:7.6 (*i.e.*, 1:3.8) and 2:13 (*i.e.*, 1:6.5) protein to Mal lipid respectively instead of the 2:1 ratio. Conjugations were performed with the addition of TCEP as described in section 2.6.4. The composition of formulations is shown in Table 4.1. The conjugates were purified using SEC and eluted in PBS. The amount of protein conjugated onto liposomes was assessed using a BCA assay. -MalLipo was used as a negative control for the BCA assay. The amount of protein that was successfully conjugated on MalLipo is stated in Table 4.2.

Table 4.1- Components used to perform conjugations of CD630_08730 and CD630_TcdB-RBD to MalLipo for *in vivo* studies.

MalLipo volume (ml)	Concentration of MalLipo (mM)	Protein conjugated	Amount of protein used (mg)	Ratio of protein to Mal lipid (µmol)	TCEP amount (mg)
1.55	13.9	CD630_08730	20	2:7.6	0.315
1.25	17.9	CD630_TcdB-RBD	20	2:13	0.188

Table 4.2- Total amount of CD630_08730 and CD630_TcdB-RBD conjugated onto MalLipo.

Protein conjugated	Concentration of protein (mg/ml)	Total amount of protein conjugated (mg)
CD630_08730	1.0775	12.98
CD630_TcdB-RBD	0.7512	15.22

From a total of 20 mg of protein used for conjugation reactions 12.98 mg of CD630_08730 and 15.22 mg of CD630_TcdB-RBD were successfully conjugated onto liposomes. These conjugates were then aliquoted such that each contained a total of 1 mg of protein.

4.3.3 Preparation of *in vivo* samples lyophilised with trehalose

It was reported using 10X the mass of trehalose relative to the total average amount of lipid present resulted in liposomes maintaining their size and PDI, therefore trehalose was used as a cryoprotectant for lyophilisation. All samples prepared for the *in vivo* study with total amounts to be used per immunisation are stated in Table 4.3. Suspensions in PBS of; MalLipo containing 1 mg protein, 1 mg of purified protein

alone, and -MalLipo using a similar concentration of liposomes to that of aliquoted conjugates were all mixed with trehalose as stated in Table 4.3.

Table 4.3- Volumes and amounts of liposomal formulation and protein used per dose for *in vivo* immunisation for each hamster group.

***In vivo* samples to be used per immunisation**					
Hamster group components	**Volume of suspension (ml)**	**Amount of protein (mg)**	**Mole of total liposomal lipid (µmol)**	**Total amount of liposomal lipid (mg)**	**Amount of trehalose**
-MalLipo	1.13	-	1.38	0.728	7.28
MalLipo + CD630_08730	1.33	1	1.42	0.846	8.46
MalLipo + CD630_ TcdB-RBD	0.982	1	1.66	0.990	10
CD630_08730	1	1	-	-	10
CD630_TcdB-RBD	1	1	-	-	10

All suspensions in PBS were aliquoted into 1.5 ml Eppendorf's and trehalose mixed in thoroughly until completely dissolved. All samples were lyophilised by overnight freeze-drying then stored at room temperature until ready to be packed into capsules.

4.4 Discussion

Attachment of *C. difficile* proteins onto liposomes via conjugation to a synthetic lipid was successfully achieved in this study. The lipid moiety containing a maleimide head group, Mal lipid, was formulated with the liposomal lipids to create MalLipo for subsequent conjugation of recombinant proteins via covalent attachment of the N-

terminal cysteine residue to the maleimide head group of protruding lipids. Specifically liposomes were formulated with 25% (w/v) DPPS, 25% (w/v) DPPC, 10% (w/v) Mal lipid and 40% (w/v) cholesterol based on previous work by Han *et al* (1997). The phospholipids chosen are known to have immunostimulatory activity, for example DPPS is a natural inner cell membrane component that can act as a danger signal facilitating antigen uptake by DCs via specific DPPS receptors (Wang *et al*., 2019; Freeman *et al*., 2010). Different molar ratios of lipids to cholesterol were tested by our collaborators before final selection of ratios. The use of cholesterol has an impact on morphology and stability of liposomes by allowing formation of dense packing of phospholipids (Bozzuto and Molinari, 2015). It was reported that with DLS and TEM analysis conducted by Panayiota Palazi, 40% cholesterol provided better desired size of liposomes of approximately 100-200 nm and a more spherical morphology compared to other ratios. Having lower or higher concentrations of cholesterol showed less spherical morphology. The concentration dependent impact of cholesterol was previously studied by Nomura *et al* (2005) showing that increasing cholesterol from 20 to 50% molar concentration can change the size and morphology of giant liposomes that form tube structures and networks to smaller liposomes of spherical morphology (Nomura *et al*., 2005). However, testing the optimal concentration is required as having too much of cholesterol can also increase the size of liposomes which has been shown when tested in combination with different phospholipids and can also interfere with dense packing of phospholipids which ultimately effects the stability (Duangjit *et al*., 2014; Shaker *et al*., 2015). Having a spherical structure was of importance to ensure that the Mal lipid was exposed as possible to enable sufficient conjugation of protein. In addition to the particle shape,

particle size was of importance for application *in vivo*. The sonication method used in this study to form liposomes, is a common technique used for the production of MLV. This process provided simplicity and ease of use. Although not monodisperse, this method provided a size of liposomes with a fairly low PDI and reproducibility. The main draw back in general with formulating liposomes is controlling the size and homogeneity (Leitgeb *et al*., 2020). An alternative approach for controlling the size of liposomes could have been the use of membrane extrusion in which liposomes are extruded through polycarbonate membranes with chosen pore-size (Akbarzadeh *et al*., 2013). When membrane extrusion was compared to other techniques such as sonication, ultrasonication, freeze-thaw sonication and homogenisation for size processing, it was found to be the most efficient (Ong *et al*., 2016). However, this technique can also be time-consuming and less cost-effective (Leitgeb *et al*., 2020).

In order to confirm protein conjugation to MalLipo, non-Mal containing liposomes, -MalLipo were used as a negative control for conjugations conducted with purified GFP. GFP was used since it is fluorescent and presentation on liposomes can be easily analysed by FACS. All conjugates were subjected to purification by SEC to separate successfully conjugated protein to the liposomes, from non-conjugated protein. The protein concentration in each elution was determined using a BCA assay. Different ratios of GFP to Mal lipid were used. Specifically, the FACS output was gated for expected size of liposomes and negative florescence using negative controls (Figure 4.6, A-B.) The detection of GFP would only be obtained if conjugated onto liposomes, as the size of protein alone is not sufficient for detection. Both molar ratios of 2:1 and 5:1 of GFP to Mal lipid confirmed successful conjugation as seen from the shift in fluorescence (Figure 4.6, E and F) compared with GFP bound electrostatically to -

MalLipo (Figure 4.6, D) and further indicated that an excess of protein had been used in both cases due to the insignificant shift in fluorescence between the two ratios used. The slight shift in fluorescence with the -MalLipo + GFP control (Figure 4.6, D) could be explained by potential electrostatic interactions between the liposomal lipids and GFP (Torchilin and Klibanov, 1981). The other possible explanation could have been insufficient separation during SEC purification. However, the likelihood of this would be very minimal as there is a large difference in MW between GFP conjugated liposomes and GFP alone. Hence, this gives more of an indication of electrostatic binding. Results of the BCA assay also confirmed trace amounts of protein. The data encouragingly confirmed that it was indeed the Mal lipid that allowed the majority of GFP included in the conjugation reaction to attach covalently to liposomes. As the FACS data shows each single event of liposome passing through and the median fluorescence intensity, the histogram displaying GFP conjugated to MalLipo (Figure 4.6, F and G) revealed a narrow peak which indicates, that the loading of GFP per liposome was of similar amount.

Since there is a requirement for the liposomal formulations to be lyophilised in order to be encapsulated, trehalose served as a cryoprotectant which maintained liposomal integrity following lyophilisation. CD630_08730 and CD630_TcdB-RBD were successfully conjugated to MalLipo. MalLipo + CD630_08730 and MalLipo + CD630_TcdB-RBD conjugates were aliquoted such that each suspension contained 1 mg of protein. Trehalose was mixed into each suspension until fully dissolved prior to lyophilisation.

Chapter 5

Enteric capsule coating

5 Enteric capsule coating

5.1 Introduction

Oral vaccines are administered in the form of liquid suspensions, or dried formulation in tablets or capsules (Vela Ramirez *et al*., 2017). The majority consist of live attenuated or inactivated whole cell microorganisms combined with subunit components (Chapter 1). The first oral vaccine licenced was the live-attenuated polio vaccine (OPV) in 1962 which successfully resulted in the control of the virus (Strebel *et al*., 1992). However rare cases of reversion of the attenuated strain to the virulent form were reported that led to vaccine-associated paralytic poliomyelitis (VAPP) (Strebel *et al*., 1992). The general concern of reversion of attenuated pathogens to the virulent form is what led to the shift towards subunit vaccines that contain defined antigens (Davitt and Lavelle, 2015). However, the major hurdle with delivery of subunit protein/peptides is the need for adjuvant to render them sufficiently immunogenic. Additionally, any oral formulation requires protection from the low pH and digestive enzymes within the stomach that will affect stability. Tablets and capsules can be delivered orally by first applying enteric coating that allows delivery of the encapsulated formulation to the small intestine whilst providing resistance to the stomach (pH 1.5-3) (Beasley *et al*., 2015). Advantages of enteric coating of capsules over tablets are their resistance to spoilage, less need for compression and fewer steps in manufacturing (Mahdi, 2015). Hard gelatin capsules are widely used for pharmaceutical dosing and can be enteric coated with a wide range of available material for targeted site delivery (Mahdi, 2015).

Since the site of infection of *C. difficile* is the colon, targeting the small intestine to stimulate mucosal antibody responses could potentially lead to local protection in the colon. In order to encapsulate the lyophilised formulation of *C. difficile* antigens attached to the surface of liposomes via the Mal lipid or lyophilised protein alone, commercially available gelatin capsules were used.

5.1.1 Enteric coating of gelatin capsules

Polymers with pH dependent solubility are typically used for enteric coating combined with plasticisers, solubilisers, colorants and anti-adhesion agents (Mahdi, 2015; Thoma and Bechtold, 2000). Such polymers resist the low pH of gastric fluid by remaining unionised and therefore insoluble. However, they become soluble and disintegrate at higher pH within the intestine as the carboxylic groups are ionised by the intestinal fluid (Kapoor *et al*., 2020). Commonly used pharmaceutical enteric coating polymers include shellac, cellulose products, polymethacrylates and polyvinyl derivatives which have different pH dissolution properties (Table 5.1) (Hussan *et al*., 2012; Kapoor *et al*., 2020). The majority of enteric coatings are dissolved in organic solvents, however there is a tendency to shift towards using aqueous solutions due to factors such as high cost of solvents, potential insufficient evaporation of solvents and safety (Kapoor *et al*., 2020). Although the use of aqueous solutions is deemed safer, the drying process of film coating is time-consuming and requires heating which increases the expense.

Table 5.1- Polymers used for enteric coating and pH-dependent dissolution.

Polymers	Dissolution pH
Shellac	7
Cellulose products	
Cellulose acetate phthalate (CAP)	6.2
Cellulose acetate trimellitate (CAT)	5
Hydroxypropylmethylcellulose phthalate (HPMCP)	5-5.5
Hydroxypropylmethylcellulose acetate succinate (HPMCAS)	5-7
Polyvinyl derivatives	
Polyvinyl acetate phalate (PVAP)	5
Polymethacrylates	
methacrylic acid ethacrylate poly (MA-EA)	
-Eudragit® L 30 D/ Eudragit L 10-55 (trade name)	5.5
methacrylic acid methyl methacrylate poly (MA-MMA)	
-Eudragit® L (trade name)	6
- Eudragit® S (trade name)	7

5.1.2 Plasticisers

Plasticisers are included to increase flexibility and aid polymer film distribution. Plasticisers work by interacting with polymer side chains lowering polymer-polymer interactions. The other importance of plasticisers is the ability to lower the glass transition temperature (T_g) resulting in a more soft and rubbery film, making the polymer less brittle (Foroughi-Dahr *et al*., 2017; Snejdrova and Dittrich, 2012). Plasticisers include organic esters, glycerol esters, polyhydric alcohols and water (Table 5.2) (Foroughi-Dahr *et al*., 2017; Snejdrova and Dittrich, 2012; Thoma and Bechtold, 2000). The choice of plasticiser depends on compatibility with the polymer and their effect on capsule dissolution and drug release.

Table 5.2- Category of plasticisers used for enteric coating.

Category	Plasticiser
Organic esters	**Citric acid esters:** triethyl citrate (TEC), tributyl citrate (TBC), acetyl triethyl citrate (ATEC) and acetyl tributyl citrate (ATBC)
	Phthalic acid esters: diethyl phthalate (DEP) and dibutyl phthalate (DBP)
	Tataric acid ester: dibutyl tartate (DBT)
	Sebatic acid ester: dibutyl sebacate (DBS)
Glycerol esters	Castor oil, sesame oil, fractionated coconut oil, acetylated mono glycerides, glycerol diacetate and glycerol triacetate
Polyhydric alcohols	Polyethylene glycols (PEG), propylene glycol, 1-2 propylene glycol glycerol

5.1.3 Eudragit® L100

Eudragit® (Evonik industries, Germany) is the brand name given to several synthetic polymethacrylate co-polymers composed of methacrylic acid (MA) and the esters; methyl methacrylate (MMA) and ethyl acrylate (EA). Several variations of Eudragit® co-polymers exist and their dissolution properties depend on the presence of varying ratios of carboxylic groups to the ester groups (Thoma and Bechtold, 2000). A range of commercially available drugs have employed Eudragit® polymers mainly for colonic targeting (Thakral *et al*., 2013). The expected range of human small intestinal pH is between 6.5 and 7 and the colon pH is between 7 and 8. Eudragit® L100 is the most common formulation used for enteric coating for delivery to the small intestine and S100 is used for deliver to the colon (Bando and McGinity, 2006; Thakral *et al*., 2013). Eudragit® L100 is an anionic powdered formulation based on the co-polymerisation of methacrylic acid and methyl methacrylate and can be dissolved in either isopropanol or acetone (Cetin *et al*., 2010). A combination of both acetone and

isopropanol with or without the addition of water can be used (Thakral *et al*., 2013; Thoma and Bechtold, 2000). The most commonly used plasticiser for Eudragit® polymers is TEC at a recommended minimum of 10% (Kadian and Harikumar, 2009).

In this study targeted delivery to the small intestine is crucial, therefore Eudragit® L100 was selected. Staelens *et al* (2016) and co-workers had previously shown $BaSO_4$ filled gelatin capsules (size 9) coated with 12.5% Eudragit® L100 combined with 10% TEC were able to bypass the stomach and dissolve in the small intestine of hamsters as visualised by CT imaging. We have chosen to use powdered L100 that could be solubilised in isopropanol and 10% TEC added as previously conducted by Staelens *et al* (2016) but was aware that commercial solutions of Eudragit® L100 contain 3% H_2O, therefore sought to test the importance of including H_2O. Following *in vitro* dissolution tests, the optimal coating established could then be assessed *in vivo* using CT imaging to track the release of encapsulated $BaSO_4$. Using CT imaging provides a three-dimensional image of dense matter in which heavy contrasting agents that are safe, such as barium or iodine can be utilized (Staelens *et al*., 2016). Other imaging techniques to track capsules such as optimal imaging which rely on bioluminescence and florescence are less capable of visualisation through the thick skin and fat of hamsters (Studwell and Kotton, 2011). We therefore used $BaSO_4$. CT imagining of $BaSO_4$ packed coated capsules were conducted by Jeni Luckett with the assistance of Michelle Kelly for hamster gavaging, anesthetising and handling.

5.1.4 Aims of this study

The aim of this study was to optimise and determine the enteric coating which would enable targeted release of vaccine formulations in the small intestine for use *in vivo*. This will be determined firstly by testing *in vitro* the dissolution of coated capsules in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.7) using 12.5% Eudragit® L100 with or without TEC and with or without water. In order to test this, capsules will be packed with dye followed by coating and absorbance measurements will be taken over time from the surrounding solution upon immersion into simulated gastric fluid followed by simulated intestinal fluid. The formulation showing the best *in vitro* dissolution profile, *i.e.*, stable in simulated gastric fluid and readily dissolved in simulated intestinal fluid could then be tested in hamsters by tracking dissolution of $BaSO_4$ packed capsules coated with this formulation using CT imaging.

5.2 *In vitro* pH dissolution of enteric coated capsules

Optimising polymer coating of gelatin capsules for targeted release of formulation to the small intestine.

5.2.1 *In vitro* dissolution of capsules coated with Eudragit® L100

Eudragit® L100 with or without TEC and/or H_2O was tested (Table 5.3). Dissolution was visualised by the release of bromophenol blue/glucose packed capsules, size 9 (Torpac®), coated with the formulations listed in Table 5.3 (section 2.7.1 and 2.7.2). For each polymer mixture, one coat versus two was tested. The capsules were immersed in simulated gastric fluid (pH 1.2) for 5 hours followed by simulated intestinal fluid (pH 6.7). Absorbance measurements were taken every 30 minutes at 450 nm and 590 nm wavelengths respectively (Figure 5.1).

Table 5.3- Variations of Eudragit® L100 tested in combinations with TEC and H_2O.

Combinations	Composition
L100	12.5% (w/v) Eudragit® L100 + isopropanol
L100 + H_2O	12.5% (w/v) Eudragit® L100 + 3% (v/v) H_2O + isopropanol
L100 + TEC	12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + isopropanol
L100 + TEC + H_2O	12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O + isopropanol

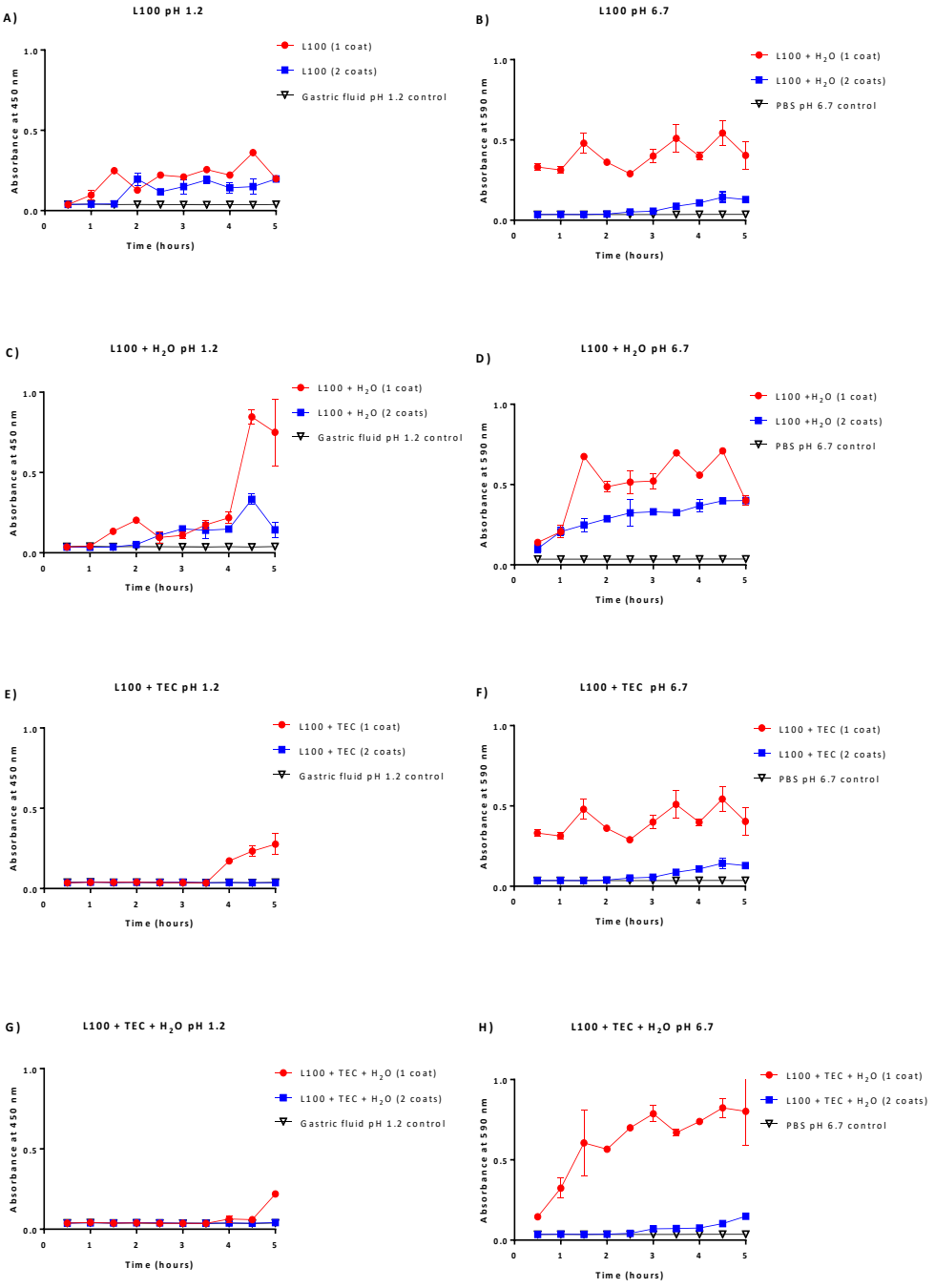

Figure 5.1- Dissolution of enteric coated capsules *in vitro* in simulated gastric fluid followed by simulated intestinal fluid over time.

Dissolution of capsules (1 coat or 2 coats) coated in Eudragit® L100 with and without triethyl citrate (TEC) and with and without water added in simulated gastric fluid pH 1.2 (left panel) and simulated intestinal fluid pH 6.7 (right panel) (n=2). The changing absorbance of surrounding solution due to the release of bromophenol blue from the capsules was measured for each capsule every 30 minutes at 423 nm wavelength in pH 1.2 and at 590 nm wavelength in pH 6.7 with two biological replicates. Error bars represent standard error of the mean (SEM).

The combinations of different enteric coatings were tested for stability in pH 1.2 and dissolution in pH 6.7. Capsules dip-coated once or twice in L100 alone or L100 + H_2O showed premature dissolution in simulated gastric fluid (pH 1.2), (Figure 5.1, A and C) compared to formulations containing the TEC plasticiser. Both showed a similar pattern in that with 1 coat, dissolution occurred following 1 hour incubation and with 2 coats the dissolution occurred following 2 hours incubation (Figure 5.1, A and C). Following 4 hours incubation in pH 1.2, capsules dip-coated once in L100 + H_2O showed the greatest dissolution (Figure 5.1, C). With the exception of L100 + H_2O (Figure 5.1, D), all capsules dip-coated twice showed incomplete dissolution in pH 6.7. Capsules dip-coated once in L100 + TEC showed dissolution after 3.5 hours (Figure 5.1, E) however capsules dip-coated once in L100 + TEC + H_2O (Figure 5.1, G) showed even greater delayed dissolution *i.e.*, at 4.5 hours. Although some disintegration of L100 + TEC + H_2O (1 coat) occurred following 5 hours in pH 1.2, rapid dissolution subsequently occurred *i.e.*, by 1.5 hours in pH 6.7 and by 3 hours the majority of bromophenol blue was released (Figure 5.1, H). Therefore, the polymer mixture of L100 + TEC + H_2O formulation was taken forward for *in vivo* testing in hamsters.

5.3 *In vivo* CT scanning of capsule containing $BaSO_4$, coated in optimal enteric polymer

The 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O enteric polymer was tested *in vivo*. Golden Syrian hamsters (n=4) were administered orally with a single capsule packed with $BaSO_4$ using two different Luer lock dosing applicators size 9 and

9hEC. Each hamster was imaged by CT scanning over 5 hours (section 2.7.1 and 2.7.3) (Figure 5.2).

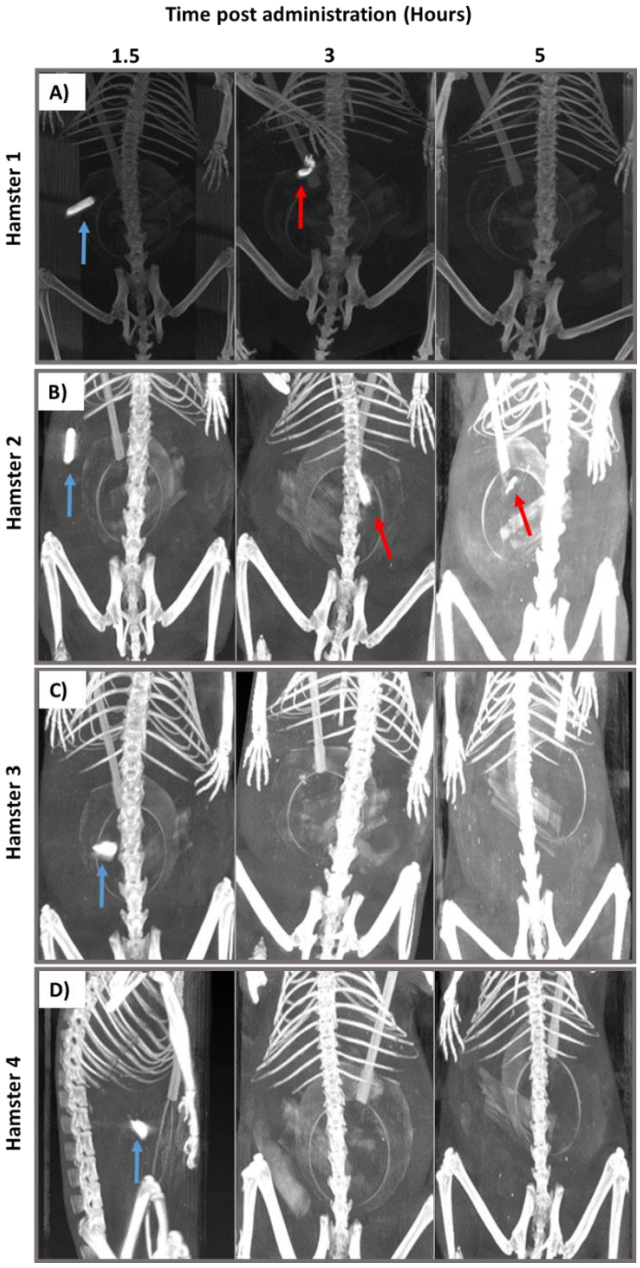

Figure 5.2- Dissolution of enteric coated capsules *in vivo* visualised using CT imaging over time.

Localisation and dissolution of optimal 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O enteric coated capsules containing barium sulphate ($BaSO_4$) over time following oral administration in hamsters (n=4). Two Luer lock dosing applicator were used for oral administration; A-B) device 9 (n=2) and C-D) device 9hEC (n=2) and then anaesthetised by inhalation using 1.5% (v/v) Isoflurane prior to each time point 1.5, 3, 5 hours post administration at which Computed Tomography (CT) images were taken. Blue arrows indicating capsule in the stomach and red arrow indicating the capsule in the small intestine.

The coated capsules were clearly visualised using CT imaging. The two hamsters gavaged using the Luer lock dosing applicator devise 9 (Figure 5.2, A and B) showed the coated capsules to be completely intact in the stomach at 1.5 hours and partially dissolved at 3 hours in the small intestine. At 5 hours in hamster 1, the capsule had completely disintegrated with $BaSO_4$ fully dispersed throughout the small intestine (Figure 5.2, A). In hamster 2, remnants of the capsule were visualised at 5 hours (Figure 5.2, B). Conversely, the two hamsters gavaged with the dosing applicator devise 9hEC (Figure 5.2, C and D), showed slight disintegration of the capsule in the stomach at 1.5 hours with some $BaSO_4$ release. Complete dissolution of the capsule was observed in the small intestine in these 2 hamsters, at 3 hours. By 5 hours, the $BaSO_4$ was seen throughout the entire small intestine.

To summarise, the data shows that gelatin capsules dip-coated once in a polymer consisting of 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O resists the acidity of the stomach reasonably well and undergoes dissolution at the higher pH of the small intestine, the target site. Dosing orally with the size 9 applicator proved more effective than dosing with the 9hEC applicator as the size 9 applicator provided a better fit for the capsules.

5.4 Discussion

Delivering an oral vaccine against *C. difficile* designed to target the small intestine could be achieved utilising pH-dependent enteric coated capsules. In this study gelatin capsules were coated with Eudragit® L100, with or without the plasticisers TEC and/or H_2O: L100, L100 + H_2O, L100 + TEC and L100 + TEC + H_2O (Table 5.1) and

first tested for pH dissolution *in vitro*. The most suitable combination of this formulation was then tested *in vivo* in hamsters using CT imaging.

Visualising the release of bromophenol blue/glucose dye by eye during *in vitro* dissolution testing immediately informed us of the rapidity of dissolution. However, for specific quantification, absorbance readings were taken (Mercier *et al*., 2007; Miller *et al*., 2015). Glucose was mixed with bromophenol blue to minimise the intensity which could lead to saturation during measurements. Although the use of a dye provided good visualisation of release and therefore dissolution of the capsule, fluctuations were observed in the absorbance measurements. For example, in some cases later time point measurements revealed lower absorbance readings compared to a previous time points of higher absorbance. It was observed that precipitates had formed in the wells of the 96-well plate following collection of solution, giving rise to fluctuated reads despite gentle pipetting up and down before sample collection. Alternative available dyes such as ponceau, bromocersol green and bromothymol blue was tested following this observation, which also revealed to form precipitates. In order to determine if the addition of glucose may have contributed to precipitation, bromophenol blue with and without glucose was also tested which gave similar results. However, as bromophenol blue revealed less fluctuations compared to the other dyes tested, this dye was therefore selected for use. We suspect that the volume the dye would have been released in may have not been sufficient enough to solubilise the dye. An alternative approach more commonly used for testing dissolution *in vitro* is by determining the percentage of compound released by measuring the concentration (Bruxelle *et al*., 2018; Sandolo *et al*., 2011). GFP was considered for use to firstly be able to visualise the release and enable

concentration measurements, however loss of fluorescence over time would have been an issue. Nevertheless, using the dye method provides a simple and inexpensive way of measuring several different enteric coatings for their dissolution *in vitro* and visualisation of complete disruption (Miller *et al*., 2015). Using smaller quantities of dye with more excipient and larger dispersion volumes could overcome the shortcomings of this method.

It has previously been shown that TEC forms a better seal between the top and bottom half of the capsule when applied to several different polymers compared to polymers without TEC analysed by TEM (Fu *et al*., 2020). Therefore, the observed delayed release *in vitro* at pH 1.2, with the addition of TEC in this study supports the importance of inclusion of plasticiser (Bando and McGinity, 2006; Zhu *et al*., 2006). Without the addition of plasticisers like TEC, the polymer appears to become brittle with cracks forming which could result in lack of resistance in low pH as observed with the Eudragit® L100 formulation without TEC in this study (Figure 5.1, A and C) (Fu *et al*., 2020; Thoma and Benchtold, 2000). The addition of H_2O also caused delayed disintegration of the capsule in pH 1.2 (Figure 5.1, E). Although only 3% H_2O was used, this certainly helped to initially dissolve the polymer prior to coating which may have contributed to even coating of the polymer. Out of all the formulations tested in this study, capsules dip-coated once in 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O resisted dissolution the greatest at pH 1.2 (Figure 5.1, G) and showed the highest rate of dissolution at pH 6.7 (Figure 5.1, H). As the hamster stomach emptying could be anywhere between 2-6 hours as observed by Staelens *et al* (2016) testing the gastric resistance of the formulations for 5 hours in pH 1.2

seemed reasonable. However, CT tracking performed in this study indicated that hamster stomach emptying occurred between 1.5 and 3 hours.

In order to verify the dissolution of 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O coated capsules in hamsters (n=4), $BaSO_4$ was used as a contrasting agent. Four capsules were packed with $BaSO_4$ then dip-coated once. Two dosing applicators, device 9 and 9hEC, were utilised to orally gavage the hamsters with a single capsule and CT images were taken at 1.5, 3 and 5 hours post dosing to track the release of $BaSO_4$. Dosing 2 animals using the device 9 dosing applicator resulted in the capsule remaining intact in the stomach at 1.5 hours, movement of the capsule to the small intestine with partial dissolution at 3 hours and release of $BaSO_4$ and almost complete disintegration by 5 hours in the small intestine (Figure 5.2, A and B). Conversely dosing 2 animals with the 9hEC dosing applicator resulted in some release of $BaSO_4$ at 1.5 hours in the stomach then complete disintegration of the capsule by 3 hours in the small intestine (Figure 5.2, C and D). The difference in the capsule dissolution in the 2 hamster groups could be explained by the following. Firstly, the 9hEC device had a larger diameter potentially releasing the capsule prematurely as opposed to further down the oesophagus resulting in more time in the hamster saliva which is at a more neutral pH (6.3-9) (Charlton *et al*., 1971). The other explanation could be the lack of consistency when coating these capsule. Capsule coating in pharmaceutical settings employ techniques such as spray drying which enables even coating and drying (Foroughi-Dahr *et al*., 2017). The manual dip coating process employed in this study is generally used only for small scale laboratory studies.

Several *in vitro* and *in vivo* studies have shown success of Eudragit® L100 enteric coating for antigenic and whole-cell bacterial preparations as oral vaccines against various infectious diseases (dea-ayuela *et al*., 2006; Saleem *et al*., 2019; Tan *et al.,* 2019; Xu *et al*., 2018). The work in this study contributes to these findings of the potential of Eudragit® L100 as an enteric coat. Another highly studied enteric coat for vaccine release into small intestine is the cellulose based enteric coating HPMCP which could either be utilised as a pre-coat or alone (Edwards *et al*., 2009; Mercier *et al*., 2007; Singh *et al*., 2015). HPMCP forms a rough surfaces which enhances adhesion of subsequent coating (Cole *et al*., 2002). The licenced oral typhoid vaccine Vivotif® also utilises HPMCP coated capsules which highlights the success of this coating for targeted vaccine delivery. Initially it was intended to also test HPMPC in addition to Eudragit® L100 as both have shown great potential however, due to time-frame restrictions of funding and the delay in HPMCP delivery by manufacturers testing this enteric coating was not possible.

The *in vitro* and *in vivo* dissolution profile of capsules coated in 12.5% (w/v) Eudragit® L100 + 10% (v/v) TEC + 3% (v/v) H_2O showed that this formulation is capable of resisting the acidity of gastric fluid and is readily dissolved at the higher pH of the small intestine and thus potentially effective for targeted release of formulations to the small intestine. Dip-coating once with this formulation was conducted for targeted delivery of the vaccine formulations developed in this study against *C. difficile.*

Chapter 6

Testing the immunogenicity and protective efficacy of *C. difficile*-directed oral vaccine formulations in hamsters

6 Testing the immunogenicity and protective efficacy of *C. difficile*-directed oral vaccine formulations in hamsters

6.1 Introduction

With *C. difficile* spore germination occurring in the small intestine and colonisation within the colon, the oral route of administration will potentially generate an effective local mucosal response in the gut where infection would otherwise develop (Kochan *et al*., 2018). Oral immunisation elicits a mucosal immune response by targeting the PPs found within the lower small intestine. Specialised M cells associated with the PPs can take up antigens and deliver them to DCs which reside in this site (Dillon and Lo, 2019). Antigen recognition and processing in DCs is facilitated by the PPRs found on DCs. DCs then present processed antigen to T cells which subsequently activate B cells. B cell activation leads to the generation of sIgA producing plasma cells (Figure 1.5). sIgA plays a crucial role in preventing adhesion of pathogens to the mucosal epithelium and is the first line of defence against mucosal pathogens (Kim and Jang, 2014). Activated B cells migrate to peripheral mucosal sites throughout the small intestine and large intestine due to their expression of gut homing receptors, $\alpha_4\beta_7$ and CCR9 (Kunkel and Butcher, 2003; Nizard *et al*., 2014). In addition to this first line of defence, a systemic IgG immune response may also be generated (Kang *et al*., 2018). Importantly oral formulations must successfully exit the stomach and reach the lower small intestine intact then cross the mucosal barrier to elicit an effective immune response.

In this chapter we first set out to test the immunogenicity of CD630_08730 involved in colonisation and the C-terminal portion of TcdB, CD630_TcdB-RBD (Kovacs-Simon

et al., 2014; Liu *et al*., 2015) either given alone or conjugated onto liposomes. Specifically the antigens were conjugated to a synthetic Mal lipid, a TLR2 agonist, formulated onto liposomes as a delivery vehicle (chapter 4). The presentation of recombinant proteins on the surface of liposomes would in theory mimic the presentation of lipoproteins on bacterial surfaces via anchorage of their lipid moiety into the phospholipid bilayer of the bacterial membrane. In order to protect the formulations from degradation in the stomach and enable their release in the small intestine, the formulations were packed into enteric capsules, following lyophilisation, then dip-coated once in the optimised polymer mixture (12.5% Eudragit® L100 + 10% TEC + 3% H_2O).

The hamster model was chosen as *C. difficile*-induced enterocolitis in hamsters, particularly in Syrian hamsters, closely resembles pseudomembranous colitis in humans (Best *et al*., 2012). In humans, disruption of the normal gut-flora with antibiotics is a major risk factor for *C. difficile* colonisation and thus CDI (Bignardi, 1998; Thibault *et al*., 1991). This phenomenon is also seen in hamsters, with strong susceptibility to CDI induced following clindamycin treatment. The major symptom of CDI in humans is inflammation and damage to the colon and severe diarrhoea. In hamsters, the caecum in particular becomes inflamed, enlarged with fluid build-up and damaged and diarrhoea is manifested by loose faeces and wet tail (Best *et al*., 2012). The onset of CDI in hamsters is rapid with animals typically succumbing to infection and death within 2 to 3 days post oral challenge with spores. The hamster lethality model is therefore an effective model for testing the efficacy of vaccines against *C. difficile* as an increase in the time to the humane end point is strongly indicative of protection (Bruxelle *et al*., 2018; Libby *et al.,* 1982; Sandolo *et al*., 2011).

The main drawback with using hamster models however is the lack of reagents commercially available for detecting immunological markers. Also, the use of clindamycin prior to challenge with *C. difficile* strains such as strain 630 sometimes can result in failure of the strain to induce CDI due to some sensitivity of *C. difficile* to the antibiotic (Best *et al*., 2012). Specifically, the antibiotic may prevail in the GI tract up to 11 days following treatment and kill some of the inoculum which is given 5 days after the antibiotic. The reduced inoculum can lower the chance of *C. difficile* establishing infection (Larson and Borriello, 1990). To avoid this problem and ensure that infection is induced, the challenge strain chosen was R20291*ermB*. This strain was derived from the hypervirulent strain R20291 and contains the *ermB* gene integrated in its chromosome (Kelly *et al*., 2016). Not only is this strain highly virulent, producing the binary toxin in addition to TcdA and TcdB, the presence of *ermB* which confers resistance to erythromycin confers some resistance to clindamycin (Kelly *et al*., 2016).

6.1.1 Aims of this study

The aims of this study was to assess the immunogenicity *in vivo* of selected *C. difficile* antigens and compare them to their synthetically lipidated counterparts, formulated onto liposomes and use this data to test the protective efficacy. The immunogenicity will be determined *in vitro* by the presence of antibodies in intestinal fluid and serum of vaccinated animals compared to the placebo control group and further tested for the functionality of these antibodies. For formulations demonstrating a successful immune response, challenge studies will be followed to assess the protective efficacy from CDI.

6.2 Immunogenicity profile of *C. difficile*-directed oral vaccine in hamsters

Female Golden Syrian hamsters were immunised as described in section 2.8.1. Hamster groups included control groups; naïve group (n=2) (non-immunised), trehalose only (n=2), -MalLipo (n=2) and experimental groups; CD630_08730 (n=4), MalLipo + CD630_08730 (n=4), CD630_TcdB-RBD (n=4) and MalLipo + CD630_TcdB-RBD (n=4) (Table 6.1). The above components were lyophilised and packed into capsules dip-coated once with 12.5% Eudragit® L100 + 10% TEC + 3% H_2O (section 2.6.6 and 2.7.1). A single capsule was given orally on days 1, 15 and 30 (section 2.7.4 and 2.8.1). Serum and intestinal fluid were harvested at the experimental end point, 2 weeks after the third immunisation and analysed (2.8.1.1).

Table 6.1- Hamster study groups used for immunisation.

Hamster study group	Encapsulated components	No. of group (n)	Total immunisation
Naïve	Non-immunised	n= 2	-
Trehalose only	10 mg trehalose	n= 2	3
-MalLipo	-MalLipo liposome + 7.28 mg trehalose	n=4	3
CD630_08730	1 mg CD630_08730 + 10 mg trehalose	n=4	3
MalLipo + CD630_08730	MalLipo + 1 mg CD630_08730 + 8.46 mg trehalose	n=4	3
CD630_TcdB-RBD	1 mg CD630_TcdB-RBD + 10 mg trehalose	n=4	3
MalLipo + CD630_TcdB-RBD	MalLipo + 1 mg CD630_TcdB-RBD + 10 mg trehalose	n=4	3

6.2.1 Detection of sIgA in immunised hamsters by Western immunoblotting

A commercial anti-hamster IgA secondary antibody was custom-produced by Brookwood Biomedical. Specifically a rabbit anti-hamster IgA antibody specific to the heavy chain was purified from rabbit anti-hamster IgM, IgG, IgA cocktail by cross adsorption against hamster IgG and IgM. A Western immunoblot was performed with this antibody to detect IgA in the intestinal fluid of each hamster used in the study. SDS-PAGE was conducted to fractionate intestinal fluid diluted 1:2 in sample buffer from each hamster group. The membrane was incubated with rabbit anti-hamster IgA antibody (1:1000) (Brookwood Biomedical) followed by incubation with anti-rabbit IgG HRP antibody (1:1000) (CST) (section 2.5.7). Detected bands were visualised using TMB (Figure 6.1). Western immunoblot analysis was not conducted for intestinal fluid samples of hamsters immunised with CD630_TcdB-RBD formulations due to conclusions of parallel analysis of the serum by ELISA.

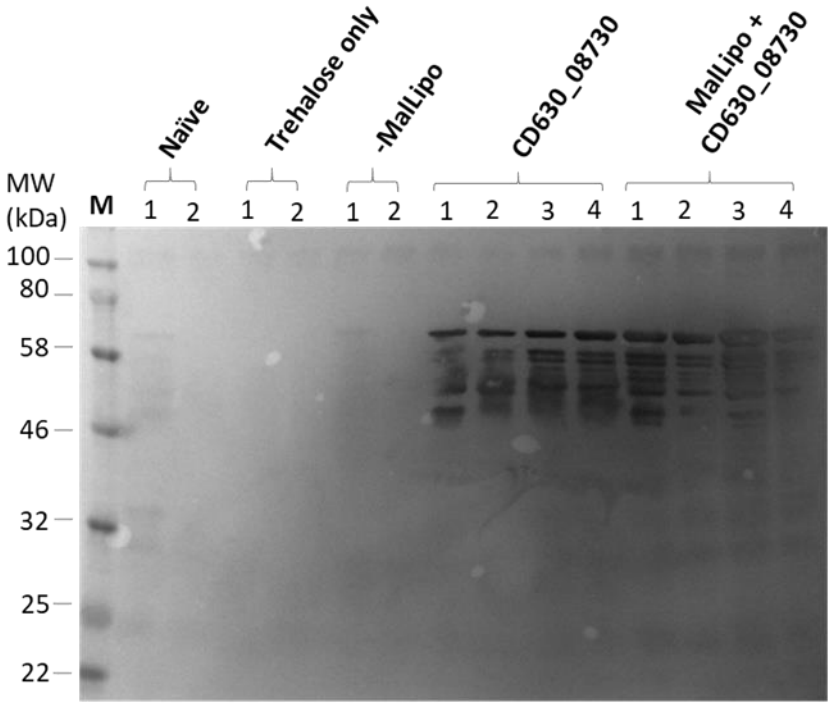

Figure 6.1- Detection of intestinal fluid sIgA in orally immunised or naïve hamsters by Western immunoblotting.
Intestinal fluid harvested from naïve (n=2), trehalose only (n=2), -MalLipo (n=2) CD630_08730 (n=4) and MalLipo + CD630_08730 (n=4) hamsters was tested for presence of sIgA. Following flushing of the small intestine with 5 ml of PBS containing protease inhibitors, the intestinal fluid was diluted 1:2 and 5 µl of intestinal fluid from each hamster, was fractionated using 10% (w/v) Tris-glycine SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was probed with rabbit anti-hamster IgA antibody (1:1000) (Brookwood Biomedical) followed by anti-rabbit IgG HRP antibody (1:1000) (CST) and visualised by 3, 3', 5, 5'-Tetramethylbenzidine (TMB). M: Colour Pre-stained Protein standard (NEB).

Due to fractionation on a denaturing gel, the heavy and light chains of antibodies separate. The antibody used for the Western immunoblot specifically detects the heavy chain of sIgA. The heavy chain of hamster sIgA was shown previously to migrate between 51 kDa and 86 kDa (Leher *et al*., 1998). A strong immuno-reactive band of approximately 60 kDa was detected in the intestinal fluid of hamsters vaccinated with CD630_08730 and MalLipo + CD630_08730 formulations, unlike in

control groups (Figure 6.1). Due to the anti-IgA antibody being suitable for Western immunoblotting only and not suitable for use in ELISA, it was not possible to quantitate the titre of antibody, or accurately compare the levels between the 2 immunised groups.

6.2.2 Serum IgG titres detected in immunised hamsters by ELISA

The titre of antigen-specific serum IgG was compared between groups by using serum from each group and quantifying the binding of IgG to CD630_08730 and CD630_TcdB-RBD by indirect ELISA (section 2.8.1.2). 96-well plates were coated with 2.5 µg/ml of each purified antigen. Plates were incubated with serum diluted 1:10. This was followed by incubation with anti-hamster IgG (H +L) highly cross adsorbed-Biotin antibody produced in goat (Sigma-Aldrich) at a 1:20 000 dilution followed by incubation with Streptavidin-HRP (RD Systems-Fisher Scientific) at 1:200 dilution. Absorbance measurements were taken using TMB at 650 nm wavelength (Figure 6.2).

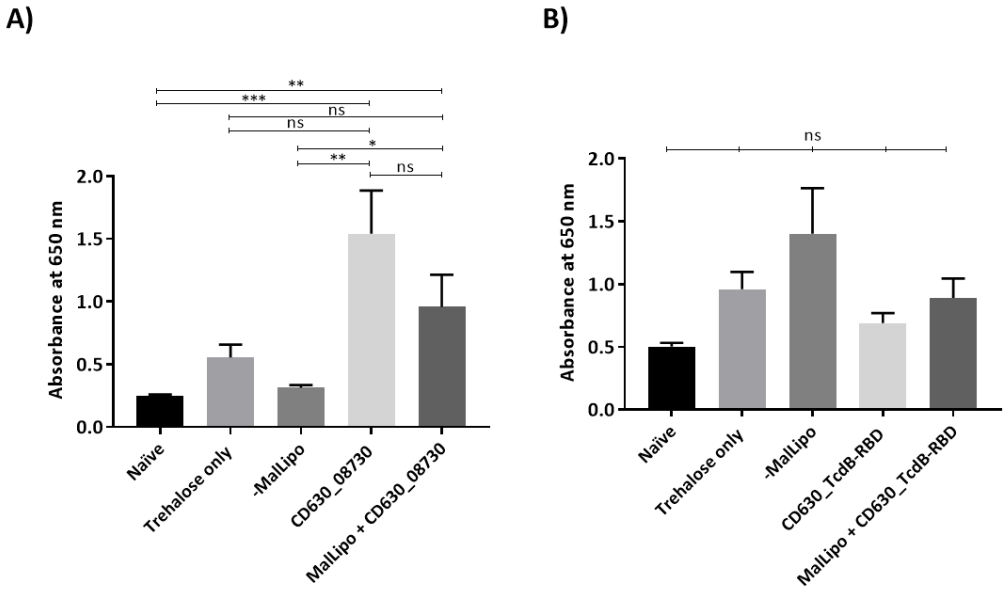

Figure 6.2- Serum IgG responses from orally immunised or naïve hamsters tested by indirect ELISA.

Serum was tested for IgG by indirect ELISA using 1:10 diluted sera from naïve (n=2) or orally immunised trehalose only (n=2), -MalLipo (n=4), CD630_08730 (n=4), MalLipo + CD630_08730 (n=4), CD630_TcdB-RBD (n=4) and MalLipo + CD630_TcdB-RBD (n=4) hamster groups. Using ELISA plates coated with 2.5 µg/ml of recombinant protein A) CD630_08730 and B) CD630_TcdB-RBD, serum IgG responses were detected with goat anti-hamster IgG (H +L) highly cross adsorbed-Biotin antibody (1:20 000) (Sigma-Aldrich) followed by incubation with Streptavidin-HRP (1:200) (RD Systems- Fisher Scientific). Absorbance measurements were taken using 3, 3', 5, 5'-Tetramethylbenzidine (TMB) at 650 nm wavelength. Two independent experiments were performed with three technical replicates. A non-parametric Kruskal-Wallis test was performed followed by Dunn's multiple comparison test to compare means of control groups with experimental groups. Error bars represent standard error of the mean (SEM). Statistical difference *p*-value; (ns) not significant, (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

The titre of serum IgG in hamsters immunised with CD630_08730 and CD630_TcdB-RBD, either alone or conjugated to liposomes was compared in addition to that of the control groups. Using the non-parametric Kruskal-Wallis test, mean absorbance values were compared. The CD630_08730 and MalLipo + CD630_08730 immunised

groups showed a significant IgG response relative to the naïve group (p = 0.0009 and p = 0.0037 respectively) and relative to the -MalLipo control group (p = 0.0029 and p = 0.0152 respectively) with no significance compared to the trehalose only group (p > 0.9999). Surprisingly, for the CD630_08730 immunised group, the antigen-specific IgG detected was higher than that of the MalLipo + CD630_08730 immunised group however, this was non-significant (p > 0.9999) (Figure 6.2, A). For the CD630_TcdB-RBD immunised group, Figure 6.2 (B), the MalLipo + CD630_TcdB-RBD group showed a slightly higher response compared to the CD630_TcdB-RBD group however neither experimental group showed a significantly higher titre of antigen-specific serum IgG compared to the control groups.

6.2.3 Toxin neutralisation ability of immunised hamster serum and intestinal fluid

Despite the non-significance in IgG titre observed, a toxin neutralisation assay was performed to test the ability of serum and intestinal fluid from the immunised groups; CD630_TcdB-RBD and MalLipo + CD630_TcdB-RBD to neutralise the cytotoxicity of FL TcdA and FL TcdB (50 ng/ml and 0.25 ng/ml, respectively) (Public Health England) on Vero cells. Using a series of 2-fold dilutions, serum and intestinal fluid was pre-incubated with FL TcdA and FL TcdB and added to Vero cells seeded onto 96-well plates at a density of 1 x 10^5/ml (section 2.8.1.3).

The colorimetric detection used for this assay is an MTT solution (yellow) which is reduced to formazan crystals (purple) in the presence of metabolically active cells.

Absorbance measures were taken at 570 nm wavelength. As an initial test, immunised hamster serum/intestinal fluid groups used for the assay were; naïve (n=1), CD630_TcdB-RBD (n=2) and MalLipo + CD630_TcdB-RBD (n=2) (Figure 6.3 and

6.4). Vero cells incubated with FL TcdA and FL TcdB only were used as a positive control for cytotoxicity, and Vero cells only were used as a negative control for cytotoxicity. The degree of toxin neutralisation by antibodies present in biological fluids was measured against these controls. Each individual serum and intestinal fluid samples were tested on separate 96-well plates containing Vero cells, and are therefore presented on separate graphs (Figure 6.3 and 6.4).

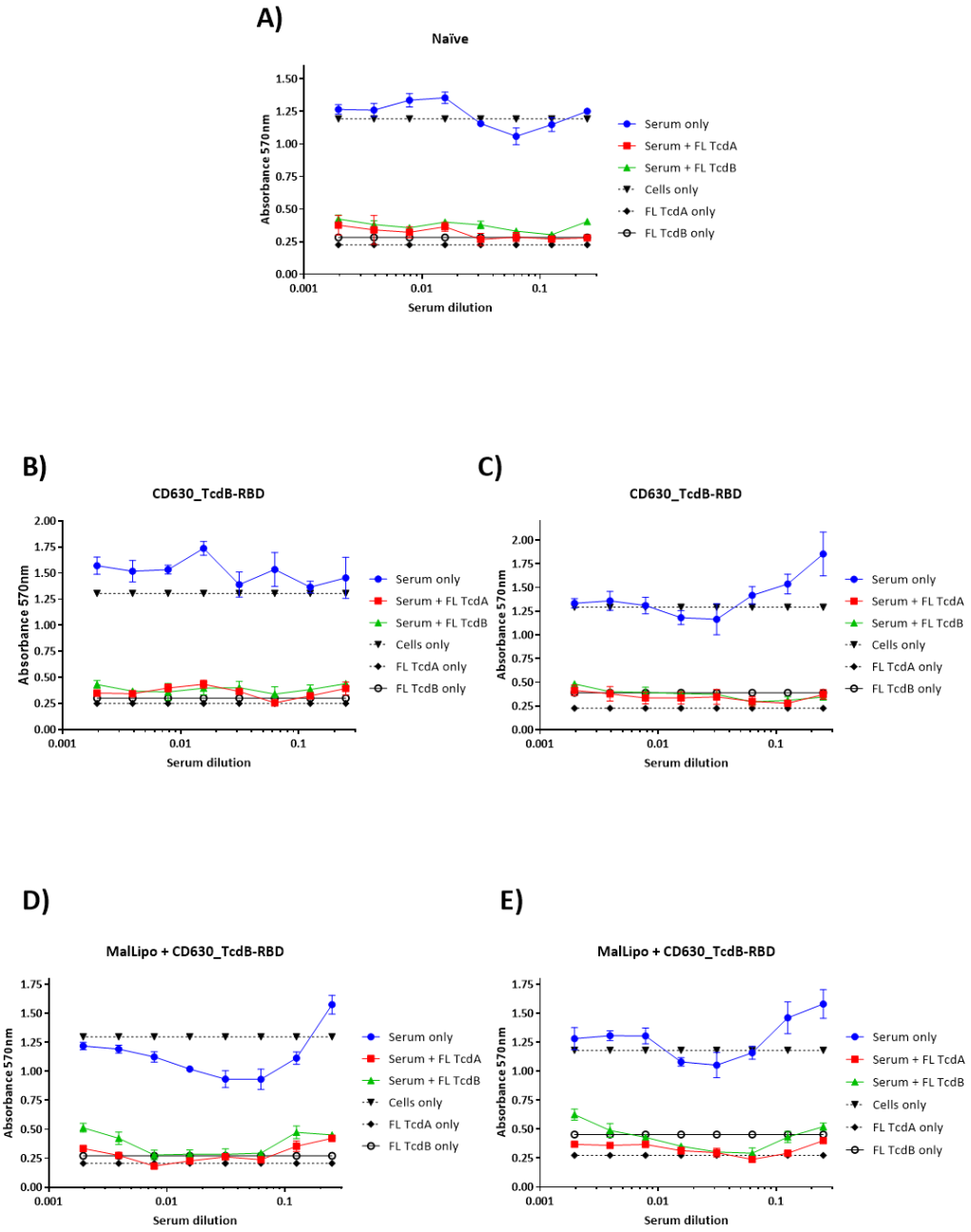

Figure 6.3- MTT assay to measure toxin neutralisation ability of orally immunised or naïve hamster serum using Vero cells.

Serum dilutions (1:4 to 1:512) of hamster groups; A) naïve (n=1) and orally immunised B-C) CD630_TcdB-RBD (n=2) and D-E) MalLipo + CD630_TcdB-RBD were pre-incubated with FL TcdA (50 ng/ml) (used to test cross reactivity) and FL TcdB (0.25 ng/ml) (Public Health England) were added to Vero cells (1 x 10^5/ml). Serum only and Cells only as negative control and FL TcdA and FL TcdB as positive controls were included. All sample dilutions were tested in triplicate. Cell rounding caused by FL TcdA/FL TcdB cytotoxicity was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution at 570 nm wavelength.

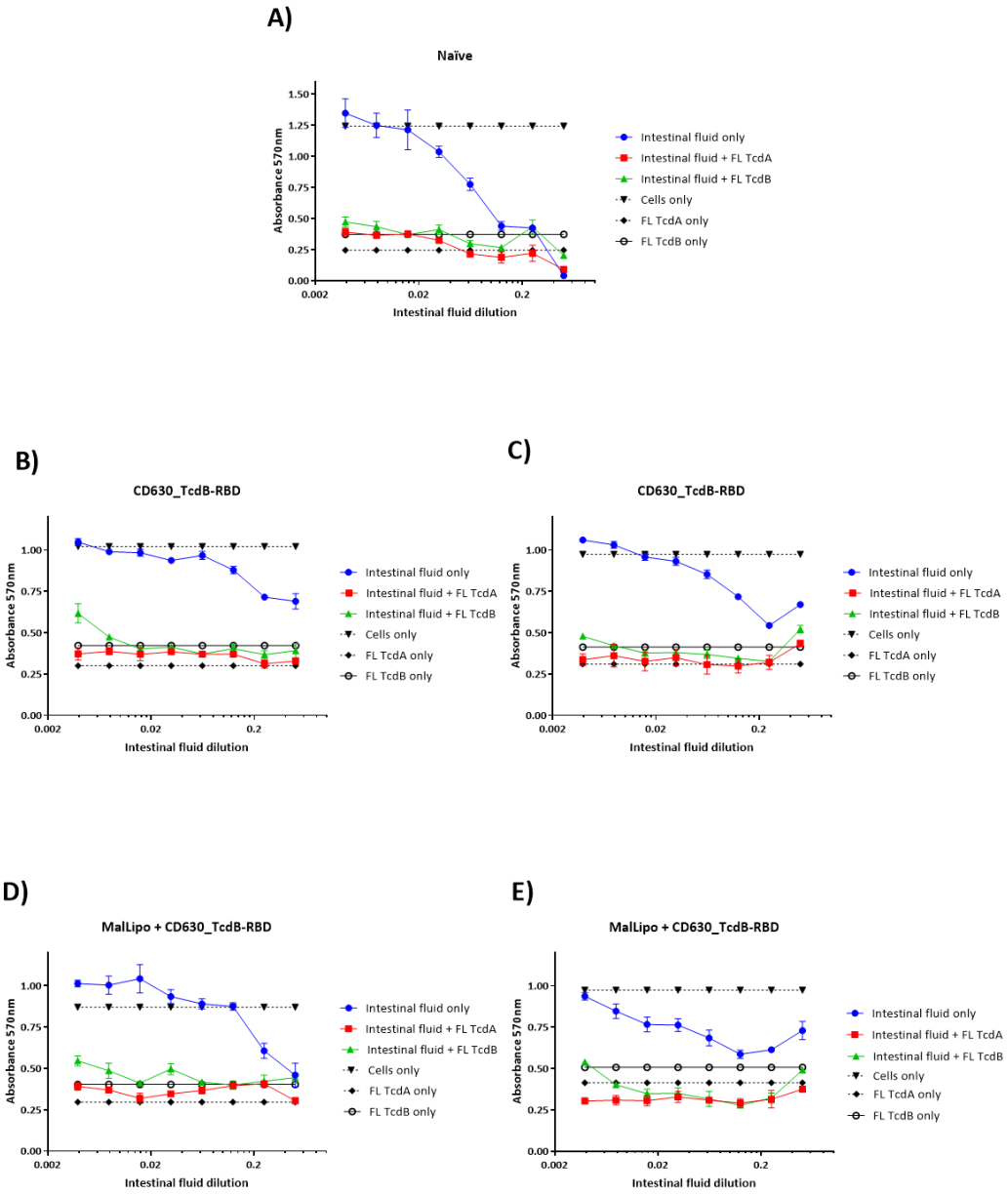

Figure 6.4- MTT assay to measure toxin neutralisation ability of orally immunised or naïve hamster intestinal fluid using Vero cells.

Intestinal fluid dilutions (1:2 to 1:128) of hamster groups; A) naïve (n=1) and orally immunised B-C) CD630_TcdB-RBD (n=2) and D-E) MalLipo + CD630_TcdB-RBD were pre-incubated with FL TcdA (50 ng/ml) (used to test cross reactivity) and FL TcdB (0.25 ng/ml) (Public Health England) were added to Vero cells (1 x 10^5/ml). Intestinal fluid only and Cells only as negative control and FL TcdA and FL TcdB as positive controls were included. All sample dilutions were tested in triplicate. Cell rounding caused by FL TcdA/FL TcdB cytotoxicity was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution at 570 nm wavelength.

All serum, Figure 6.3 (A-E) and intestinal fluid, Figure 6.4 (A-E) samples tested showed no ability to neutralise FL TcdA or FL TcdB. As seen with Figure 6.3 (A), no difference was observed between the sera of the CD630_TcdB and MalLipo + CD630_TcdB-RBD groups compared to that of the naïve group. Each of these groups showed a similar absorbance reading relative to Vero cells incubated with FL TcdA and FL TcdB only (positive control for cytotoxicity) (Figure 6.3, B-E). The Vero cells which were incubated with the higher concentration of serum only (with no toxin added) showed an increase in absorbance compared to the cells only control (Figure 6.3). This indicates that the serum caused an increase in metabolically active cells visualised by the further reduction of the MTT solution to formazan. However unlike the increase in absorbance caused by the serum only control, for the intestinal fluid only control, specifically the higher concentrations of intestinal fluid, cell death was observed (Figure 6.4). This was visualised by cell rounding and cells lifting off the plate and also by the decrease in absorbance reading compared to the cells only control with less reduction of the MTT solution to formazan. Thus growth promoting factors in the serum and cell damaging factors in intestinal fluid skew the results of this assay.

6.2.4 Adherence blocking of *C. difficile* to Caco-2 cells caused by immunised hamster serum and intestinal fluid

In order to test the ability of antibodies in serum and intestinal fluid of immunised groups; CD630_08730 and MalLipo + CD630_08730, to block *C. difficile* adherence to epithelial cells, an adherence blocking assay was performed using Caco-2 cells. Cells of *C. difficile* were pre-incubated with serum or intestinal fluid prior to their addition to Caco-2 cells. Adhered bacteria were determined by CFU/ml using MOI of 1:20 and

1:5 Caco-2 cells to bacteria with serum and intestinal fluid respectively (section 2.8.1.4) (Figure 6.5).

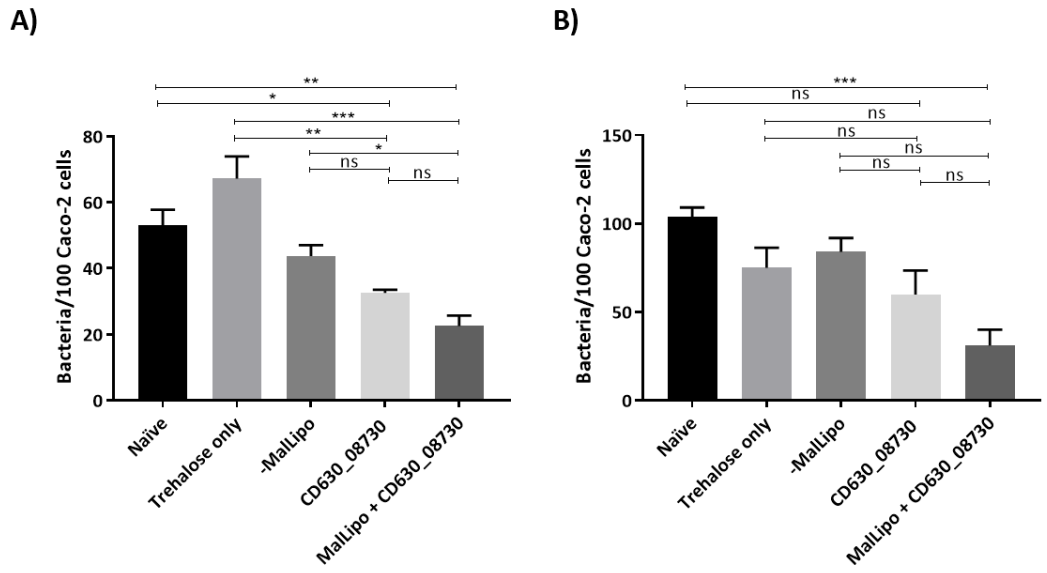

Figure 6.5- Reduced adherence of *C. difficile* strain 630 to Caco-2 cells by CD630_08730 immunised hamster antibodies.
Adherence of *C. difficile* to Caco-2 cells when pre-incubated with A) serum (1:5) and MOI (1:20) and B) intestinal fluid (1:2) and MOI (1:5) from naïve (n=2) and orally immunised trehalose only (n=2), -MalLipo (n=2), CD630_08730 (n=4) and MalLipo + CD630_08730 (n=4) hamster groups. Caco-2 cells were washed and mean numbers of adherent bacteria enumerated by CFU/100 Caco-2 cells represented. This assay was performed using three technical replicates. A non-parametric Kruskal-Wallis test was performed followed by a Dunn's multiple comparison test to compare means of control groups with experimental groups. Error bars represent standard error of the mean (SEM). Statistical difference *p*-value; (ns) not significant, (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

The number of bacterial cells pre-incubated with serum from the CD630_08730 immunised group that adhered to Caco-2 cells was significantly reduced relative to that of the naïve group ($p = 0.0364$) and trehalose only group ($p = 0.0056$) (Figure 6.5, A). Although a reduction relative to the MalLipo control group, this was not significant ($p > 0.9999$). An even greater reduction in adherence was observed with

serum from the MalLipo + CD630_08730 group relative to the naïve (p = 0.0011), trehalose only (p = 0.0001) and -MalLipo (p = 0.0492) control groups (Figure 6.5, A). Although further reduction in adherence was caused by the MalLipo + CD630_08730 group compared to the CD630_0830 group, this was not significant (p > 0.9999) (Figure 6.5, A). A similar pattern in bacterial adherence reduction was observed with intestinal fluid (Figure 6.5, B). Some degree of reduction in bacterial cell binding to Caco-2 cells was observed with the CD630_08730 intestinal fluid group compared to the control groups, however this reduction was not significant relative to any of the control groups; naïve (p = 0.1808), trehalose only and MalLipo groups (p > 0.9999 respectively). On the other hand, as observed with the MalLipo + CD630_08730 serum group, the intestinal fluid revealed a significant reduction in bacterial cell binding compared to the naïve control (p = 0.0021) (Figure 6.5, B). However this reduction was not significant relative to the trehalose only (p = 0.3076), -MalLipo control (p = 0.1743) and the CD630_08730 (p = 0.9951) groups (Figure 6.5, B).

The further reduced binding caused by the serum and intestinal fluid from the MalLipo + CD630_08730 group compared to the CD630_08730 indicates enhanced immunogenicity with higher serum IgG and mucosal sIgA and increased binding of antibodies capable of blocking *C. difficile* binding to Caco-2 cells.

6.3 Protective efficacy of *C. difficile*-directed oral vaccine in hamsters

Due to the encouraging immunogenicity data observed with all formulations containing CD630_08730, a pilot study was conducted in hamsters to test the ability of this antigen per se to protect against CDI in the hamster model.

6.3.1 *In vivo* immunisation

Female Golden Syrian hamsters were immunised as described in section 2.8.1. Hamster groups included control groups; naïve group (n=2) (non-immunised) and toxoids (i.m.) (mock Sanofi Pasteur vaccine) (n=2) and experimental group; CD630_08730 (oral) (n=4) (Table 6.2). The toxoids (FL TcdA and FL TcdB) (The Native Antigen Company, Oxfordshire, UK) were adjuvanted with Alhydrogel® adjuvant 2% ALUM (InvivoGen, Toulouse, France) and administered as an additional control (Aboudola *et al*., 2003; Anosova *et al*., 2013). The toxoids group was given an i.m. injection following anaesthesia using 1.5% (v/v) Isoflurane on the same days as groups orally administered with CD630_08730. As before, 1 mg of antigen in excipient, trehalose was lyophilised and packed into a gelatin capsule and dip-coated once with 12.5% Eudragit® L100 + 10% TEC + 3% H_2O (section 2.6.6 and 2.7.1). A total of 3 immunisations were administered at 14 day intervals.

Table 6.2- Hamster study groups immunised for *C. difficile* challenge study.

Hamster study group	Delivered components	Route of delivery	No. of group (n)	Total immunisation
Naïve	Non-immunised	-	n= 2	-
CD630_08730 (oral)	1 mg CD630_08730 + 1 mg trehalose	oral	n=4	3
Toxoids (i.m.)	3 µg Toxoid A + 2 µg Toxoid B + 5 µg ALUM + 15 µl PBS	i.m.	n=2	3

Abbreviation 2: intramuscular; i.m., ALUM: aluminium hydroxide

6.3.2 Oral delivery of CD630_08730 provides partial protection from *C. difficile* infection in hamsters

Following immunisations, hamsters were treated with clindamycin then infected with 10^3 spores of *C. difficile* strain R20291*ermB* (section 2.8.2) (Figure 6.6). Hamsters were monitored every 4 hours and weight measured daily. The caecum and large intestine were taken at the humane or experimental end point, 2 weeks post challenge. In order to determine bacterial burden in challenged animals, faecal pellets were taken daily and spores enumerated. Faecal pellets were processed as stated in section 2.8.2.1 and *C. difficile* strain R20291*ermB* spores were enumerated by plating faecal pellets onto TCCFA agar (Table 2.2 and 2.3). Data is presented as CFU/mg (Figure 6.7).

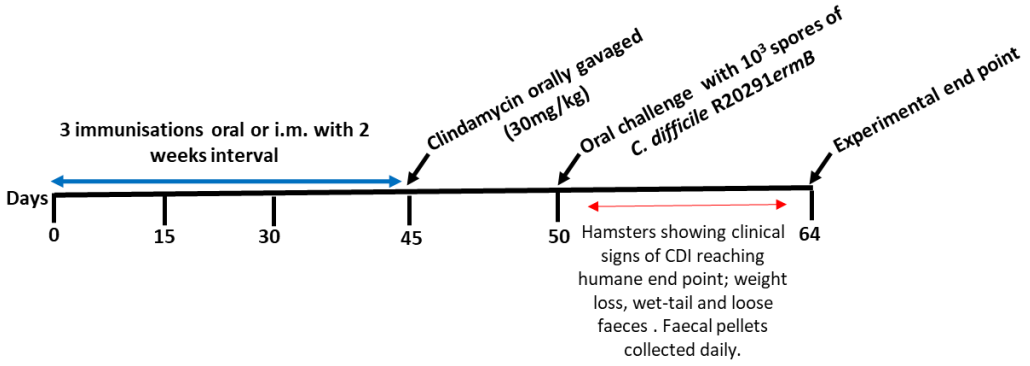

Figure 6.6- Schematic diagram of immunisation and *C. difficile* challenge protocol used in hamsters.
Hamsters were immunised on days 1, 15 and 30 either orally or by i.m. injection. Hamster were then orally gavaged with clindamycin (30 mg/kg), 2 weeks post final immunisation on day 45 and orally challenged 5 days later with 10^3 spores of *C. difficile* strain R20291*ermB* on day 50. Hamsters showing clinical signs of CDI reaching humane end point such as weight loss, wet-tail, loose faeces were euthanised. Hamsters that survived 2 weeks post challenge were euthanised at the end point of study.

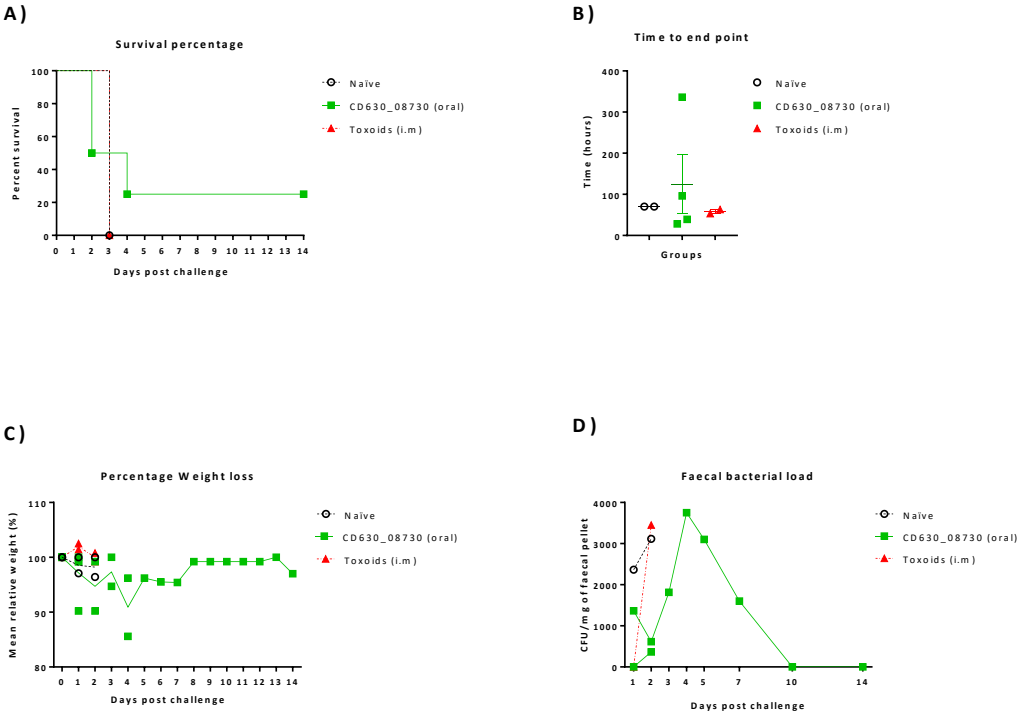

Figure 6.7- Clinical outcome of hamsters orally immunised with recombinant CD630_08730 followed by challenge with hypervirulent strain of *C. difficile*.
Hamster groups naïve (n=2) or immunised CD630_08730 (oral) (n=4) and toxoids (i.m.) (n=4) receiving dosing on days 1, 15 and 30 were treated orally with clindamycin (30 mg/kg) on day 45 and challenged 5 days later with 10^3 spores of *C. difficile* strain R20291*ermB* on day 50. Clinical outcome post challenge showing A) survival percentage of hamsters, B) time to end point for each hamster, C) individual percentage weight loss from starting weight with lines representing means for each group and D) *C. difficile* spore counts shed in faeces from individual hamsters where detected. Data for the CD630_08730 immunised group was compared with the naïve group to investigate significance. For analysis of A) Kaplan-Meier survival graph a log-rank Mantel-Cox test was performed and for B-D) a Mann-Whitney U test was performed. Non-significance (p > 0.05) was reported for all data sets. Error bars represent standard error of the mean (SEM).

The percentage survival of hamsters orally immunised with CD630_08730 was 25% at day 14 post *C. difficile* strain R20291*ermB* spore challenge (Figure 6.7, A). The surviving hamster displayed no clinical signs of infection and was euthanised on day 14 at the experimental end point. Its caecum appeared normal and non-enlarged post mortem. The remaining hamsters including the control groups, naïve and

toxoids had all reached the humane end point by day 3, post challenge (Figure 6.7, A). The toxoids provided no protection in i.m.-injected hamsters. Of the CD630_08730 immunised group, another hamster showed mild symptoms with low clinical scoring, compared to the rest of the hamster groups but was euthanised on day 4. Post-mortem analysis of the caecum of this hamster revealed only minor swelling. The mean time to end point of the CD630_08730 group was 80% higher than the naïve group (Figure 6.7, B). All hamsters showed progressive weight loss at each daily measurement post infection (Figure 6.7, C). The surviving CD630_08730 immunised hamster although showing a 10% weight loss over the first 4 days post challenge, regained its weight over the next 4 days. Direct comparison of faecal spore counts between each hamster proved difficult as sometimes no spores were detected (Figure 6.7, D). However, on day 2, where spores from each group were detected, the mean spore counts for the CD630_08730 immunised group was lower relative to the naïve and toxoids–injected groups. By day 4, an increase in counts was observed for the CD630_08730 immunised survivor above that of all other animals that were culled at this point. Interestingly, from day 5, a decrease in spore counts was observed and by day 10, no spores were detected indicating clearance of infection (Figure 6.7, D).

6.3.3 PCR confirmation of *C. difficile* strain R20291*ermB* from challenged hamsters

Colonies obtained from faecal plating onto TCCFA plates were used as template DNA to confirm the presence of the infecting strain by PCR amplification. In addition to this, caecum content homogenates were also plated onto TCCFA plates for PCR confirmation where faecal colonies were not detected (section 2.8.2.1). The *C.*

difficile strain R20291*ermB* specific primers used were; cdi-630-pyrD-sF1 and ermBHindII-R (Kelly *et al*., 2016) and the 16S specific primers used were; Uni-00027-F and Uni-1492-R (Table 2.11) (Figure 6.8). The same 16S specific primers used for PCR were subsequently used for sequencing.

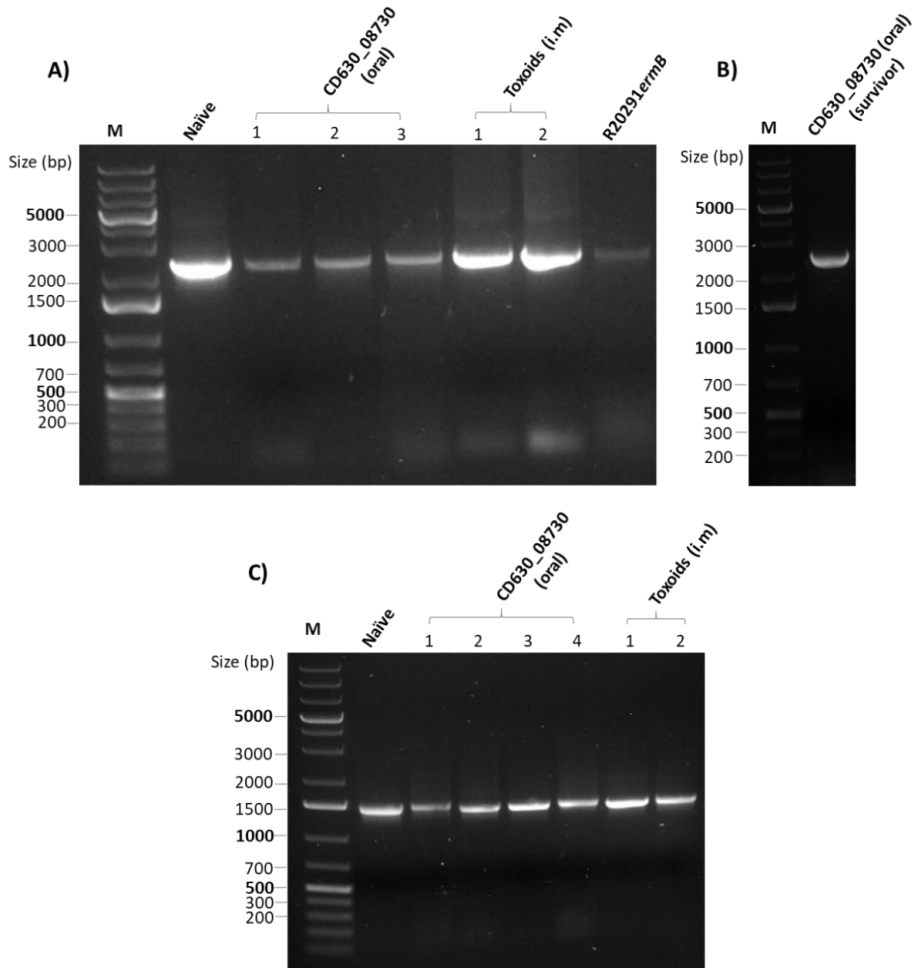

Figure 6.8- PCR confirmation of *C. difficile* strain R20291*ermB* from challenged hamsters.

Agarose gel electrophoresis showing PCR products obtained using A) *ermB* specific primers cdi-630-pyrD-sF1 and ermBHindII-R (Kelly *et al*., 2016) from hamster groups; naïve control (n=1), CD630_08730 (oral) (n=3), toxoids (i.m.) (n=2) and the *C. difficile* strain R20291*ermB* was used as a positive control and B) CD630_08730 (oral) (survivor) (n=1) and C) PCR products using 16S specific primers for sequencing. All PCR products were obtained directly from either faecal pellets or caecum content homogenates from challenged hamsters using colony PCR.

For all challenged hamsters, PCR confirmed the presence of the infecting strain, R20291*ermB.* Genomic DNA of this strain was used as template for a positive control for PCR (Figure 6.8, A-B). As no colonies were detected for 1 hamster from the naïve group using either the faecal or caecum contents for plating, we were unable to perform PCR confirmation (Figure 6.8, A). Importantly though we were able to confirm the challenged survivor was indeed infected with the *C. difficile* strain R20291*ermB* using faecal pellet homogenates as DNA template (Figure 6.8, B). The caecum plating for this survivor also revealed no spores, confirming clearance of infection as observed with faecal plating from days 10-14 of challenge. Additional PCR amplification of the 16S region was conducted and the infecting strain confirmed by sequencing following BLASTn® analysis (section 2.2.1) (Figure 6.8, C).

6.3.4 Histopathology assessment showing reduced pathology in CD630_08703 immunised hamsters

Histopathology of challenged hamsters was assessed for sections taken from the caecum. The most prominent clinical manifestation of *C. difficile*-infected hamsters is the enlargement of the caecum due to oedema, however pathology in the large intestine can sometimes be observed (Goulding *et al*., 2009). Caecal tissue samples were collected at the end point and immediately fixed in 10% (v/v) NBF. Histopathological assessment of the tissue samples was conducted in a blinded fashion by Philip Kaye (section 2.8.2.4). Tissues were sectioned at 5 µm, H&E stained and imaged using the Nanozoomer (Hamamatsu Photonics) slide scanner. Tissue sections were scored for oedema (0-3), neutrophil infiltration (0-3) and epithelial tissue damage (0-3) using the scoring system described by Pawlowski *et al* (2010)

where 0 is normal and 3 is severe. The mean accumulative score for each group is shown in Figure 6.9 for the caecal tissue.

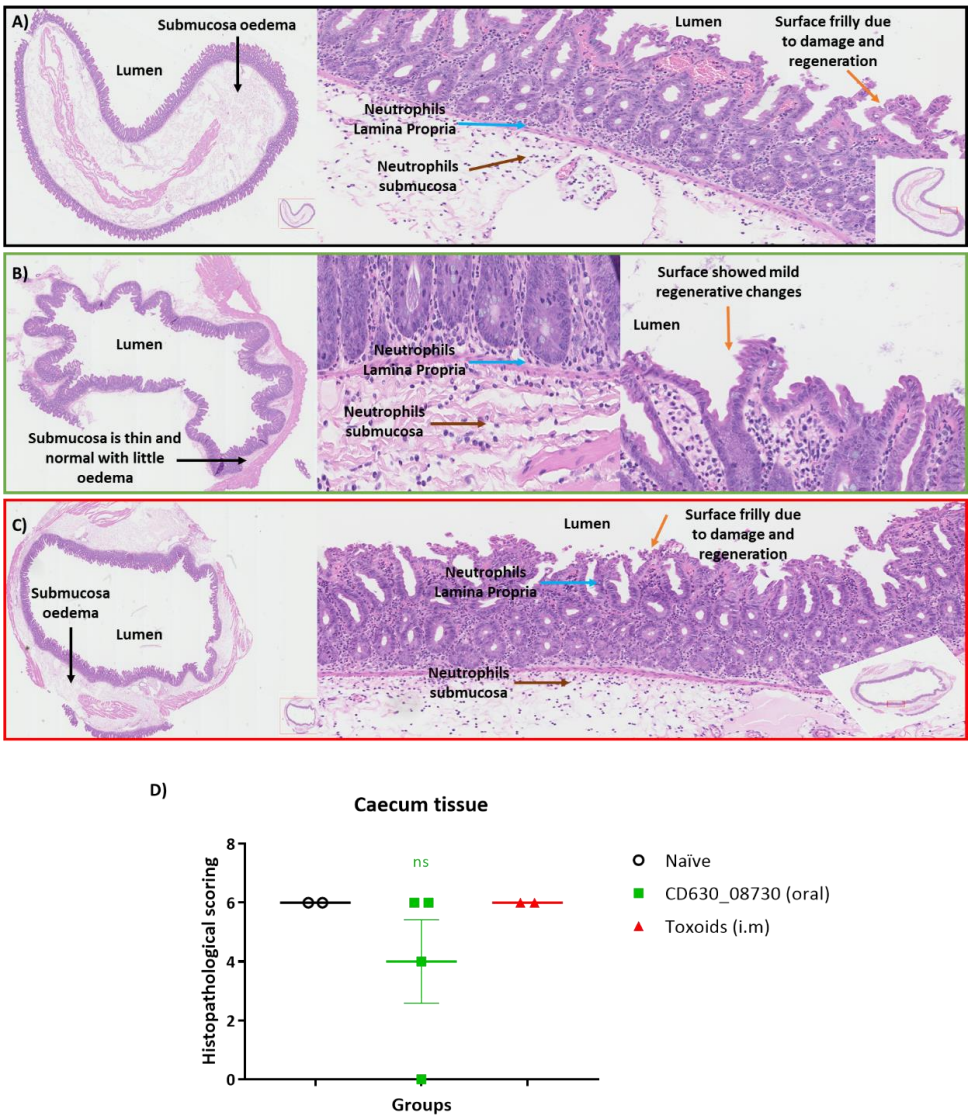

Figure 6.9- Histopathology of hamster caeca following oral immunisation with recombinant CD630_08730 and challenge with hypervirulent strain of *C. difficile*.
End point caecal tissue sections from all hamsters were fixed in 10% (v/v) neutral buffered formalin (NBF), sectioned at 5 µm and haematoxylin and eosin (H&E) stained. H&E stained images of caecum from A) naïve (n=1) and immunised B) CD630_08730 (oral) (survivor) (n=1) and C) toxoids (i.m.) (n=1). Blue and brown arrows indicate neutrophils in the Lamina Propria and submucosa respectively. Black arrows show the submucosa oedema and orange the epithelium. D) Caecal sections for all hamsters; naïve (n=2) and immunised CD630_08730 (oral) (n=4) (survivor n=1, hamster showing mild symptoms n=1 and others n=2) and toxoids (i.m.) (n=2) were scored for oedema (0-3), neutrophil infiltration (0-3) and tissue damage (0-3) with 0 indicating normal and 3 severe and presented as combined scores for individual hamsters. Combined scores of the naïve group were compared with the CD60_08730 immunised group using a Mann-Whitney U test. Error bars represent the standard error of mean (SEM). Non-significance ($p > 0.05$).

Representative images of H&E stained sections of the experimental group CD630_08730 survivor compared with that of naïve and toxoids groups are shown in Figure 6.9, A-C. The CD630_08730 immunised hamster (Figure 6.9, B), protected from CDI, showed remarkably reduced pathology compared to the naïve (Figure 6.9, A) and toxoids (Figure 6.9, D) group. This is shown with the reduced submucosa oedema, a relatively preserved epithelium and reduced neutrophil infiltration in the Lamina Propria and submucosa layer (Figure 6.9, A). Conversely the naïve and toxoids immunised hamsters showed substantial oedema, greater epithelium damage and neutrophil abundance in the Lamina Propria and the submucosa layer (Figure 6.9, A and C). Histopathological assessment of caecum for hamster groups was scored on oedema (0-3), neutrophil infiltration (0-3) and tissue damage (0-3) (Figure 6.9, D). The mean combined scores for all 3 criteria scoring out of 9 was compared for each hamster group. The CD630_08730 immunised group scored a mean of 4 out of 9 compared to the naïve and toxoids group which both scored a mean of 6 out of 9 (Figure 6.9, D). Although not significant, the CD630_08730-immunised group had a lower mean pathology score for the caecum compared to the control groups.

6.4 Discussion

The immunogenicity of selected *C. difficile* antigens alone and synthetically lapidated onto liposomes was assessed in this study. The antigens were conjugated, via their N-terminal cysteine, to the maleimide head group of a synthetic lipid, formulated in liposomes, MalLipo, for adjuvancy. Formulations were lyophilised then encapsulated and dip-coated once in optimised enteric polymer. Hamsters were immunised orally 3 times over 30 days. Experimental groups; CD630_08730, MalLipo + CD630_08730,

CD630_TcdB-RBD and MalLipo + CD630_TcdB-RBD were compared with the non-immunised (naïve), the trehalose only and -MalLipo groups (Table 6.1).

Two weeks following immunisation, hamster serum and intestinal fluid were collected for analysis. For oral vaccines, detection of intestinal sIgA is of prime importance (Corthésy, 2013). However, due to the absence of commercially available anti-hamster IgA antibody suitable for ELISA, a Western immunoblot of intestinal fluid fractionated on a denaturing gel was probed with a custom-produced anti-hamster IgA antibody. In all hamsters immunised with formulations based on CD630_08730, a distinct band corresponding to the MW of the heavy chain of IgA was observed. Despite the intestinal fluid being heavily diluted *i.e.*, in 5 ml of PBS and further diluted 1:2 in sample buffer, the IgA band was prominent suggesting an abundance of this antibody in immunised animals. Conversely, sIgA was either not detected or barely detected in animals from the control groups. The antibody concentrations could have been quantified by using Western immunoblotting for comparison between immunised groups however, this would require the use of purified hamster IgA of known concentration as a marker control which unfortunately was not commercially available and neither were we able to obtain this from other groups within the University of Nottingham. Hong *et al* (2017) previously showed the use of a secondary anti-mouse IgA HRP (Sigma) antibody for detection of sIgA in hamster faecal samples indicating cross reactivity of this mouse antibody to hamster antibodies. We also tested this secondary anti-mouse IgA antibody by ELISA using immunised hamster faecal and intestinal fluid samples. However, we were unable to detect any signal by using this antibody. Perhaps further optimisation would have been required when using this antibody which was a

limitation as sample volumes were not sufficient enough, particularly those of control hamsters or this antibody was unable to bind to hamster IgA. Animals immunised with CD630_08730 based formulations additionally showed an antigen-specific systemic IgG response. Following quantification by ELISA, the IgG titre in the serum of immunised animals was significantly higher than that of the naïve and -MalLipo groups (Figure 6.2, A). Interestingly, the IgG response was greater in the CD630_08730 immunised group than in the MalLipo + CD630_08730 group. Considering all samples used for immunisation contained the same amount of trehalose, we suspect the non-significance observed between the trehalose only and the CD630_08730 based formulations could have been a result of some contamination causing an antibody response.

Conversely for hamsters immunised with CD630_TcdB-RBD or MalLipo + CD630_TcdB-RBD a non-significant antigen-specific serum IgG titres were detected. This indicates that an immune response towards the RBD of TcdB was not successful in this study. We speculate that the recombinant protein CD630_TcdB-RBD may have not reconstituted in the intestinal fluid of immunised hamsters based on its poor solubility. For lyophilised protein to successfully induce antibody responses, the protein must regain its structure and immunologically important epitopes re-formed to be recognised.

Although non-significant serum IgG titres were observed for groups given CD630_TcdB-RBD-based formulations, a toxin neutralisation assay was nonetheless performed to test for any ability of these antibodies to neutralise FL TcdB. As TcdA and TcdB share 68% amino acid homology, neutralisation of both toxins was assessed

(Pruitt *et al*., 2010). No toxin neutralisation was observed with serum or intestinal fluid of groups immunised with CD630_TcdB-RBD-based formulations. Supporting the ELISA data, we conclude that insufficient sIgA or IgG was generated to achieve any toxin neutralisation.

As CD630_08730 is a known colonisation factor, an adherence blocking assay was performed with serum and intestinal fluid from animals immunised with CD630_08730-based formulations. Significant blocking of *C. difficile* cells to Caco-2 cells was observed relative to control groups with vaccinated serum proving to be the more effective than vaccinated intestinal fluid. Both serum and intestinal fluids from the MalLipo + CD630_08730 vaccinated group, resulted in greater adherence blocking than that observed for the CD630_08730 immunised group despite a slightly higher titre of serum IgG observed for the latter. The difference in adherence blocking between these two groups however was not significant. The biggest difference was seen with the intestinal fluid of these two immunised groups. The data suggest that either the lipidation of CD630_08730 per se, or the delivery of CD630_08730 on liposomes or both resulted in an enhanced B cell response. Indeed palmitoylation of antigens, whether native or synthetic is known to enhance antibody responses by the lipid moiety acting as a TLR2 agonist (Chen *et al*., 2009; Huang *et al*., 2015; Jackson *et al*., 2004; Khan *et al*., 2008; Moyle *et al*., 2014). Together, with the ELISA data, this study further confirms that oral delivery of enteric coated capsules encapsulated with antigens can successfully be used to deliver vaccine components. Moreover, the use of semi-synthetic lipoproteins presented on liposomes could serve a mucosal delivery platform for enhancing immunogenicity as

observed from the further reduced binding of *C. difficile* to Caco-2 cells with MalLipo + CD630_08730 immunised hamster serum and intestinal fluid.

To test the inherent protective efficacy of antigen CD630_08730, an immunisation-challenge study was conducted. Groups included naïve control, CD630_08730 given orally and a mock Sanofi Pasteur toxoids vaccine given via the i.m. route as an additional control (Anosova *et al*., 2013) (Table 6.2). Hamsters were immunised as before, treated with clindamycin then challenged with *C. difficile* strain R20291*ermB*. This strain was specifically selected as it confers resistance to erythromycin as well as clindamycin thus minimising sensitivity of the inoculum at the time of infection which has previously been reported (Kelly *et al*., 2016). In addition to this using this strain benefits from PCR confirmation of *ermB* using faecal homogenates. Surface-exposure of CD630_08730 on the strain R20291*ermB* was confirmed by whole cell Immuno-dot blotting using anti-CD0873 antibody prior to challenge in hamsters (section 2.5.7) (Figure A4) (Kovacs-Simon *et al*., 2014). The experimental group CD630_08730 showed 25% protection against *C. difficile* challenge with no survivors from the naïve control and toxoids hamster groups (Figure 6.7, A). The faecal spore shedding profile for this hamster that showed protection, was low compared the control groups at day 2 and this shedding peaked at day 4 (Figure 6.7, B). However, by day 7 a gradual decrease was observed with infection cleared as no spores were detected from day 10 until the experimental end point, day 14 (Figure 6.7, A). This spore shedding profile inversely correlated with the 10% weight loss observed on day 4 and weight regained by day 8 (Figure 6.7, C). For control group hamsters from which no spores could be detected in faecal pellets, yet CDI was clearly manifest, it may be that their culling within 3 days post challenge was not sufficient time for germinated

vegetative cells to have sporulated. As only spores were enumerated rather than vegetative cells, tracking bacterial burden was not possible for all animals. The increase in time to end point in the vaccinated group was 80% compared to the naïve group (Figure 6.7, B). The data in this study suggests that CD630_08730 delivered orally mounts mucosal antibody responses that inhibit colonisation as observed with the *in vitro* adherence blocking assay (Figure 6.5) and thus interferes with an early stage of pathogenesis. Indeed markedly reduced pathology of the caecum was observed for this hamster compared to that of control groups (Figure 6.9). This hamster scored a total of 4 out of 9 following histopathological assessment in comparison to the 2 other hamsters in this group which scored 6. This includes the hamster which showed milder symptoms and a slightly delayed time to end point compared to all other animals. However, 1 hamster of the CD630_08730 immunised group scored 0, despite having the earliest time to end point. This hamster showed haemorrhage rather than inflammation. As this hamster presented with some weight loss prior to challenge, it was suspected that other underlining conditions may have resulted in the observed early end point for this hamsters. The pilot study conducted showed a clear trend for the protective efficacy of CD630_08730 against CDI despite the small n values used and suggests that toxin mediated damage is intercepted either by sIgA coating of *C. difficile*, blocking adhesion thus preventing close proximity of toxins to epithelial cells or by directly blocking the secretion of these toxins or even directly neutralising these toxins.

To conclude given the ability of orally delivered CD630_08730 antigen to elicit mucosal and systemic immune responses as well as provide partial protection against *C. difficile*, the potential for CD630_08730 as an oral vaccine candidate is highlighted.

The logical next step is to assess whether greater protection is offered by the lipidated antigen presented on liposomes or given alone as a micelle formulation. With the data generated from this pilot study, power calculations can be performed to determine minimum but relevant group sizes to establish the degree of significance regarding the protective efficacies of these formulations. Modifications to the experimental design would be to lower the spore inoculum to resemble more realistically natural infection. Due to the lockdown and restrictions with using other buildings post lockdown, it was unfortunately not possible to generate any more liposomal formulations and test their protective efficacy.

Chapter 7

General discussion

7 General discussion

C. difficile is a gut pathogen which is the leading cause of nosocomial antibiotic-associated diarrhoea spread by the transmission of ingested spores (Wang *et al*., 2018). Symptoms range from mild diarrhoea to severe pseudomembranous colitis, toxic mega colon, sepsis and death (Riley *et al*., 2019; Walker *et al*., 2012). Predisposition of CDI is associated with the use of antibiotics and proton pump inhibitors, advanced age, immunosuppressive treatment and other underlying gastrointestinal conditions (Bignardi, 1998; Hung *et al*., 2013; Loo *et al*., 2011; Thibault *et al*., 1991; Trifan *et al*., 2017). Recurrence of CDI is the major concern which is defined by CDI symptoms appearing 8 weeks following treatment. The relapse percentage in patients treated for an initial episode of CDI is 15-35% and up to 60% in patients who have suffered two or more episodes (Singh *et al*., 2019). The risk of relapse is even greater in patients infected with hypervirulent strains 027/BI/NAPI, since these strains are more difficult to control with conventional treatment; oral vancomycin and/or fidaxomicin/FMT (McDonald *et al*., 2018; Singh *et al*., 2019). Inevitably, recurrent infections can be untreatable and thus the need for prophylactics realised. Clinical trials for preventative measures have focused exclusively on i.m. delivery of TcdA and TcdB which causes colonic epithelium damage as it has been shown that circulating anti-toxin antibodies correlate with protection (Abouldala *et al*., 2013; Aronsson *et al*., 1985; Kyne *et al*., 2001; Leav *et al*., 2010; Wullt *et al*., 2012). However, Sanofi Pasteur's vaccine in phase III clinical trials was terminated when it was concluded that protection against CDI could not be achieved by this approach. This has raised concerns for two other very similar vaccines in trials, with Valneva and Pfizer utilising i.m. delivery of recombinant

chimeric toxin fusion and toxoids respectively (Riley *et al*., 2019). These vaccines aim to elicit systemic toxin-neutralising IgG responses but do not target colonisation. As *C. difficile* colonisation occurs in the colonic mucosa, oral mucosal vaccines which can target this mucosal site by production of a local intestinal immune response, specifically mucosal sIgA should be more effective against CDI. Several oral vaccines that have demonstrated strong sIgA production as well as systemic responses have shown strong protective efficacy against mucosal infections such as polio, rotavirus, adenovirus, *S. typhimurium* and *V. cholera* (Kang *et al*., 2018; Miquel-Clopés *et al*., 2018). In this project, an immunogenicity study was conducted to test oral administration of *C. difficile* recombinant proteins; colonisation factor, CD630_08730 and the terminal portion of the RDB of TcdB, CD630_TcdB-RBD administered alone and as antigens presented on liposomes by attachment to a TLR2 agonist.

7.1 Purification of recombinant *C. difficile* vaccine candidates and sero-reactivity screening

Synthetically lipidating vaccine candidates have majored on synthetic peptides to which a synthetic lipid moiety is attached via an N- or C-terminal cysteine residue. However, using peptides exploits only a small portion of the entire native protein expressed by the pathogen of interest (Malonis *et al*., 2020). The loss of important immunological epitopes and likely alterations in structure and thus epitope confirmation in the peptide itself limits the potential of peptides as vaccines (Malonis *et al*., 2020). The pTWIN1 expression vector was adapted to allow purification of recombinant antigens with an N-terminal cysteine and a C-terminal His tag. Addition of the His tag enabled purification of the target protein which was being lost in the flow-through due to premature cleavage of the intein tag and thus loss of the CBD.

The premature cleavage of the intein tag remains as a major bottleneck and was also encountered in this study (Nanda *et al*., 2020). Many attempts are currently being made to prevent premature cleavage of intein based purification systems to enable rapid, one-step purification systems of low cost that could be deployed for industrial scale production. Split-inteins have been explored to overcome this issue namely, *Ssp* DnaB and *Npu* DnaE (from *Nostoc punctiforme*) in which the N-terminal intein is fused to another tag and the C-terminal of the intein is fused to the target protein. The N-terminal intein with the tag is first immobilised and then the addition of the C-terminal intein fused to the target protein allows assembly of these intein fragments by affinity and hence the protein is released with a change in pH (Demonte *et al*., 2015; Han *et al*., 2019; Lu *et al*., 2011; Vila-Perelló *et al*., 2013). Although a very encouraging approach, this system requires the expression of two separate proteins and also depends on the binding affinity of the intein fragments (Pinto *et al*., 2020). Ultimately, the *in vivo* cleavage encountered in this study was actually put to use for the production of high yields of protein. In means of perhaps simplifying the use of the pTWIN1 vector, further studies could be conducted to eliminate the need of removing the His tag. This should include studying the impact of various amino acids closest to the *Ssp* DnaB intein tag, directly adjacent to the N-terminal cysteine of the target protein. Although this has been studied for the pTXB1/2 TWIN vectors (NEB) with the *Mxe* GyrA intein using 20 different amino acids, this has been to a lesser extent with the *Ssp* DnaB intein tag (Evans *et al.,* 1999*;* Southworth *et al*., 1999). However, for the *Ssp* DnaB intein tag, arginine following the N-terminal cysteine has been shown to not present with *in vivo* cleavage and could be deployed. Wider analysis using different amino acids should be exploited for using the pTWIN1 vector

specifically. This may also overcome the insolubility issue encountered in this study which led to incomplete cleavage of the intein tag for CD630_34640. The impact of additional amino acids will have on the protein structure and folding will need to be investigated with this. Another investigation could be further determining the expression conditions that could prevent premature cleavage. Despite, various conditions being tested in this study, a more detailed investigation of various factors could be more beneficial. Hosseini *et al* (2020) recently utilised the PTXB1 vector of the IMPACT™ Kit (NEB), which uses the *Mxe* GyrA intein for C-terminal tagging, investigating the expression of the V antigen of *Yersinia* by varying conditions such as medium, temperature and IPTG concentrations for induction based on the Taguchi statistical method. This study showed that the temperature and the medium used had the greatest impact on production of the fusion protein with no *in vivo* cleavage observed. In terms of preventing intein cleavage, these factors could be investigated. Nevertheless, the modified pTWIN1.His expression system generated in this study enabled the purification of entire proteins or large fragments of toxins with a unique N-terminal cysteine for lipid conjugation. Moreover this expression system allowed high yields of protein with a high degree of purity to be obtained (Kowalczyk *et al*., 2017).

The recombinant *C. difficile* vaccine candidates CD630_08760, CD630_08730, CD630_27190 and CD630_TcdB-RBD and the surrogate antigen GFP were successfully purified. Following immunogenicity screening using pooled *C. difficile* patient serum, proteins CD630_08730 and CD630_TcdB-RBD were chosen for investigation as they showed the highest sero-reactivity *i.e.*, binding to serum IgG and serum IgA (Figure 3.5 and A3). The sero-reactivity of CD630_08730 was also

previously shown by Wright *et al* (2008) using 6 patient serum. We therefore confirm that CD630_08730 could serve as a potential vaccine target given the observed sero-reactivity by testing a larger number of patient serum in this study indicating host recognition during infection. The protein crystal structure of CD630_08730 has been resolved by Bradshaw *et al* (2019), confirming that this lipoprotein specifically binds the amino acid tyrosine. *C. difficile* is capable of fermenting tyrosine to produce *para*-cresol which is bacteriostatic to the intestinal flora but can be highly tolerated by *C. difficile*, suggesting that this may offer an advantage to compete over other commensal bacteria enabling *C. difficile* to proliferate therefore, highlighting that CD630_08730 may play an key role in infection (Passmore *et al*., 2018; Dawson *et al*., 2011). Due to the shared homology with CD630_08730, CD630_08760 is also predicted to be a tyrosine substrate binding protein of the ABC transporter system. It was suggested by Bradshaw *et al* (2019) that as some clinical strains of ribotype 027 have previously shown convergent loss of neighbouring ABC transporter genes CD630_08760 and CD630_08770, that this deletion maybe due to selection pressure to stabilise the *para*-cresol production which may indeed be a mechanisms to ensure tolerability of *C. difficile* to this bacteriostatic compound. This may explain perhaps the lack of sero-reactivity of CD630_08760 observed in this study and therefore, limit its potential as a vaccine candidate (Steglich *et al*., 2018). The *N*-deacetylase PdaA putative lipoprotein, CD630_27190 which has been shown to be involved muramic-δ-lactam synthesis required for spore cortex hydrolysis in *C. difficile* also showed low sero-reactivity. The alternative PdaA identified is CD630_14300, in which deletion of this genes caused further defect in muramic-δ-lactam production compared to CD630_27190 however, deletion of both these genes resulted abolishment of

muramic-δ-lactam production *in vitro*. As both are required for complete disruption which leads to germination defects, it would be interesting to test the sero-reactivity of CD630_14300 using patient serum for future work (Coullon *et al*., 2018).

7.2 Protein conjugation to the surface of liposomes using a synthetic lipid adjuvant

In chapter 4, a synthetic lipid containing a maleimide head group, Mal lipid, with similar structure to that of the Pam2Cys TLR2 agonist was formulated with liposomes composed of DPPS, DPPC, Mal lipid and cholesterol to create MalLipo. Liposomes of particle size 100-200 nm in diameter were used for this study. A similar recombinant approach has previously been demonstrated by Moyle *et al* (2013) whereby a lipid adjuvant incorporating a maleimide group was attached to a recombinant *S. pyogenes* protein by a C-terminal cysteine introduced by site-directed mutagenesis. These semi-synthetic lipoproteins formed nanoparticles of 40 nm and when delivered via the i.m. route in mice showed enhanced immunogenicity compared to antigen received alone. However, bacterial lipoproteins are lipidated at the N-terminus therefore by expressing recombinant proteins with a unique N-terminal cysteine provides a closer mimic of native bacterial lipoproteins and also eliminates the need for site-directed mutagenesis for the production of proteins with a cysteine residue. When considering delivery of protein vaccine candidates using liposomal technology, it could be argued that encapsulation of proteins within the liposomal core could provide advantage in prevention of degradation. However, this may limit the presentation of proteins to APC for efficient immune stimulation. Barnier-Quer *et al* (2013) showed that when the influenza protein hemagglutinin was coupled to the liposomal surface, this proved to be more immunogenic with subcutaneous

delivery in mice when compared to being encapsulated in the liposomal core. Thus, by targeting M cells with the use of liposomes with surface-exposed antigens that are linked to a TLR2 agonist could provide enhanced immunogenicity.

In this study, GFP was used as a protein control to determine the minimum ratio of moles of protein to Mal lipid required for conjugation as determined by FACS. A comparison with formulations lacking the synthetic Mal lipid allowed us to quantify the fluorescence attributed to conjugation versus fluorescence attributed to electrostatic interactions of GFP with liposomes. This study confirmed that the majority of the conjugated GFP contributed to the Mal lipid permitting covalent attachment of GFP to the liposomal surface. The use of the Mal lipid formulated into liposomes provides a sufficient way of lapidating recombinant proteins to provide adjuvancy as well as to be incorporated to the surface of liposomal delivery vehicles minimising the steps required using chemical synthesis of non-native peptides (Kowalczyk *et al*., 2017). In addition to this, adjuvants are generally co-administered with vaccine antigens, however adjuvants can be dissociated from the antigen upon administration. By using liposomes which can serve as targets for M cells and increase the interaction on the mucosal surface, covalently attaching a TLR2 agonist to the antigen ensures DC activation (Xu and Moyle, 2018). This study also showed that lyophilisation of liposomes with the use of trehalose as a cryoprotectant, the liposomal integrity was maintained. This is of importance as it eliminates the issue of cold chain storage and transportation of vaccines, particularly in developing countries which can ultimately lead to spoilage and waste of vaccines (Miquel-Clopés *et al*., 2019). The selected *C. difficile* recombinant proteins; CD630_08730 and CD630_TcdB-RBD were successfully conjugated to MalLipo.

The Mal lipid synthesised for use in this project was based on the structure of the Pam2Cys adjuvant. However, in this study the ester-bonds which connects the fatty acid lipids to a glycerol backbone were replaced with more stable ether-bonds. There has been some controversy over the replacement of ester-bonds with for example more stable amide-bonds. Using an IL-8 assay to assess TLR2 signalling, Zeng *et al* (2010) showed that substitution of one ester-bound lipid with an amide-bond reduced IL-8 secretion indicating reduced TLR2 activity when compared to a Pam3Cys derivative. However, Guo *et al* (2017) recently showed that substitution of both ester-bonds with amide-bonds tested *in vivo* enhanced immune responses by increased DCs activation compared to a Pam3Cys derivative. Pam2Cys/Pam3Cys adjuvants that have ester-bound lipids can be subjected to esterification in plasma or intestinal fluid, hence can reduce the TLR2 mediated response (Zeng *et al*., 2010). Therefore, the observed enhanced immune responses was thought to be attributed to the presences of more stable amide-bonds. *In vivo* studies showing antibody response are most revealing of the potential of adjuvants by comparing vaccines candidates with and without. In means of determining the TLR2 dependent activity of the Mal lipid, further studies should be conducted. The release of IL-8 and induction of NF-κB are ways to determine TLR2 signalling (Buwitt-Beckmann *et al*., 2005; Kang *et al*., 2009; Zeng *et al*., 2010). Therefore, further *in vitro* studies using cells lines co-transfected with plasmids containing human TLR2 or NF-κB luciferase reporter genes could be utilised with Mal lipid incubations and compared to that of incubations with commercially available Pam2Cys with known adjuvant properties. Another approach could be to look at DC activation through upregulation of MHC class II molecules on DCs (Guo *et al*., 2017; Zeng *et al*., 2010). These studies will

further contribute to the understanding of the Mal lipid-TLR2 interaction for potential use as a mucosal adjuvant.

It would also be beneficial to study the exact uptake of liposomal formulations in the PPs. Specifically the uptake via M cells, which is of importance for induction of mucosal responses, could provide insights for alterations. To this end, the *in vitro* model for studying M cell uptake has been conducted using differentiated M cell-like cells induced by co-culturing Caco-2 cell lines with B cell lymphoma Raji cells (Gullberg *et al*., 2000). More recently, with concerns over varying phenotypic display of differentiated Caco-2 cells, differentiated M cell monolayers derived from human stem cell ileal enteroids have been deployed (Fasciano *et al*., 2019; Rouch *et al*., 2016; Tong *et al*., 2020). By utilising such models, M cell facilitated liposomal uptake could be determined and thus further tailored for a more targeted approach.

7.3 Enteric capsule coating

In order to protect the formulations from the harsh environment of the stomach and target the small intestine, the formulations were encapsulated and coated in enteric polymer. In chapter 5, we tested various combinations of the polymer 12.5% (w/v) Eudragit® L100 mixed with or without 10% (v/v) TEC and/or 3% (v/v) H_2O dip-coated either once or twice. The dissolution profile of these polymer mixtures to resist low pH and disintegrate at higher pH releasing encapsulated bromophenol blue was assessed *in vitro* over 5 hours in each solution. The optimal polymer mixture observed from this study was 12.5% Eudragit® L100 + 10% TEC + 3% H_2O dip-coated once which provided the greatest resistance in simulated gastric fluid (pH 1.2) and subsequently showed the fastest dissolution in simulated intestinal fluid (pH 6.7).

Staelens *et al* (2016) previously tested capsules dip-coated with a single layer of 12.5% Eudragit L100® and 10% TEC *in vitro* and showed resistance in pH 1 and some dissolution observed when the capsule was left at this pH for longer than 5 hours. This was also observed in this study, however resistance was retained up to 4.5 hours in the low pH. This study confirms previous finding of resistance of Eudragit® L100 coated capsules for at least 2 hours in low pH which is the typical length of preclinical dissolution profile testing (Bruxelle *et al*., 2018). The rapid dissolution of 1 coat of Eudragit® L100 in pH 1.2 was of surprise as previously Saleem *et al* (2019) tested a lyophilised compressed Bacillus Calmette-Guérin (BCG) vaccine coated with this polymer and showed greater durability in low pH when tested *in vitro*. This could also be related to the increased adhesion of the polymer with a tablet form compared to a gelatin capsule. It has also been shown that mannosylated chitosan nanoparticles formed by ionic gelation method with the additive tripolyphosphate which were subsequently coated with Eudragit® L100 retained good stability in low pH and release in higher pH when tested *in vitro*. Again, the electrostatic interaction between the polymer and the additive may have provided an enhanced coating property. The use of 2 coats of Eudragit® L100 in this study showed lack of disintegration, therefore the addition of TEC was thought to have played a role in both enhanced interaction of the polymer with the capsule as well as flexibility to allow for 1 coat of the polymer to be utilised. This is beneficial as 1 coat of enteric formula minimises the time required for coat drying. Although 12.5% Eudragit® L100 + 10% TEC + 3% H_2O dip-coated once showed good stability and release profiles, it would be useful to compare the profile of Eudragit® L100 with the use of different ratios of TEC and compare the disintegration profile *in vitro* (Fu *et al*., 2020).

Although not applicable for this study due to lack of dissolution testing equipment, besides testing the dissolution of enteric polymers merely with different pH solutions, it would also be useful to compare the impact of temperature and shaking which could be conducted with more pronounced equipment known as a paddle apparatus (for example USP type-I and type-II), which provides more of a mimic of the GI tract pH, body temperature and peristaltic movement (Begum *et al*., 2012). The optimal polymer mixture was used to dip-coat capsules packed with $BaSO_4$ for tracking *in vivo* using CT imaging with oral delivery to hamsters. 2 out of 4 hamsters showed complete resistance of the capsule with release of $BaSO_4$ in the small intestine following 3 hours post administration. However, the other 2 hamsters showed partial disintegration of the capsule in the stomach at 1.5 hours administration and complete disintegration in the small intestine at 3 hours. The observed difference was thought to be due to the different dosing applicators used. Another explanation could be differences in the consistency of polymer coating. Capsule products developed for humans are far more advanced than for rodents and are widely used thus in considering oral vaccination against *C. difficile*, the use of capsules to target the small intestine is promising.

7.4 Testing immunogenicity and protective efficacy of *C. difficile*-directed oral vaccine formulation in hamsters

In this chapter, a pilot study was conducted using 1 mg of protein CD630_08730 and CD630_TcdB-RBD alone and 1 mg of protein conjugated to MalLipo delivered orally in capsules to hamsters. Analysis of the intestinal fluid and serum showed that CD630_08730 alone and MalLipo + CD630_08730 generated mucosal sIgA and systemic antigen-specific IgG which reduced the binding of *C. difficile* to Caco-2 cells

significantly compared to control groups. Our findings corroborate those reported by Kovacs-Simon *et al* (2014) whereby mouse anti-CD0873 sera was shown to block *C. difficile* strain 630Δ*erm* adherence to Caco-2 cells. Our results therefore confirm that CD630_08730 plays an important role in colonisation. The reduction caused by the MalLipo + CD630_08730 immunised group was greater compared to the CD630_08730 immunised group in the case of both serum and intestinal fluid which highlights that the MalLipo vehicle which contains the Mal lipid does enhance the immunogenicity of the attached protein and therefore could serve as a potential adjuvant (Figure 6.5). This could provide ease of attachment of recombinant proteins without the need for synthesising peptides and additional requirement of modification to amino acid residues for attachment of immunostimulating components (Moyle *et al*., 2014). Additionally having CD630_08730 presented on the surface of liposomes may successfully mimic its display on cells of *C. difficile* as intended. Mucosal delivery of protein based vaccines without nanoparticle encapsulation have mainly been investigated for i.n. administration as the nasal mucosa is less harsh of an environment compared to the gut mucosa (Aramaki *et al*., 1993; Borges *et al*., 2008; Childers *et al*., 2000; Morris *et al*., 2000; Petersson *et al*., 2010; Wang *et al*., 2015). Here we show, that all CD630_08730-formulations induced mucosal and systemic responses when delivered orally. Conversely, no response was observed for CD630_TcdB-RBD formulations as shown by ELISA and toxin neutralisation assays. It is speculated that the insolubility of this protein encountered during its purification may account for the lack of immunogenicity observed *in vivo*. Importantly we show in this study the safety of all formulations tested as no adverse reactions were detected from histopathological analysis of intestinal tissue.

Following evidence of both mucosal and systemic responses to formulations containing CD630_08730, a pilot study was conducted to test the protective efficacy of CD630_08730 alone. For the challenge study the, hypervirulent 027 *C. difficile* strain R20291*ermB* was used. Oral immunisation with CD630_08730 showed partial protection of 25% in hamsters with a mean time to end point 80% greater than the naïve group (Figure 6.7, A). The initial 10% weight loss observed in the surviving hamster was regained, and this weight profile inversely correlated with an initial increase in bacterial shedding followed by complete clearance of the infection (Figure 6.7, D). The caecum of this hamster showed markedly reduced pathology compared to the control groups which all succumbed to infection (Figure 6.9, B). An additional hamster in the CD630_08730 immunised group showed mild symptoms compared to the control groups with a later end point at day 4 compared to control groups which was day 3 and a non-enlarged caecum post-mortem. In this study we further show that hamsters immunised with i.m. toxoids, as a control, had no protection and were fully colonised to a similar level as that of naïve animals which is consistent with previous findings by Hong *et al* (2017). Bradshaw *et al* (2019) previously assessed CD630_08730 as a vaccine candidate in mice dosed via the i.p. route then challenged with *C. difficile* strain 630Δ*erm*. Although end point survival percentage was not stated for challenged mice, vaccinated animals did not present with clinical signs of diarrhoea and had lower weight loss compared to non-vaccinated hamsters. This was associated with significant decrease in *C. difficile* gut colonisation at day 13 post challenge following plating of faecal pellets and caecum content compared to non-vaccinated mice. This correlated with the significant increase in anti-CD630_08730 IgG and IgA levels in the serum and in the faeces respectively compared to pre-

immunisation (Bradshaw *et al*., 2019). The observation by Bradshaw *et al* (2019) that both immunised and non-immunised mice survived up to the experimental end point (13 days post challenge) suggests that refinements to this model is required to make it more of a lethality model. More importantly we question whether parenteral routes of immunisation can ever elicit local protection in the gut.

As the caecum of the surviving hamster displayed markedly reduced toxin mediated damage compared to that of control hamsters (Figure 6.9), we propose that the sIgA generated by oral immunisation with CD630_08730 blocks the binding of *C. difficile* to enterocytes reducing the proximity of TcdA and TcdB to host cells and may even block their secretion or neutralise their activity. Previous studies have highlighted that sIgA and the secretory component alone from human milk directly binds to TcdA thus, preventing binding to hamster intestinal brush border membranes. It was proposed that the abundance of sIgA conferred from the mother to breast fed infants intercepts or neutralises TcdA as infants remain asymptomatic of CDI yet show high rates of colonisation (Dallas and Rolfe, 1998; Kim *et al*., 1984). Currently available mucosal vaccines against rotavirus, polio, *S. typhimurium*, *V. cholera* have highlighted the correlation of sIgA as well as serum IgG in providing protection against these mucosal infections (Kang *et al*., 2018). In a study conducted by Permpoonpattana *et al* (2011) oral delivery of *B. subtilis* spores expressing the RBD of TcdA, was able to elicit both sIgA and IgG capable of neutralising both TcdA and TcdB with 75% protection in a challenge study and complete protection following re-challenge. It was shown that the sIgA was correlated with protection as mice that received i.p. delivery of these antigens alone which showed no sIgA, failed to survive. Similarly, FliC, which has also been shown to act as an adjuvant through TLR5 interaction was

encapsulated using pectin beads for oral delivery in hamsters which showed a 20% increase in survival compared those animals that received free-FliC via the i.p. route. Despite the superior serum IgG observed in the hamsters receiving free-FliC via the i.p. route, it was speculated that the mucosal response generated led to the difference in the survival, however due to lack of anti-hamster IgA antibody this was not verified (Bruxelle *et al*., 2018; Bruxelle *et al*., 2017). Given the mucosal and systemic responses observed in this study, our findings coincide with reports of the importance of sIgA in addition to IgG to provide protection. A mucosal route of delivery and the inclusion of colonisation factors to target earlier stages of CDI infection in addition to later toxin-secreting stages could be a plausible solution for targeting *C. difficile*.

7.5 Limitations and future work

In evaluating the protective efficacy of CD630_08730 from this study, a few points are noteworthy. Although a clear trend in protection is evident, non-significance was observed. This was not surprising since this was a pilot study using small *n* values. Further evaluation would require greater statistical power using larger *n* values. Another point to make is the variation observed in hamsters, which is not uncommon. The enteric polymer and the dip-coating process for rodent capsules of such small size used in this study is not advanced. Therefore, variation in delivery to the small intestine in hamsters is expected. Although 3 oral doses were used it is not known how many capsules successfully made it to the small intestine. Other coatings could be tested for improved delivery to the small intestine such as the pre-made organic solution of Eudragit® L100 and cellulose acetate phthalate and other

plasticisers tried such as PEG 6000 for more consistent coating (Hussan *et al*., 2012; Kapoor *et al*., 2020; Thoma and Benchtold, 2000). In addition using more sophisticated coating methods such as the spray drying like that is used in pharmaceutical settings may improve the consistency of coating (Mittal, 2017).

Another limitation in this study was the lack of commercially available hamster-specific reagents for immunological assays. Using the mouse model for which many immunological detection reagents are readily available and using the specific model whereby mice develop fulminant CDI like hamsters could be more beneficial. However the susceptibility of mice to *C. difficile* can vary and models that predispose mice to fulminant infection are more complex than hamster models requiring antibiotic cocktails (metronidazole, vancomycin, kanamycin, gentamicin, colistin and clindamycin) (Chen *et al*., 2008; Pawlowski *et al*., 2010). When considering mouse models, susceptibility to CDI varies in different strains which may reflect differences between strains in their gut microbiota (Hutton *et al*., 2014). The *C. difficile* strain and the size of the spore inoculum used in this study could also be altered to mimic a more realistic infection as this may have overwhelmed the immune system of immunised hamsters.

As CD630_08730 formulated onto liposomes using the Mal lipid showed further reduced binding of *C. difficile* to Caco-2 cells, the next step would be to test the ability of MalLipo + CD630_08730, to provide protection against CDI when delivered orally. It would also be of interest to test the immunogenicity and protective efficacy of an oral CD630_08730 in its native lipidated form which would naturally form self-adjuvanting micelles. In addition, combining CD630_08730 with other more soluble,

immunogenic portions of TcdA and TcdB for targeting later stages of pathogenesis may provide greater protection.

7.6 Concluding remarks

To conclude this study highlights the potential of using a synthetic lipid adjuvant for conjugation of proteins to the liposomal surface as an oral delivery platform to enhance immunogenicity. This study also highlights CD630_08730 as a promising oral vaccine candidate that can be administered as a lyophilised recombinant full-length protein. As a strong sIgA response was observed in all immunised hamsters, this study demonstrates the excellent bioavailability of CD630_08730 in the gut mucosa. Importantly the intestinal sIgA response elicited by oral immunisation with CD630_08730 reduces colonisation of *C. difficile* and potentially intercepts and neutralises its toxins.

Appendix

8 Appendix

8.1 Supplementary material chapter 2

Animal Monitoring Sheet. Hamster/Mouse

PPL Number:	19b ref:	PPL Holder:		PIL Holder:	
Species: Hamster	Strain: Syrian	Animal Identification: C. diff	Lab Track:	Weight at issue:	Weight at 20% loss:
Species: Mouse	Strain: Balb/c or C57/Bl6	Animal Identification: C. diff	Lab Track:	Weight at issue:	Weight at 20% loss:

Complete the following sheet as appropriate:

Date																
Time																
Presence/degree of change in parameter:	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Normal activity	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3
Loose faeces present	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0
Wet tail/perineum	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0
Diarrhoea	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0
Hunched posture	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0
Starey coat	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0
Sunken eyes	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0
Response to stimulus	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3	Yes 0	No 1,2,3
Weight loss	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0	Yes 1,2,3	No 0
Total Score																
Weight																
Room Temperature																
Assessed by:																
Comments:																

Key: 0=no change, 1=Minor change, 2=Moderate change, 3=Major change.
If any 2 parameters score 3, add 1 to the total score. Euthanase when score = >15.
Any animal showing an unsteady gait accompanied with a head bob will be monitored every 2 hours and if showing a reduction in activity along with the unsteady gait and head bob over 2 consecutive checks will be euthanised using a Schedule 1 method.

Actual severity: Mild, Moderate, Severe

Figure A1- Hamster monitoring scoring system.
Hamsters were closely monitored daily using the scoring system from 1-3 (1-being mild and 3- being severe) for each of the criteria with weight changes recorded. Any hamster that had a total score of 15 was euthanised. In addition to this any hamster that showed a 20% weight loss between consecutive checks or a 10% weight loss from 2 consecutive checks was euthanised.

8.2 Supplementary material chapter 3

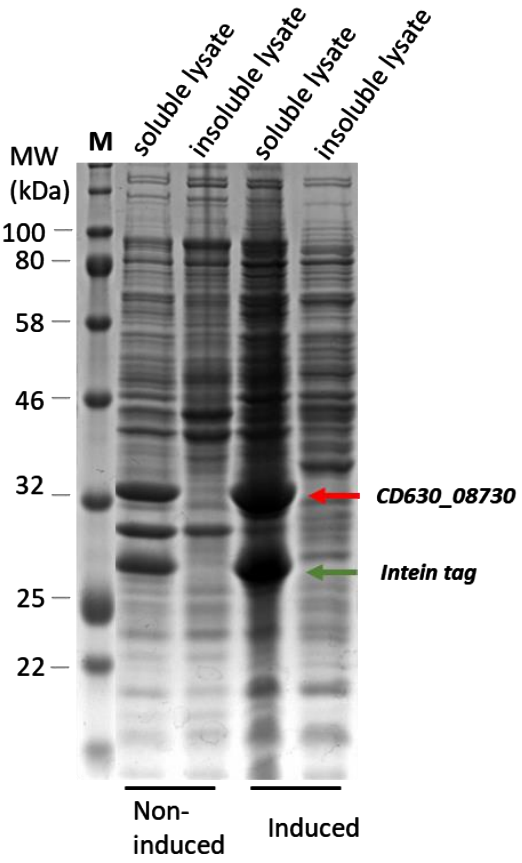

Figure A2- SDS-PAGE showing *in vivo* cleavage of the intein tag following expression.

Cell lysates of T7 Express cells harbouring the pTWIN1-CD630_08730 construct revealed cleavage of the intein tag when induced at room temperature following fractionation using 10% (w/v) Tris-glycine SDS-PAGE. Intein tag cleaved during expression at room temperature indicated with green arrow and recombinant CD630_08730 without the intein tag indicated with red arrow.

8.2.1 DNA sequencing alignments for pTWIN1.His constructs

pTWIN1.His constructs were sequenced for forward and reverse reads using the primers Ssp intein For or His check seq Rev or Cwp84 CO internal for and Cwp84 CO internal Rev for pTWIN-CD630_cwp84 construct. Sequence alignments were performed with the forward and reverse sequences using the Clustal Omega tool

(section 2.2.5) for multiple alignments against template contracts A) pTIWN1.His, B) pTWIN1-CD630_08760.His, C) pTWIN1-CD630_08730.His, D) pTWIN1-CD630_27190.His, E) pTWIN1-CD630_34640.His, F) pTWIN1-CD630_TcdA-RBD.His, G) pTWIN1-CD630_TcdB-RBD.His, H) pTWIN1-CD630_cwp84.His. Sequences highlighted in yellow indicate the start of the *C. difficile* codon optimised gene cloned and the end of the gene is highlighted in green. For represents forward sequence and Rev represents reverse sequence.

A)

pTWIN1.His

```
pTWIN1.His   CTGGGACTCCATCGTTTCTATTACGGAGACTGGAGTCGAAGAGGTTTTTGATTTGACTGT   720
Rev          ----------------------------ACCTGGGATTAAAAGGTTTTTTGATTTGACTGT   33
                                          ****  *  **  * ****************

pTWIN1.His   GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACGGAAGAGCCATGGG   780
Rev          GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACGGAAGAGCCATGGG   93
             ************************************************************

pTWIN1.His   CGGCCGCGAATTCCTCGAGGGCTCTTCCTGCATCACGGGAGATGCACTAGTTGCCCTACC   840
Rev          CGGCCGCGAATTCCTCGAGGGCTCTTCCTGCATCACGGGAGATGCACTAGTTGCCCTACC   153
             ************************************************************

pTWIN1.His   CGAGGGCGAGTCGGTACGCATCGCCGACATCGTGCCGGGTGCGCGGCCCAACAGTGACAA   900
Rev          CGAGGGCGAGTCGGTACGCATCGCCGACATCGTGCCGGGTGCGCGGCCCAACAGTGACAA   213
             ************************************************************

pTWIN1.His   CGCCATCGACCTGAAAGTCCTTGACCGGCATGGCAATCCCGTGCTCGCCGACCGGCTGTT   960
Rev          CGCCATCGACCTGAAAGTCCTTGACCGGCATGGCAATCCCGTGCTCGCCGACCGGCTGTT   273
             ************************************************************

pTWIN1.His   CCACTCCGGCGAGCATCCGGTGTACACGGTGCGTACGGTCGAAGGTCTGCGTGTGACGGG   1020
Rev          CCACTCCGGCGAGCATCCGGTGTACACGGTGCGTACGGTCGAAGGTCTGCGTGTGACGGG   333
             ************************************************************

pTWIN1.His   CACCGCGAACCACCCGTTGTTGTGTTTGGTCGACGTCGCCGGGGTGCCGACCCTGCTGTG   1080
Rev          CACCGCGAACCACCCGTTGTTGTGTTTGGTCGACGTCGCCGGGGTGCCGACCCTGCTGTG   393
             ************************************************************

pTWIN1.His   GAAGCTGATCGACGAAATCAAGCCGGGCGATTACGCGGTGATTCAACGCAGCGCATTCAG   1140
Rev          GAAGCTGATCGACGAAATCAAGCCGGGCGATTACGCGGTGATTCAACGCAGCGCATTCAG   453
             ************************************************************

pTWIN1.His   CGTCGACTGTGCAGGTTTTGCCCGCGGAAAACCCGAATTTGCGCCCACAACCTACACAGT   1200
Rev          CGTCGACTGTGCAGGTTTTGCCCGCGGGAAACCCGAATTTGCGCCCACAACCTACACAGT   513
             ************************** ******************************

pTWIN1.His   CGGCGTCCCTGGACTGGTGCGTTTCTTGGAAGCACACCACCGAGACCCGGACGCCCAAGC   1260
Rev          CGGCGTCCCTGGACTGGTGCGTTTCTTGGAAGCACACCACCGAGACCCGGACGCCCAAGC   573
             ************************************************************

pTWIN1.His   TATCGCCGACGAGCTGACCGACGGGCGGTTCTACTACGCGAAAGTCGCCAGTGTCACCGA   1320
Rev          TATCGCCGACGAGCTGACCGACGGGCGGTTCTACTACGCGAAAGTCGCCAGTGTCACCGA   633
             ************************************************************

pTWIN1.His   CGCCGGCGTGCAGCCGGTGTATAGCCTTCGTGTCGACACGGCAGACCACGCGTTTATCAC   1380
Rev          CGCCGGCGTGCAGCCGGTGTATAGCCTTCGTGTCGACACGGCAGACCACGCGTTTATCAC   693
             ************************************************************

pTWIN1.His   GAACGGGTTCGTCAGCCACGCTACTGGCCTCACCGGTCTGAACTCAGGCCTCACGACAAA   1440
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Rev          GAACGGGTTCGTCAGCCACGCTACTGGCCTCACCGGTCTGAACTCAGGCCTCACGACAAA   753
             ************************************************************

pTWIN1.His   T-CCTGGTGTATCCGCTTGGCAGGTCAACACAGCTTATACTGCGGGACAATTGGTCACAT   1499
Rev          TCCCTGGTGTATCCGCTTGGCAGGTCAACACAGCTTATACTGCGGGACAATTGGTCACAT   813
             * ************************************************************

pTWIN1.His   ATAACGGCAAGACGTATAAATGTTTGCAGCCCCACACCTCCTTGGCAGGATGGGAACCAT   1559
Rev          ATAACGGCAAGACGTATAAATGTTTGCAGCCCCACACCTCCTTGGCAGGATGGGAACCAT   873
             ************************************************************

pTWIN1.His   CCAACGTTCCTGCCTTGTGGCAGCTTCAACTGC-AGGAAGGGGATCCGCTGGTGCCACGC   1618
Rev          CCAACGTTCCTGCCTTGTGGCAGCTTCATGACTGCAGGAAGGGATCCGCTGGTGCCACGC   933
             ****************************        * * ********************

pTWIN1.His   GGTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAAGCTGAGCAATAACTA   1678
Rev          GGTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAAGCTGAGCATA-ACTA   992
             ************************************************   ****

pTWIN1.His   GCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACT   1738
Rev          ACATAACCCCTTTGGGCCTCTAAACGGGTCTTGAAGGGTTTTTTGCTGAAAGAAGAACTA   1052
             *********** ******************** ***************** ** *

pTWIN1.His   AT-------------------------------------------------------------   1740
Rev          TATCCCGAATACTTACGTCAGTGGAACTTTTCCGGGAAATGTGCCCCGGAAACCCTATAT   1112


pTWIN1.His   ----------------------------------------     1740
Rev          TGGTTTTATTTTTTCCAAAAACATTTCCAAATATTGAAT     1151
```

B)

pTWIN1-CD630_08760.His

```
Rev          ------------------------------------------------------------   0
p.08760.His  CTGGGACTCCATCGTTTCTATTACGGAGACTGGAGTCGAAGAGGTTTTTGATTTGACTGT   720
For          ----------------------------GTTGTTGAATTGAGAGGTTTTTGATTTGACTGT   33

Rev          ------------------------------------------------------------   0
p.08760.His  GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTTCTCAAAATGA   780
For          GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTTCTCAAAATGA   93


Rev          ------------------------------------------------------------   0
p.08760.His  TGGCTCCAATGCTTCAAATGAAAATAAAGAAACAGATAGCAAAAAACAAAAAAATATAGG   840
For          TGGCTCCAATGCTTCAAATGAAAATAAAGAAACAGATAGCAAAAAACAAAAAAATATAGG   153


Rev          ------------------------------------------------------------   0
p.08760.His  TATTACTCAATTAGTGGAACATCCATCTCTGGATAAAGCAAAGAAAGGATTCATCAAAGC   900
For          TATTACTCAATTAGTGGAACATCCATCTCTGGATAAAGCAAAGAAAGGATTCATCAAAGC   213


Rev          ------------------------------------------------------------   0
p.08760.His  ACTCGAAGATAAAGGCTATAAAGATGGAGATAATATAAAAATAGATTTCCAAAATGCACA   960
For          ACTCGAAGATAAAGGCTATAAAGATGGAGATAATATAAAAATAGATTTCCAAAATGCACA   273


Rev          ----------------------------GCTAGTAAGGTTGTATTCCGACAAAAAAAAGA   32
p.08760.His  AAATGATATGCCTACTACACAAAGTATTGCTAGTAAGTTTGTATCCGACAAAAA----AG   1016
For          AAATGATATGCCTACTACACAAAGTATTGCTAGTAAGTTTGTATCCGACAAAAA----AG   329
                                     ********* ****** *    ****

Rev          TTTGAATTTATGTTATATTTTACTTCCATTTTGCCCAGGCAGGTTTTAAATTGCTATTAA   92
p.08760.His  ATTTGATATATGCTATA--TCTACTCCAT-CTGCACAAGCAGC--TTATAATGCTACTAA   1071
For          ATTTGATATATGCTATA--TCTACTCCAT-CTGCACAAGCAGC--TTATAATGCTACTAA   384
              **  ** **** ****  *    *****  *** ** ****   *** * ***** ***

Rev          AGGATATTCAGAAAATTAATGACTGGTTGTTAAAAGACCCTATAGAAGCTGAACTTGTTA   152
p.08760.His  AG-ATATTCCG---ATAATAATGACTGCTGTTACAGACCCTGTAGAAGCTGGACTTGTTA   1127
For          AG-ATATTCCG---ATAATAATGACTGCTGTTACAGACCCTGTAGAAGCTGGACTTGTTA   440
             ** ****** *   ** *           * * ******* ********* ********

Rev          AATCTCTTGAAAAAACCAGGTGGGAAATTGTTTTCTGGTACATTGGATTATCTTTCAAAT   212
p.08760.His  AATCTCTTGAAAAACCAGGTGGAAAT----GTTTCTGGTACATCTGATTATCTTTCAATT   1183
```

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For          AATCTCTTGAAAAACCAGGTGGAAAT----GTTTCTGGTACATCTGATTATCTTTCAATT   496
             ************** *  *  *  *      ************  ************* *

Rev          TGATAAAAACATTTAGAATTGGGTAAAAAACATTGACTCCCAAAAAGCAAAAGAAAATAG   272
p.08760.His  GATAAAAC---ATTAGAATTGGTTA--AAACATTGACT----CCAAAAGCAAAGAAAATA   1234
For          GATAAAAC---ATTAGAATTGGTTA--AAACATTGACT----CCAAAAGCAAAGAAAATA   547
                  ***     ********** **  ***********      **    **  ***

Rev          GGGGTTATATACAATACTAGTGAAGTTAATTCAAAAAATCCCAAGTTGATTTTTATACAT   332
p.08760.His  GGGGTTATATACAATACTAGTGAAGTTAATTCAAAAATC---CAAGTTGATTCTCTACAT   1291
For          GGGGTTATATACAATACTAGTGAAGTTAATTCAAAAATC---CAAGTTGATTCTCTACAT   604
             ************************************      *  *   ** * *****

Rev          GATTAGGCTAAGAAAAATAATTATGAAGTAGTTGAAAAAGGAATCAGCTTTTTCAAGTGA   392
p.08760.His  GATTATGCTAAGAAAAATAATTATGAAGTAGTTGAAAAAGGAATCAGCT-CTTCAAGTGA   1350
For          GATTATGCTAAGAAAAATAATTATGAAGTAGTTGAAAAAGGAATCAGCT-CTTCAAGTGA   663
             ***** ****************************************  *********

Rev          AGTTAACCAAGCTATTTCTAGTTTAGTTGGCAAAATAGATGTTTTATATGTCCCTACTGA   452
p.08760.His  AGTTAACCAAGCTATTTCTAGTTTAGTTGGCAAAATAGATGTTTTATATGTCCCTACTGA   1410
For          AGTTAACCAAGCTATTTCTAGTTTAGTTGGCAAAATAGATGTTTTATATGTCCCTACTGA   723
             ************************************************************

Rev          CAATTTAATAGTTTAAAAAAAAAAAAAAATAGTTTCTAAAGTTGCTAATGAAAACAAGATT   512
p.08760.His  CAATTTAATAGTTTCTTCTA--TGCCAATAGTTTCTAAAGTTGCTAATGAAAACAAGATT   1468
For          CAATTTAATAGTTTCTTCTA--TGCCAATAGTTTCTAAAGTTGCTAATGAAAACAAGATT   781
             **************     *      *********************************

Rev          CCTATAATAGCCTCTGAAGAAGGTTCTGTATCTTCTGGTGCTTTAGCTTGTTGTGGAATA   572
p.08760.His  CCTATAATAGCCTCTGAAGAAGGTTCTGTATCTTCTGGTGCTTTAGCTTGTTGTGGAATA   1528
For          CCTATAATAGCCTCTGAAGAACGTTCTGTATCTTCTGGTGCTTTAGCTTGTTGTGGAATA   841
             ********************* ************************************

Rev          GATTATGAAAAACTAGGTTATAAGGCTGGAGAACTTGCTATTGAAGTATTAGAAGGAAAA   632
p.08760.His  GATTATGAAAAACTAGGTTATAAGGCTGGAGAACTTGCTATTGAAGTATTAGAAGGAAAA   1588
For          GATTATGAAAAACTAGGTTATAA-GCTGGAGAACTTGCTATTGAAGTATTAGAAGGAAAA   900
             ********************** ***********************************

Rev          TCTGTTGGCGATATACCAGTTACTACATTAGATGAAACTGAAATAATAATTAACGAAGAT   692
p.08760.His  TCTGTTGGCGATATACCAGTTACTACATTAGATGAAACTGAAATAATAATTAACGAAGAT   1648
For          TCTGTTGGCGATATACCAGATACTACATTAGATGAAACTGAAATAATAATTAACGAAGAT   960
             ******************* ****************************************

Rev          ACACTAAAAGCACTAGACATGCAAAAGTTATCAGCAGATAATATAAAGTATATAAAGTCA   752
p.08760.His  ACACTAAAAGCACTTGACATGCAAAAGTTATCAGCAGATAATATAAAGTATATAAAGTCA   1708
For          GCACTAAAAGCACTTGACATGCAAAAGTCACCACCAGACAATATAAAGTACAATAAAGCC   1020
              ************* ************* * ** **** *********** *  **  *

Rev          GATGAAAATGCAAAATCTGCAAATTTTCTGTAGG-GATCCGCTGGTGCCACGCGGTAGTT   811
p.08760.His  GATGAAAATGCAAAATCTGCAAACTGCAGGAAGGGGATCCGCTGGTGCCACGCGGTAG--   1766
For          CGATGAAAATGCAAATCTGCAAAACTGCCGTAGGGGATCCGCTTGGGGCACCGCAGGTAG   1080
                  ***    ***********      * *** ******** * * ***

Rev          CCGCCCCCTCCTCCATCATCACCATCACCACC----------ACTAAGCTGAGCAATAAC   861
p.08760.His  TTCCGCTCATCACCACCATCATCACCATCA-------CCACCACTAAGCTGAGC-AATAA   1818
For          TTCCTGTCTATAA-CCACAGATCATCATCTGCTGGCCACGCCACTAAGCTGGAGCAATAT   1139
                *   *            * ** ** *             *********    *  *

Rev          TA---GCATAACCCCT-----GGGGGCTTTAAAAACGTGTAA------------------   895
p.08760.His  CT--AGCATAACCCCTT----GGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAA   1872
For          CTTCGCGGTAACCCGTGAAGGCCACACTCCTAACGGGTCTGGAAGGGTTTTGCGTTGAAA   1199
                      ****** *          **   **   ** *

Rev           -----------    895
p.08760.His    GGAGGAACTAT    1883
For            GGGGGTTCCC-    1209
```

C)

pTWIN1-CD630_08730.His

```
Rev         ------------------------------------------------------------    0
p.08730.His CTGGGACTCCATCGTTTCTATTACGGAGACTGGAGTCGAAGAGGTTTTTGATTTGACTGT    720
For         ----------------------------GTCTATGAATTAAAAGTTTTTTGATTTGACTGT    33
```

```
Rev          ------------------------------------------------------------   0
p.08730.His  GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTAGCCAAGGTGG   780
For          GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTAGCCAAGGTGG   93


Rev          ------------------------------------------------------------   0
p.08730.His  TGATAGCGGTAATAGCAAACAAGAAAGCAACAGCAAAGACAAAGAGGTGAAAAAAATCGG   840
For          TGATAGCGGTAATAGCAAACAAGAAAGCAACAGCAAAGACAAAGAGGTGAAAAAAATCGG   153


Rev          -------------------------------------------ACCGGGTTTGGTTAAAGG   18
p.08730.His  TATTACCCAGCTGGTTGAACATCCGGCACTGGATGCAACCCGTACCGGTTTTGTTAAAGC   900
For          TATTACCCAGCTGGTTGAACATCCGGCACTGGATGCAACCCGTACCGGTTTTGTTAAAGC   213
                                                       * ***** *******


Rev          CATGGGAAAAAAACGCCTTTTAAAGGAGGGGGAAAACCTGGACATCGATTTTCAGAATGC   78
p.08730.His  ACTGGA---AAAAAACGGCTTTAAAGATGGCGAAAACATCGACATCGATTTTCAGAATGC   957
For          ACTGGA---AAAAAACGGCTTTAAAGATGGCGAAAACATCGACATCGATTTTCAGAATGC   270
               ***    ****  *   ** ** ** ** ****** * ********************


Rev          ACCGGATGATATGCCGACCCACCCGGAGCCTTTGCAAGTTAATTTGCAAGCGACAAAAAA   138
p.08730.His  ACAGAATGATATGCCGACCACACAGAG--CATTGCAAGTAAATTTGCAAGCGACAA-AAA   1014
For          ACAGAATGATATGCCGACCACACAGAG--CATTGCAAGTAAATTTGCAAGCGACAA-AAA   327
             ** * **************   * *    * ******** ***************** ***


Rev          GGATTTGATCTTTGCAATTGGCACCCCGGAGCGCTCAGGGCAGCATTTATGCACCCAAAG   198
p.08730.His  GGATCTGATCTTTGCAATTAGCACCCCG-AGCGCACAGGCAGCATTTAATGCAACCAAAG   1073
For          GGATCTGATCTTTGCAATTAGCACCCCG-AGCGCACAGGCAGCATTTAATGCAACCAAAG   386
             **** ************** ******** ***** ****      ** ***** ******


Rev          ATATTCCGGTTTCTGATTTACCGCAGTTAGCGAT-CCGGTGCAGCAGG-TCTGTTTAAAC   256
p.08730.His  ATATTCCGATTCTG-ATT-ACCGCAGTTAGCGATCCGGTTGCAGCAGGTCTGGTTAAAAC   1131
For          ATATTCCGATTCTG-ATT-ACCGCAGTTAGCGATCCGGTTGCAGCAGGTCTGGTTAAAAC   444
             ******** **    *** *************** * * *********    *** ****


Rev          CTTAGAAAAACCGGGTACAAATGTTAGCGGCACCAGCGATT-TTTTAGCGTTGATAAAGA   315
p.08730.His  CTTAGAAAAACCGGGTACAAATGTTAGCGGCACCAGCGATTTTGTTAGCGTTGATAAAGG   1191
For          CTTAGAAAAACCGGGTACAAATGTTAGCGGCACCAGCGATTTTGTTAGCGTTGATAAAGG   504
             ************************************** * ***************


Rev          TCTGGAACTGTTGAAAATCTTTGCACCGAAAGCAAAAACCATTGGCGTGATGTATAATTC   375
p.08730.His  TCTGGAACTGCTGAAAATCTTTGCACCGAAAGCAAAAACCATTGGCGTGATGTATAATAC   1251
For          TCTGGAACTGCTGAAAATCTTTGCACCGAAAGCAAAAACCATTGGCGTGATGTATAATAC   564
             ********** ********************************************** *


Rev          CAGCGAAGT-AATAGCAAAGTTCAGGTTGATGCCCTGAAAGAATATGCCAGCAAAAATGG   434
p.08730.His  CAGCGAAGTGAATAGCAAAGTTCAGGTTGATGCCCTGAAAGAATATGCCAGCAAAAATGG   1311
For          CAGCGAAGTGAATAGCAAAGTTCAGGTTGATGCCCTGAAAGAATATGCCAGCAAAAATGG   624
             ********* ************************************************


Rev          TTTTAAAGTGGTGGAAAAAGGCATCACCACCAGTAATGAAGTTAATCAGGGTATTAGCAG   494
p.08730.His  TTTTAAAGTGGTGGAAAAAGGCATCACCACCAGTAATGAAGTTAATCAGGGTATTAGCAG   1371
For          TTTTAAAGTGGTGGAAAAAGGCATCACCACCAGTAATGAAGTTAATCAGGGTATTAGCAG   684
             ************************************************************


Rev          CCTGGTGGGTAAAATTGATGTTCTGTATGTTCCGACCGATAATCTGGTTGCAAGCAGCAT   554
p.08730.His  CCTGGTGGGTAAAATTGATGTTCTGTATGTTCCGACCGATAATCTGGTTGCAAGCAGCAT   1431
For          CCTGGTGGGTAAAATTGATGTTCTGTATGTTCCGACCGATAATCTGGTTGCAAGCAGCAT   744
             ************************************************************


Rev          GCCGATTGTTAGCAAAATTGCAACCGAAAATAAGATCCCGGTTATTGCAGCAGAAAGCGG   614
p.08730.His  GCCGATTGTTAGCAAAATTGCAACCGAAAATAAGATCCCGGTTATTGCAGCAGAAAGCGG   1491
For          GCCGATTGTTAGCAAAATTGCAACCGAAAATAAGATCCCGGTTATTGCAGCAGAAAGCGG   804
             ************************************************************


Rev          TCCGGTTGAAAAAGGTGCACTGGCAGCACAGGGTATCAATTATGAAAAACTGGGTTATAA   674
p.08730.His  TCCGGTTGAAAAAGGTGCACTGGCAGCACAGGGTATCAATTATGAAAAACTGGGTTATAA   1551
For          TCCGGTTGAAAAAGGTGCACTGGCAGCACAGGGTATCAATTATGAAAAACTGGGTTATAA   864
             ************************************************************


Rev          GACCGGTGAGATGGCAGTGAAAATTCTGAATGGTGAAAGCGTTAGCGATATG---CCGGT   731
p.08730.His  GACCGGTGAGATGGCAGTGAAAATTCTGAATGGTGAAAGCGTTAGCGATAT---GCCGGT   1608
For          GACCGGTGAGATGGCAGTGAAAATTCTGAATGGGTGAAAGCCGTTAGCAATATGCCCGGT   924
             ********************************   **        *  **    *****


Rev          TGCCACCAGTGATGATACCGATATTATTGTGAACGAGGATATTCTGGAAGCCCTTGGTAT   791
p.08730.His  TGCCACCAGTGATGATACCGATATTATTGTGAACGAGGATATTCTGAAAGCCCTTGGTAT   1668
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For          TGCCACCAGTGATGATACCCGATATTATTTGTGAAC------------------------   960
             ******************     *  * **

Rev          GGAAAAACCGAGCAATGAAAACATCAGCTACGTGAAAACCAAGCAAGAGCTGCAGGTTAG   851
p.08730.His  GGAAAAACCGAGCAATGAAAACATCAGCTACGTGAAAACCAAGCAAGAGCTGCAGGAA-G   1727
For          ------------------------------------------------------------   960


Rev          AAAATCCGCTGGTGCCACGCGGTAGTTCCGCTCATCACCACCATCATCACCATCACCACC   911
p.08730.His  GGGATCCGCTGGTGCCACGCGGTAGTTCCGCTCATCACCACCATCATCACCATCACCACC   1787
For          ------------------------------------------------------------   960


Rev          ACTAAGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTTCTTAAACCGGTTTTT-----   966
p.08730.His  ACTAAGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTT   1847
For          ------------------------------------------------------------   960


Rev           ----------------------- 966
p.08730.His   TTTTGCTGAAAGGAGGAACTATA 1870
For           ----------------------- 960
```

D)

pTWIN1-CD630_27190.His

```
Rev          ------------------------------------------------------------   0
p.27190.His  CTGGGACTCCATCGTTTCTATTACGGAGACTGGAGTCGAAGAGGTTTTTGATTTGACTGT   720
For          -------------------------CCCGGGGGGCCGAAGAAGTTTTTTGATTTTGACTGT   36


Rev          ------------------------------------------------------------   0
p.27190.His  GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGCAGCAACAGCCA   780
For          GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGCAGCAACAGCCA   96


Rev          ------------------------------------------------------------   0
p.27190.His  GAATAATCAGAATGAAAACCAGAACAAAGAAACCCAGCTGCAAGAGGATAAAGAGAAAAT   840
For          GAATAATCAGAATGAAAACCAGAACAAAGAAACCCAGCTGCAAGAGGATAAAGAGAAAAT   156


Rev          ------------------------------------------------------------   0
p.27190.His  TGATAGCGGTAAAGATACCAGCAACGTGATTGTTAGTGATGGCACCGATAAACCGAGCAA   900
For          TGATAGCGGTAAAGATACCAGCAACGTGATTGTTAGTGATGGCACCGATAAACCGAGCAA   216


Rev          ------------------------------------------------------------   0
p.27190.His  AGCAACCACCAATAACGATAACAATAAACTGGATGTTAGCAGCCTGGATAATACCACACT   960
For          AGCAACCACCAATAACGATAACAATAAACTGGATGTTAGCAGCCTGGATAATACCACACT   276


Rev          ------------------------------------------------------------   0
p.27190.His  GGATTGGTTTTATATCCCGAACAACAAACACAAAACACCGGAAGTGAATACCGACATCGA   1020
For          GGATTGGTTTTATATCCCGAACAACAAACACAAAACACCGGAAGTGAATACCGACATCGA   336


Rev          ------------------------------------------------------------   0
p.27190.His  ATTCAAATTCAGCGATTATGATGCCCTGTATAATGGTCCGACCAAAGATGGTCAGAAAAC   1080
For          ATTCAAATTCAGCGATTATGATGCCCTGTATAATGGTCCGACCAAAGATGGTCAGAAAAC   396


Rev          ------------------------------------------------------------   0
p.27190.His  CCTGTATCTGACCTTTGATGAAGGTTATGAAAATGGCTACACCACCAAAATTCTGGATAC   1140
For          CCTGTATCTGACCTTTGATGAAGGTTATGAAAATGGCTACACCACCAAAATTCTGGATAC   456


Rev          ------------------------------------------------------------   0
p.27190.His  CCTGAAACAGAATCAGGTGAAAGCCGTTTTTTTTGTTACCGCACCGTATATCAAAGAGAA   1200
For          CCTGAAACAGAATCAGGTGAAAGCCGTTTTTTTTGTTACCGCACCGTATATCAAAGAGAA   516


Rev          ------------------------------------------------------------   0
p.27190.His  CAAGGATCTGGTTAAACGCATGGTTAGCGAAGGTCATATTGTTGGCAATCATAGCAAAAC   1260
For          CAAGGATCTGGTTAAACGCATGGTTAGCGAAGGTCATATTGTTGGCAATCATAGCAAAAC   576
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Rev          ------------------------------------------------------------   0
p.27190.His  CCATCCGAGCATGCCGACCAAAACCAGCAATCTGAAAAACTTTAACGATGAGCTGTACGA   1320
For          CCATCCGAGCATGCCGACCAAAACCAGCAATCTGAAAAACTTTAACGATGAGCTGTACGA   636

Rev          ---------------------------------------------AGTTTTTTCGTCCGCC   16
p.27190.His  CGTGGAAAAACTGTATAAAGATGTTACCGGCAAAGATATGGTGAAGTTTTTTCGTCCGCC   1380
For          CGTG-----------------------------------------------------------   640

Rev          TATGGGTAAATATAGCGAAAAAAGCCTGGCCATGACCAAAAATCTGGGTTATAAAACCGT   76
p.27190.His  TATGGGTAAATATAGCGAAAAAAGCCTGGCCATGACCAAAAATCTGGGTTATAAAACCGT   1440
For          ------------------------------------------------------------   640

Rev          GTTTTGGAGCTTTGCATATCGTGATTGGGATACAGATAAACAGCCGAGCCATGAAGAAGC   136
p.27190.His  GTTTTGGAGCTTTGCATATCGTGATTGGGATACAGATAAACAGCCGAGCCATGAAGAAGC   1500
For          ------------------------------------------------------------   640

Rev          AACCCAGAAAATTATGGATAATCTGCATGATGGTAGCATCCTGCTGCTGCATGCCGTTAG   196
p.27190.His  AACCCAGAAAATTATGGATAATCTGCATGATGGTAGCATCCTGCTGCTGCATGCCGTTAG   1560
For          ------------------------------------------------------------   640

Rev          CAAAACAAGCACCGAAATTCTGAATGACTTTATCAGCAATGCACGCAAACTGGGCTATGA   256
p.27190.His  CAAAACAAGCACCGAAATTCTGAATGACTTTATCAGCAATGCACGCAAACTGGGCTATGA   1620
For          ------------------------------------------------------------   640

Rev          ATTTGAGCTGCTGGAATATCTGCAGGAAGGGGATCCGCTGGTGCCACGCGGTAGTTCCGC   316
p.27190.His  ATTTGAGCTGCTGGAATATCTGCAGGAAGGGGATCCGCTGGTGCCACGCGGTAGTTCCGC   1680
For          ------------------------------------------------------------   640

Rev          TCATCACCACCATCATCACCATCACCACCACTAAGCTGAGCAATAACTAGCATAACCCCT   376
p.27190.His  TCATCACCACCATCATCACCATCACCACCACTAAGCTGAGCAATAACTAGCATAACCCCT   1740
For          ------------------------------------------------------------   640

Rev          TGGGCCTCTAAAAACGTTCAG-----------------------------   397
p.27190.His  TGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTAT   1791
For          ---------------------------------------------------   640
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E)

pTWIN1-CD630_34640.His

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For          ------------------------------------------------------------   0
p.34640.His  CGGCAAGACGTATAAATGTTTGCAGCCCCACACCTCCTTGGCAGGATGGGAACCATCCAA   240
Rev          ----------------------CGTTCCCATAGTACCTTGGGGGCAGG------------   26

For          ------------------------------------------------------------   0
p.34640.His  CGTTCCTGCCTTGTGGCAGCTTCAAAACAACGGT-AACAACGGTCTCGAACTGCGCGAGT   299
Rev          ---------GCTTTC---TAAAAAACAAACCGGTAACAACGGTTCTCGGAATGCGCGAGT   74

For          ------------------------------------------------------------   0
p.34640.His  CCGGAGCTATCT---CTGGC--GATAGTCTGATCAGCCTGGCTAGCACAGGAAAAAGAGT   354
Rev          CCACGGAGCAATTCTCTGACAGAAAGTTGATACAGGCGGGCTGCACCCGGAAAAAAGAGT   134

For          ------------------------------------------------------------   0
p.34640.His  TTCTATTAAAGATTTGTTAGATGAAAAAGATTTTGAAATATGGGCAATTAATGAACAGAC   414
Rev          TTCTATTAAAGGTTGTTTAGATAAAAAAGATTTGAAATTGGGCAATAAAGA-----CAGG   189

For          ------------------------------------------------------------   0
p.34640.His  GATGAAGCTAGAATCAGCTAAAGTTAGTCGTGTATTTTGTACTGGCAAAAAGCTAGTTTA   474
Rev          CGAGAAGCTAGGATCAGCTAAATTTAGTGG---GTATTTGTCTGGCAAAAAGCT--AGTT   244

For          ------------------------------------------------------------   0
p.34640.His  TATTCTAAAAACTCGACTAGGTAGAACTATCAAGGCAACAGCAAATCATAGATTTTTAAC   534
Rev          TATTTTTAAAAACTGACTAGGTAGAACTATCAAGGCAACAGCAAATCATAGATTTTTAAC   304
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For          ------------------------------------------------------------   0
p.34640.His  TATTGATGGTTGGAAAAGATTAGATGAGCTATCTTTAAAAGAGCATATTGCTCTACCCCG   594
Rev          TATTGAGGGTGGAAAAAGATTAGATGAGCTATCCTTAAAAGAGCATATTGCTTTCCCCCG   364


For          ------------------------------------------------------------   0
p.34640.His  TAAACTAGAAAGCTCCTCTTTACAATTGTCACCAGAAATAGAAAAGTTGTCTCAGAGTGA   654
Rev          TAAACTAGAAAGCTCCTCTTTACAATTGTCCCCAGAAATAGAAAAAGTGTCTCAGAGTGA   424


For          ----------------------------------CATTGTTGAATCGAAAAAGTTTTTT   25
p.34640.His  TATTTACTGGGACTCCAT-CGTTTCTATTAC-GGAGACTGGAGTCG-AAG--AGGTTTTT   709
Rev          TATTTACTGGGGACTCCATCGGTTCTATTACCGGAGACTGGAGTCG-AAAGAGGTTTTTT   483
                                            * **  *     *    * *****

For          GATTTGAC-TGTGCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTG   84
p.34640.His  GATTTGACTGT-GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTG   768
Rev          GATTTGACTGTGCCAGGACCCACATAACTTTGTCGCGAATGACATCATTGTACACAACTG   543
             ********     *  *  ****************************************

For          TCAGAAACGTCAGAGCACCAAAGAAGAGGTGTATAAAGATTTCCAAAAGCAGATCAGCGA   144
p.34640.His  TCAGAAACGTCAGAGCACCAAAGAAGAGGTGTATAAAGATTTCCAAAAGCAGATCAGCGA   828
Rev          TCAGGAACGTCAGAGCACCAAAGAAGAGGTGTATAAAGATTTCCAAAAGCAGATCAGCGA   603
             **** ************************************************************

For          TATGAACTATTATAGCGCAAAAGCCGAAGTTGAAGTGGTGGGTAATAAAAGTCCGCATAA   204
p.34640.His  TATGAACTATTATAGCGCAAAAGCCGAAGTTGAAGTGGTGGGTAATAAAAGTCCGCATAA   888
Rev          TATGAACTATTATAGCGCAAAAGCCGAAGTTGAAGTGGTGGGTAATAAAAGTCCGCATAA   663
             ************************************************************

For          CTATGTTCTGATCCACACCTACAAAAAGACCGACAACTATAAACTGGAAGTGATCAGCCC   264
p.34640.His  CTATGTTCTGATCCACACCTACAAAAAGACCGACAACTATAAACTGGAAGTGATCAGCCC   948
Rev          CTATGTTCTGATCCACACCTACAAAAAGACCGACAACTATAAACTGGAAGTGATCAGCCC   723
             ************************************************************

For          GAAACATCTGAAAGGTAAAAGCATTGAATATCAGGGCGATAAGATCCTGGTGAAAAACCC   324
p.34640.His  GAAACATCTGAAAGGTAAAAGCATTGAATATCAGGGCGATAAGATCCTGGTGAAAAACCC   1008
Rev          GAAACATCTGAAAGGTAAAAGCATTGAATATCAGGGCGATAAGATCCTGGTGAAAAACCC   783
             ************************************************************

For          GAAAATTAGTGATGTTGTTGAACTGCCGAATACCGGCAAAAACAATCAGTACCTGTTTGT   384
p.34640.His  GAAAATTAGTGATGTTGTTGAACTGCCGAATACCGGCAAAAACAATCAGTACCTGTTTGT   1068
Rev          GAAAATTAGTGATGTTGTTGAACTGCCGAATACCGGCAAAAACAATCAGTACCTGTTTGT   843
             ************************************************************

For          GGGTGACTTCATCAAAAACTATCTGCAGAACGAAGAGATGAAAGTGAAACTGAGCAAAGG   444
p.34640.His  GGGTGACTTCATCAAAAACTATCTGCAGAACGAAGAGATGAAAGTGAAACTGAGCAAAGG   1128
Rev          GGGTGACTTCATCAAAAACTATCTGCAGAACGAAGAGATGAAAGTGAAACTGAGCAAAGG   903
             ************************************************************

For          TCATCTGGTTCTGGAAACATTTATTCCGGGTGACAACAAGTACTTCAATAAACAGGTTCT   504
p.34640.His  TCATCTGGTTCTGGAAACATTTATTCCGGGTGACAACAAGTACTTCAATAAACAGGTTCT   1188
Rev          TCATCTGGTTCTGGAAACATTTATTCCGGGTGACAACAAGTACTTCAATAAACAGGTTCT   963
             ************************************************************

For          GTATGTGAACGCCGATACCAAAAATCCGGAAAAAATGGAAGTGCTGGATAAAGAAGGTGT   564
p.34640.His  GTATGTGAACGCCGATACCAAAAATCCGGAAAAAATGGAAGTGCTGGATAAAGAAGGTGT   1248
Rev          GTATGTGAACGCCGATACCAAAAATCCGGAAAAAATGGAAGTGCTGGATAAAGAAGGTGT   1023
             ************************************************************

For          TCCGCGTTTTACCGTGAAATACAAAGATTTTGAGTATCGCAAGGATCCGCTGGTGCCACG   624
p.34640.His  TCCGCGTTTTACCGTGAAATACAAAGATTTTGAGTATCGCAAGGATCCGCTGGTGCCACG   1308
Rev          TCCGCGTTTTACCGTGAAATACAAAGATTTTGAGTATCGCAAGGATCCGCTGGTGCCACG   1083
             ************************************************************

For          CGGTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAAGCTGAGCAATAACT   684
p.34640.His  CGGTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAAGCTGAGCAATAACT   1368
Rev          CGGTAGTTCCGCTCATCTCCACCATCATCACCATCACCACCACTAAGCTGAGCAATAACT   1143
             ***************** ******************************************

For          AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAAC   744
p.34640.His  AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAAC   1428
Rev          AGCATAACCCCTTGGGCCTCAAAACGTCAGCCCCATTCTTTTAG----------------   1187
             *************** *    **      *   *    ***
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F)

pTWIN1-CD630_TcdA-RBD.His

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For          ----------------------------CCGCTCTGATGAGAGGTTTTTGATTTGACTGT   32
p.TcdA.His   CTGGGACTCCATCGTTTCTATTACGGAGACTGGAGTCGAAGAGGTTTTTGATTTGACTGT   720
Rev          ------------------------------------------------------------   0

For          GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTAAAGCAGTTAC   92
p.TcdA.His   GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTAAAGCAGTTAC   780
Rev          ------------------------------------------------------------   0

For          CGGTTGGCAGACCATTGATGGCAAAAAATACTATTTCAACCTGAACACCGCAGAAGCAGC   152
p.TcdA.His   CGGTTGGCAGACCATTGATGGCAAAAAATACTATTTCAACCTGAACACCGCAGAAGCAGC   840
Rev          ------------------------------------------------------------   0

For          AACAGGCTGGCAGACAATCGACGGTAAAAAGTATTACTTTAATCTGAACACAGCCGAGGC   212
p.TcdA.His   AACAGGCTGGCAGACAATCGACGGTAAAAAGTATTACTTTAATCTGAACACAGCCGAGGC   900
Rev          -------CCACTTTCAGAC-GCGGTAAAAAAGTATATCCTGATTCGAACCACGCCGAGGC   52
                      *   **  *  *********    **   * **  ****   ********

For          AGCCACTGGTTGGCAAACGATCGATGGGAAGAAATATTACTTCAACACCAACACCTTTAT   272
p.TcdA.His   AGCCACTGGTTGGCAAACGATCGATGGGAAGAAATATTACTTCAACACCAACACCTTTAT   960
Rev          AGCCA---TTGTGCAAACGATCGATGG-AAGAAATATTA-CTCCACCCCATCACCTTTAT   107
             *****    *  *************** ***********  ** ** *** *********

For          TGCCAGCACCGGTTATACCAGCATTAACGGTAAACACTTCTACTTTAACACCGATGGCAT   332
p.TcdA.His   TGCCAGCACCGGTTATACCAGCATTAACGGTAAACACTTCTACTTTAACACCGATGGCAT   1020
Rev          TGCCAGC---ACATATACCAGCATGAACGGTAAACACT--CTACTTTACACCGATGGCAT   162
             *******      *********** *************      ** *************

For          TATGCAGATCGGTGTTTTTAAAGGTCCGAACGGCTTTGAATATTTTGCACCGGCAAATAC   392
p.TcdA.His   TATGCAGATCGGTGTTTTTAAAGGTCCGAACGGCTTTGAATATTTTGCACCGGCAAATAC   1080
Rev          TATGCAGATCGGTGTT-TTTAAGGTCCGAACGGCTTTGAATATTTTGCACCGGCAAATAC   221
             **************** ** ****************************************

For          CCATAACAACAATATTGAAGGTCAGGCCATTCTGTATCAGAACAAATTTCTGACCCTGAA   452
p.TcdA.His   CCATAACAACAATATTGAAGGTCAGGCCATTCTGTATCAGAACAAATTTCTGACCCTGAA   1140
Rev          CCATAACAACAATATTGAAGGTCAGGCCATTCTGTATCAGAACAAATTTCTGACCCTGAA   281
             ************************************************************

For          CGGCAAGAAGTACTACTTTGGTAGCGATAGCAAAGCCGTGACCGGTCTGCGTACAATAGA   512
p.TcdA.His   CGGCAAGAAGTACTACTTTGGTAGCGATAGCAAAGCCGTGACCGGTCTGCGTACAATAGA   1200
Rev          CGGCAAGAAGTACTACTTTGGTAGCGATAGCAAAGCCGTGACCGGTCTGCGTACAATAGA   341
             ************************************************************

For          CGGAAAGAAATACTATTTTAACACCAATACCGCAGTTGCGGTGACCGGCTGGCAAACTAT   572
p.TcdA.His   CGGAAAGAAATACTATTTTAACACCAATACCGCAGTTGCGGTGACCGGCTGGCAAACTAT   1260
Rev          CGGAAAGAAATACTATTTTAACACCAATACCGCAGTTGCGGTGACCGGCTGGCAAACTAT   401
             ************************************************************

For          AAATGGAAAGAAGTATTATTTCAATACGAATACGAGCATTGCGAGTACCGGCTATACAAT   632
p.TcdA.His   AAATGGAAAGAAGTATTATTTCAATACGAATACGAGCATTGCGAGTACCGGCTATACAAT   1320
Rev          AAATGGAAAGAAGTATTATTTCAATACGAATACGAGCATTGCGAGTACCGGCTATACAAT   461
             ************************************************************

For          TATTAGCGGCAAACACTTTTATTTCAACACGGACGGTATCATGCAGATTGGCGTGTTCAA   692
p.TcdA.His   TATTAGCGGCAAACACTTTTATTTCAACACGGACGGTATCATGCAGATTGGCGTGTTCAA   1380
Rev          TATTAGCGGCAAACACTTTTATTTCAACACGGACGGTATCATGCAGATTGGCGTGTTCAA   521
             ************************************************************

For          AGGACCGGATGGTTTTGAGTATTTCGCTCCTGCCAATACCGATGCCAATAACATCGAAGG   752
p.TcdA.His   AGGACCGGATGGTTTTGAGTATTTCGCTCCTGCCAATACCGATGCCAATAACATCGAAGG   1440
Rev          AGGACCGGATGGTTTTGAGTATTTCGCTCCTGCCAATACCGATGCCAATAACATCGAAGG   581
             ************************************************************

For          CCAGGCAATCCGTTATCAGAATCGTTTTCTGTATCTGCACGACAACATCTATTATTTCGG   812
p.TcdA.His   CCAGGCAATCCGTTATCAGAATCGTTTTCTGTATCTGCACGACAACATCTATTATTTCGG   1500
Rev          CCAGGCAATCCGTTATCAGAATCGTTTTCTGTATCTGCACGACAACATCTATTATTTCGG   641
             ************************************************************

For          CAATAATTCAAAAGCAGCCACCGGCTGGGGTTACAATTGATGGTAATCGTTATTACTTTG   872
p.TcdA.His   CAATAATTCAAAAGCAGCCACCGGCTG-GGTTACAATTGATGGTAATCGTTATTACTTTG   1559
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Rev          CAATAATTCAAAAGCAGCCACCGGCTG-GGTTACAATTGATGGTAATCGTTATTACTTTG   700
             ************************** ******************************

For          AGCCGAATACCGCAATGGGTGCCAATGGCTATAAAACCATTGACAACAAAAACTTCTAAT   932
p.TcdA.His   AGCCGAATACCGCAATGGGTGCCAATGGCTATAAAACCATTGACAACAAAAACTTCTATT   1619
Rev          AGCCGAATACCGCAATGGGTGCCAATGGCTATAAAACCATTGACAACAAAAACTTCTATT   760
             ********************************************************** *

For          TTTTCGCAATGGGCCTGCCGCAGATTGGAGTATTTTAAGGCAGCATTGGTGTCGAATACT   992
p.TcdA.His   TT---CGCAATGGCCTGCCGCAGATTGGAGTATTTAAAGGCAGCAATGGTTTCGAATACT   1676
Rev          TT---CGCAATGGCCTGCCGCAGATTGGAGTATTTAAAGGCAGCAATGGTTTCGAATACT   817
             **      *  ********************** ********* **** *********

For          TTGCCCCAGCGAATACAGATGCAAACACATT-GAGGGTCAAGCAATTCGCTATC--AAAC   1049
p.TcdA.His   TTGCCCCAGCGAATACAGATGCAAACAACATTGAGGGTCAAGCAATTCGCTATCAAAACC   1736
Rev          TTGCCCCAGCGAATACAGATGCAAACAACATTGAGGGTCAAGCAATTCGCTATCAAAACC   877
             **************************   * **********************  ** *

For          GCTTCCTGCATCTGCTTGGCAGAATTTACTATTTTTGGCTACAACACAAAGCGGTAACCT   1109
p.TcdA.His   GCTTCCTGCATCTGCTGGGCAAAATTTACTATTTTGGCAACAACAGCAAAGCGGTAACTG   1796
Rev          GCTTCCTGCATCTGCTGGGCAAAATTTACTATTTTGGCAACAACAGCAAAGCGGTAACTG   937
             *************** **** ************ *     *   ************

For          GAATGGCAAGACGATTAATGGTAAAGTGTAACTACTTCATGTCCGAATTACCGCCCATGC   1169
p.TcdA.His   GATGGC--AGACGATTAATGGTAAAGTGTACTACT-TCATGCCGGAT--ACCG-CCATGG   1850
Rev          GATGGC--AGACGATTAATGGTAAAGTGTACTACT-TCATGCCGGAT--ACCG-CCATGG   991
             **  *   **********************      ***** * **   **** *****

For          AAGCAGCAGC-GGCCCGTTTGGAAATATGAATGGGCGCGGTATATCTA------------   1216
p.TcdA.His   CAGCAGCAGGCGGTCTGTTTGAAATTGATGGCGTTATCTATTTCTTTGGTGTGGATGGTG   1910
Rev          CAGCAGCAGGCGGTCTGTTTGAAATTGATGGCGTTATCTATTTCTTTGGTGTGGATGGTG   1051
             ********  ** * ***** ** *      *    *  * * * *

For          ------------------------------------------------------------   1216
p.TcdA.His   TGAAAGCACCGGGTATTTATGGGGATCCGCTGGTGCCACGCGGTAGTTCCGCTCATCACC   1970
Rev          TGAAAGCACCGGGTATTTATGGGGATCCGCTGGTGCCACGCGGTAGTTCCGCTCATCACC   1111

For          ------------------------------------------------------------   1216
p.TcdA.His   ACCATCATCACCATCACCACCACTAAGCTGAGCAATAACTAGCATAACCCCTTGGGGCCT   2030
Rev          ACCATCATCACCATCACCACCACTAAGCTGAGCAATAACTAGCATAACCCCTTGGGCCTC   1171

For          ------------------------------------------- 1216
p.TcdA.His   CTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTAT 2073
Rev          TAAAGCTGTTTG------------------------------- 1183
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G)

pTWIN1-CD630_TcdB-RDB.His

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Rev          ------------------------------------------------------------   0
p.TcdB.His   CTGGGACTCCATCGTTTCTATTACGGAGACTGGAGTCGAAGAGGTTTTTGATTTGACTGT   720
For          --------------------------------CGTGTCGCGAGAGTTTTTGATTTGACTGT   29

Rev          ------------------------------------------------------------   0
p.TcdB.His   GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTATTACCGGTTT   780
For          GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGTATTACCGGTTT   89

Rev          ------------------------------------------------------------   0
p.TcdB.His   TGTTACCGTGGGTGATGACAAATATTACTTCAATCCGATTAATGGTGGTGCAGCAAGCAT   840
For          TGTTACCGTGGGTGATGACAAATATTACTTCAATCCGATTAATGGTGGTGCAGCAAGCAT   149

Rev          ------------------------------------------------------------   0
p.TcdB.His   TGGTGAAACCATTATTGACGACAAGAACTACTATTTCAATCAGAGCGGTGTTCTGCAGAC   900
For          TGGTGAAACCATTATTGACGACAAGAACTACTATTTCAATCAGAGCGGTGTTCTGCAGAC   209

Rev          ------------------------------------------------------------   0
p.TcdB.His   CGGTGTTTTTAGCACCGAAGATGGCTTTAAATACTTTGCACCGGCAAATACCCTGGATGA   960
For          CGGTGTTTTTAGCACCGAAGATGGCTTTAAATACTTTGCACCGGCAAATACCCTGGATGA   269
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Rev          ------------------------------------------------------------   0
p.TcdB.His   AAATCTGGAAGGTGAAGCCATTGATTTTACCGGCAAACTGATTATCGATGAGAACATCTA   1020
For          AAATCTGGAAGGTGAAGCCATTGATTTTACCGGCAAACTGATTATCGATGAGAACATCTA   329


Rev          ------------------------------------------------------------   0
p.TcdB.His   TTACTTCGACGACAATTATCGTGGTGCCGTTGAATGGAAAGAACTGGATGGTGAAATGCA   1080
For          TTACTTCGACGACAATTATCGTGGTGCCGTTGAATGGAAAGAACTGGATGGTGAAATGCA   389


Rev          ------------------------------------------------------------   0
p.TcdB.His   CTATTTTAGTCCGGAAACCGGCAAAGCGTTTAAAGGTCTGAATCAGATTGGCGACTATAA   1140
For          CTATTTTAGTCCGGAAACCGGCAAAGCGTTTAAAGGTCTGAATCAGATTGGCGACTATAA   449


Rev          ------------------------------------------------------------   0
p.TcdB.His   GTACTATTTTAACAGTGATGGCGTGATGCAGAAAGGCTTTGTTAGCATTAACGACAACAA   1200
For          GTACTATTTTAACAGTGATGGCGTGATGCAGAAAGGCTTTGTTAGCATTAACGACAACAA   509


Rev          ----------GATACCGAGGGCGTTTTGTAAAGTGGGCCTATCCGGATCGATGCCACACT   50
p.TcdB.His   ACACTATTTCGATGATTCAGGCGTTATGAAAGTGGGCTATACCGAAATTGATGGCAAACA   1260
For          ACACTATTTCGATGATTCAGGCGTTATGAAAGTGGGCTATACCGAAATTGATGGCAAACA   569
                       * **      ****** ** **   **   ** *   ** **** ** **

Rev          TTA-------TTCGCGAATGCGAAATGCAGATTCGAGCGTGTCATTACAGAAGAATGGTT   103
p.TcdB.His   CTTTTATTTCGCCGAAAATGGCGAAATGCAGATCGGCGTGTTTAATACAGAAGATGGTTT   1320
For          CTTTTATTTCGCCGAAAATGGCGAAATGCAGATCGGCGTGTTTAATACAGAAGATGGTTT   629
              *          **  ****   **       ***      * * *********  * **

Rev          CCAGTATTTCGCCCCACA----TATGAGATCTGGTATGAAGAAGGCGAAGAAAGTAGCTA   159
p.TcdB.His   CAAGTATTTCGCCCACCATAATGAAGATCTGGGTAATGAAGAAGGCGAAGAAATTAGCTA   1380
For          CAAGTATTTCGCCCACCATAATGAAGATCTGGGTAATGAAGAAGGCGAAGAAATTAGCTA   689
             * ************  **     * **  *  *  ****************** ******

Rev          TAGCGGCATTCTGAACTCAACAACCAAGATCTACTACTTGATGACAGCTTAC--CGCAGT   217
p.TcdB.His   TAGCGGCATTCTGAACTTCAACAACAAGATCTACTACTTTGATGACAGCTTTACCGCAGT   1440
For          TAGCGGCATTCTGAACTTCAACAACAAGATCTACTACTTTGATGACAGCTTTACCGCAGT   749
             ******************  *  * ***************          *    ******

Rev          TGT--GGTTGGAAGATTTAGA-AGATGGCAGCAATACTATTTTGATGAGGATACCGCAGA   274
p.TcdB.His   TGTTGGTTGGAAAGATTTAGAAGATGGCAGCAAATACTATTTTGATGAGGATACCGCAGA   1500
For          TGTTGGTTGGAAAGATTTAGAAGATGGCAGCAAATACTATTTTGATGAGGATACCGCAGA   809
             ***  * * * **********     *     ****************************

Rev          AGCCTATATTGGTCTGAGCCTGATTAATGATGGCCAGTATTATTTCAACGATGACGGCAT   334
p.TcdB.His   AGCCTATATTGGTCTGAGCCTGATTAATGATGGCCAGTATTATTTCAACGATGACGGCAT   1560
For          AGCCTATATTGGTCTGAGCCTGATTAATGATGGCCAGTATTATTTCAACGATGACGGCAT   869
             ************************************************************

Rev          TATGCAGGTTGGTTTTGTGACCATTAACGATAAGGTGTTCTATTTCAGCGACAGCGGTAT   394
P.TcdB.His   TATGCAGGTTGGTTTTGTGACCATTAACGATAAGGTGTTCTATTTCAGCGACAGCGGTAT   1620
For          TATGCAGGTTGGTTTTGTGACCATTAACGATAAG-TGTTCTATTTCAGCGACAGCGGTAT   928
             ********************************** ************************

Rev          TATTGAAAGCGGTGTGCAGAATATCGATGACAACTATTTCTACATCGACGATAATGGCAT   454
p.TcdB.His   TATTGAAAGCGGTGTGCAGAATATCGATGACAACTATTTCTACATCGACGATAATGGCAT   1680
For          TATTGAAAGCGGTGTGCAGATATCGATGAC--ACTATTTCTACATCGACGATATGC--AT   984
             ********************           *********************     **

Rev          TGTTCAGATTGGTGTGTTTGATACCAGTGACGGCTATAAGTATTTTGCCCCTGCCAATAC   514
p.TcdB.His   TGTTCAGATTGGTGTGTTTGATACCAGTGACGGCTATAAGTATTTTGCCCCTGCCAATAC   1740
For          TGTTCAGATTG---TGTGTTGATACAGTGACGGCTATAGT-ATTTTGCCCCTGCT-ATAC   1039
             ***********   *** *     **************   *************  ****

Rev          CGTGAACGATAACATTTATGGTCAGGCGGTTGAATACAGCGGTCTGGTTCGTGTTGGTGA   574
p.TcdB.His   CGTGAACGATAACATTTATGGTCAGGCGGTTGAATACAGCGGTCTGGTTCGTGTTGGTGA   1800
For          CGTGAACGAATAACAATTTATGATCAGCGTTGGATACAGGCGGATCTGGTTCGGTTGGTG   1099
             *********  *    * *         **** ******  *  *       * * *

Rev          AGATGTTTATTACTTTGGCGAAACCTATACCATTGAAACCGGCTGGATTTATGATATGGA   634
p.TcdB.His   AGATGTTTATTACTTTGGCGAAACCTATACCATTGAAACCGGCTGGATTTATGATATGGA   1860
For          AGATGTTTATTACTTTGTCTACTTTACATGAACCGCCTGAGTGTGCATT-------GGAA   1152
             ****************** * *          *  *     *  ** ***       * *

Rev          AAACGAGAGCGATAAATACTACTTTAACCCGGAAACCAAAAAAGCAGCCAAAGGCATTAA   694
p.TcdB.His   AAACGAGAGCGATAAATACTACTTTAACCCGGAAACCAAAAAAGCAGCCAAAGGCATTAA   1920
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For          ATCGTAGAGCGAAGAATACCAACTTTTAC------------------------------   1181
             *    *******  ***** *  **   *

Rev          TCTGATCGACGACATCAAGTACTACTTCGATGAAAAAGGTATTATGCGTACCGGTCTGAT   754
p.TcdB.His   TCTGATCGACGACATCAAGTACTACTTCGATGAAAAAGGTATTATGCGTACCGGTCTGAT   1980
For         ------------------------------------------------------------   1181

Rev          CAGCTTTGAAAACAATAACTATTACTTTAACGAGAACGGCGAGATGCAGTTTGGCTATAT   814
p.TcdB.His   CAGCTTTGAAAACAATAACTATTACTTTAACGAGAACGGCGAGATGCAGTTTGGCTATAT   2040
For          ------------------------------------------------------------   1181

Rev          TAATATCGAGGATAAAATGTTCTACTTCGGTGAGGATGGTGTTATGCAGATTGGAGTTTT   874
p.TcdB.His   TAATATCGAGGATAAAATGTTCTACTTCGGTGAGGATGGTGTTATGCAGATTGGAGTTTT   2100
For          ------------------------------------------------------------   1181

Rev          TAATACTCCGGACGGCTTCAAGTACTTTGCGCATCAGAACACACTGGATGAGAATTTTGA   934
p.TcdB.His   TAATACTCCGGACGGCTTCAAGTACTTTGCGCATCAGAACACACTGGATGAGAATTTTGA   2160
For          ------------------------------------------------------------   1181

Rev          AGGCGAAAGCATTAACTATACCGGTTGGCTGGATCTGGACGAAAAACGCTACTACTTCAC   994
p.TcdB.His   AGGCGAAAGCATTAACTATACCGGTTGGCTGGATCTGGACGAAAAACGCTACTACTTCAC   2220
For          ------------------------------------------------------------   1181

Rev          CGATGAATACATTGCAGCAACCGGTAGCGTGATTATTGATGGTGAAGAATATTACTTTGA   1054
p.TcdB.His   CGATGAATACATTGCAGCAACCGGTAGCGTGATTATTGATGGTGAAGAATATTACTTTGA   2280
For          ------------------------------------------------------------   1181

Rev          TCCGGATACCGCACAGCTGGTTATTAGCGAGGATCCGCTGGTGCCACGCGGTAGTTCCGC   1114
p.TcdB.His   TCCGGATACCGCACAGCTGGTTATTAGCGAGGATCCGCTGGTGCCACGCGGTAGTTCCGC   2340
For          ------------------------------------------------------------   1181

Rev          TCATCACCACGGATCATCACCATCACCACCACTAAGCTGAGCAATAACTAGCATAACCCC   1174
p.TcdB.His   TCATCACCACC-ATCATCACCATCACCACCACTAAGCTGAGCAATAACTAGCATAACCCC   2399
For          ------------------------------------------------------------   1181

Rev          TTGGGCCTCTTAAGCTTTTAG--------------------------------  1195
p.TcdB.His   TTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTAT  2451
For          ------------------------------------------------------  1181
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H)

pTWIN1-CD630_cwp84.His (using internal primers)

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For          ------------------------------------------------------------   0
p.cwp84.His  CTGGGACTCCATCGTTTCTATTACGGAGACTGGAGTCGAAGAGGTTTTTGATTTGACTGT   720
Rev          ---------------------------------GTCCGAAGGAGTTTCTGAGATGACTTG   27

For          ------------------------------------------------------------   0
p.cwp84.His  GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGCGAAAATCATAA   780
Rev          ---CAGTGCCAATTACTTGTGGGCGATAGACATCATGTAACC--AACGCGAAATTCATA-   81

For          ------------------------------------------------------------   0
p.cwp84.His  AACCCTGGATGGTGTTGAAACCGCAGAATATAGCGAAAGCTATCTGCAGTATCTGGAAGA   840
Rev          -AAA-CTGGATGGTGTGAATCCGCAGAATATAGCGAAGCTATTCTGCAGTATCTGA-AGA   138

For          ------------------------------------------------------------   0
p.cwp84.His  TGTGAAAAATGGTGATACCGCCAAATATAACGGTGTTATTCCGTTTCCGCATGAAATGGA   900
Rev          TGTGAAAAATGGTGATACGC-CAAATAATACGGTGTTAT-TCCGTTTCGCATGAAATGGA   196

For          ------------------------------------------------------------   0
p.cwp84.His  AGGTACAACCCTGCGTAATAAAGGTCGTAGCAGCCTGCCGAGCGCATATAAAAGCAGCGT   960
Rev          AGGTACAATCCTGCGTAATAAAGGTCGTAGCAG-CTGCCGAGCGCATATAAAAGCAGCGT   255
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For          ------------------------------------------------------------   0
p.cwp84.His  TGCATATAATCCGATGGATCTGGGTCTGACCACACCGGCAAAAAATCAGGGTAGCCTGAA   1020
Rev          TGCATATAATCCGATGGATCTGGGTCTGACCACACCGGCAAAAAATCAGGGTAGC-TGAA   314

For          ------------------------------------------------------------   0
p.cwp84.His  TACCGCATGGTCATTTAGCGGTATGAGCACCCTGGAAGCATATCTGAAACTGAAAGGTTA   1080
Rev          TACCGCATGGTCATTTAGCGGTATGAGCA-CCTGGAAGCATATCTGAAACTGAAAGGTTA   373

For          ------------------------------------------------------------   0
p.cwp84.His  TGGCACCTATGATCTGAGCGAAGAACATCTGCGTTGGTGGGCAACCGGTGGTAAATATGG   1140
Rev          TGGCACCTATGATCTGAGCGAAGAACATCTGCGTTGGTGGGCAACCGGTGGTAAATATGG   433

For          ------------------------------------------------------------   0
p.cwp84His   TTGGAATCTGGATGATATGAGCGGTAGCAGCAATGTTACCGCAATTGGTTATCTGACCGC   1200
Rev          TTGGAATCTGGATGATATGAGCGGTAGCAGCAATGTTACCGCAATTGGTTATCTGACCGC   493

For          ------------------------------------------------------------   0
p.cwp84His   ATGGGCAGGTCCGAAACTGGAAAAAGATATTCCGTATAACCTGAAAAGCGAAGCACAGGG   1260
Rev          ATGGGCAGGTCCGAAACTGGAAAAAGATATTCCGTATAACCTGAAAAGCGAAGCACAGGG   553

For          ------------------------------------------------------------   0
p.cwp84His   TGCAACCAAACCGAGCAATATGGATACCGCACCGACACAGTTTAATGTGACCGATGTTGT   1320
Rev          TGCAACCAAACCGAGCAATATGGATACCGCACCGACACAGTTTAATGTGACCGATGTTGT   613

For          ------------------------------------------------------------   0
p.cwp84His   TCGTCTGAACAAGGATAAAGAAACCGTGAAAAACGCCATTATGCAGTATGGTAGCGTTAC   1380
Rev          TCGTCTGAACAAGGATAAAGAAACCGTGAAAAACGCCATTATGCAGTATGGTAGCGTTAC   673

For          ------------------------------------------------------------   0
p.cwp84His   CAGCGGTTATGCACATTATAGCACCTATTTCAACAAAGACGAAACCGCATATAACGCCAC   1440
Rev          CAGCGGTTATGCACATTATAGCACCTATTTCAACAAAGACGAAACCGCATATAACGCCAC   733

For          ------------------------------------------------------------   0
p.cwp84His   CAATAAACGTGCACCGCTGAATCATGCAGTTGCAATTGTTGGTTGGGATGATAACTATAG   1500
Rev          CAATAAACGTGCACCGCTGAATCATGCAGTTGCAATTGTTGGTTGGGATGATAACTATAG   793

For          ------------------------------------------------------------   0
p.cwp84His   CAAAGACAACTTTGCCAGTGATGTTAAACCGGAAAGCAATGGTGCATGGCTGGTGAAAAG   1560
Rev          CAAAGACAACTTTGCCAGTGATGTTAAACCGGAAAGCAATGGTGCATGGCTGGTGAAAAG   853

For          ------------------------------------------------------------   0
p.cwp84His   CAGCTGGGGTGAATTTAACAGCATGAAAGGCTTTTTCTGGATCAGCTATGAAGATAAAAC   1620
Rev          CAGCTGGGGTGAATTTAACAGCATGAAAGGCTTTTTCTGGATCAGCTATGAAGATAAAAC   913

For          ------------------------------------------------------------   0
p.cwp84His   ACTGCTGACCGATACCGATAACTATGCCATGAAAAGCGTTAGCAAACCGGATAGCGACAA   1680
Rev          ACTGCTGACCGATACCGATAACTATGCCATGAAAAGCGTTAGCAAACCGGATAGCGACAA   973

For          ------------------------------------------------------------   0
p.cwp84His   AAAAATGTATCAGCTGGAATATGCAGGCCTGAGCAAAATTATGAGCAATAAAGTTACCGC   1740
Rev          AAAAATGTATCAGCTGGAATATGCAGGCCTGAGCAAAATTATGAGCAATAAAGTTACCGC   1033

For          ------------------------------------------------------------   0
p.cwp84His   AGCCAACGTGTTTGATTTTAGCCGTGATAGCGAAAAACTGGATAGCGTTATGTTTGAAAC   1800
Rev          AGCCAACGTGTTTGATTTTAGCCGTGATAGCGAAAAACTGGATAGCGTTATGTTTGAAAC   1093

For          ----------------------CAGATTTTGTTGGAGCGTGACGGATGTATGGTGTTCGC   38
p.cwp84His   CGATAGCGTTGGTGCCAAATATGAGGTTTATTATGCACCGGTTGTTAATGGTGTTCCGCA   1860
Rev          CGATAGCGTTGGTGCCAAATATGAGGTTTATTATGCACCGGTTGTTAATGGTGTTCCGCA   1153
                                  ** ***  *  *  *  *  *    *   **

For          AGACAATAGCATGACCAAACTGGCAAGCGGCACCGTTAGCTATAGCGGTTATATCAATGT   98
p.cwp84His   GAACAATAGCATGACCAAACTGGCAAGCGGCACCGTTAGCTATAGCGGTTATATCAATGT   1920
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Rev          GAACAATAGCATGACCAAACTGCAAGCGCGCCGAAGT----------------------   1190
               ******************  *    **     *

For          TCCGACCAATAGCTATAGTCTGCCGAAAGGTAAAGGTGCCATTGTTGTTGTTATTGACAA   158
p.cwp84His   TCCGACCAATAGCTATAGTCTGCCGAAAGGTAAAGGTGCCATTGTTGTTGTTATTGACAA   1980
Rev          ------------------------------------------------------------   1190

For          TACCGCAAATCCGAATCGTGAAAAAAGTACCCTGGCATATGAAACCAACATCGATGCCTA   218
p.cwp84His   TACCGCAAATCCGAATCGTGAAAAAAGTACCCTGGCATATGAAACCAACATCGATGCCTA   2040
Rev          ------------------------------------------------------------   1190

For          TTATCTGTATGAAGCAAAAGCCAATCTGGGCGAAAGTTATATCCTGCAGAACAACAAATT   278
p.cwp84His   TTATCTGTATGAAGCAAAAGCCAATCTGGGCGAAAGTTATATCCTGCAGAACAACAAATT   2100
Rev          ------------------------------------------------------------   1190

For          CGAGGACATCAATACCTATAGCGAATTCAGTCCGGCAAACTTTGTGATTAAAGCCATTAC   338
p.cwp84His   CGAGGACATCAATACCTATAGCGAATTCAGTCCGGCAAACTTTGTGATTAAAGCCATTAC   2160
Rev          ------------------------------------------------------------   1190

For          CAAAACCAGCAGCGGTCAGGCAACCAGCGGTGAAAGCCTGACCGGTGCAGATCGTTATGA   398
p.cwp84His   CAAAACCAGCAGCGGTCAGGCAACCAGCGGTGAAAGCCTGACCGGTGCAGATCGTTATGA   2220
Rev          ------------------------------------------------------------   1190

For          AACAGCAGTTAAAGTTAGCCAGAAAGGTTGGACCAGCAGCCAGAATGCCGTTCTGGTGAA   458
p.cwp84His   AACAGCAGTTAAAGTTAGCCAGAAAGGTTGGACCAGCAGCCAGAATGCCGTTCTGGTGAA   2280
Rev          ------------------------------------------------------------   1190

For          TGGTGATGCGATTGTTGATGCACTGACCGCAACACCGTTTACAGCAGCAATTGATAGCCC   518
p.cwp84His   TGGTGATGCGATTGTTGATGCACTGACCGCAACACCGTTTACAGCAGCAATTGATAGCCC   2340
Rev          ------------------------------------------------------------   1190

For          GATTCTGCTGACAGGTAAAGATAATCTGGATAGCAAAACCAAAGCAGAACTGCAGCGTCT   578
p.cwp84His   GATTCTGCTGACAGGTAAAGATAATCTGGATAGCAAAACCAAAGCAGAACTGCAGCGTCT   2400
Rev          ------------------------------------------------------------   1190

For          GGGCACCAAAAAAGTTTATCTGATTGGTGGTGAAAACTCCCTGAGTAAAAATGTGCAGAC   638
p.cwp84His   GGGCACCAAAAAAGTTTATCTGATTGGTGGTGAAAACTCCCTGAGTAAAAATGTGCAGAC   2460
Rev          ------------------------------------------------------------   1190

For          CCAGCTGAGTAATATGGGTATTAGCGTTGAACGTATTAGCGGTAGCGATCGGTATAAAAC   698
p.cwp84His   CCAGCTGAGTAATATGGGTATTAGCGTTGAACGTATTAGCGGTAGCGATCGGTATAAAAC   2520
Rev          ------------------------------------------------------------   1190

For          CAGTATTAGCCTGGCACAGAAACTGAACAGCATTAAAAGCGTGAGCCAGGTTGCAGTTGC   758
p.cwp84His   CAGTATTAGCCTGGCACAGAAACTGAACAGCATTAAAAGCGTGAGCCAGGTTGCAGTTGC   2580
Rev          ------------------------------------------------------------   1190

For          CAATGGTGTGAATGGTCTGGCAGATGCAATTAGCGTGGGTGCAGCAGCAGCCGATAATAA   818
p.cwp84His   CAATGGTGTGAATGGTCTGGCAGATGCAATTAGCGTGGGTGCAGCAGCAGCCGATAATAA   2640
Rev          ------------------------------------------------------------   1190

For          CATGCCGATTATTCTGACCAACGAAAAAAGTGAACTGCAGGGTGCAGATGATTTC-TGAA   877
p.cwp84His   CATGCCGATTATTCTGACCAACGAAAAAAGTGAACTGCAGGGTGCAGATGAATTTCTGAA   2700
Rev          ------------------------------------------------------------   1190

For          TAGCTCCAAAATCACCAAAAGCTATTATTATCGGTGGTACAGCACCCTGAGCAGCAATCT   937
p.cwp84His   TAGCTCCAAAATCACCAAAAGCTATATTATCGGTGGTACAGCAACCCTGAGCAGCAATCT   2760
Rev          ------------------------------------------------------------   1190

For          GGAAAGTAAACTGAGCATCGACACGT--CTGGCAGGTAGCATCGTATGAACGA------T   989
p.cwp84His   GGAAAGTAAACTGAGCAATCCGACACGTCTGGCAGGTAGCAATCGTAATGAAACGAATGC   2820
Rev          ------------------------------------------------------------   1190
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For          GCAAATCATCGAC--AATTCTATCGAGCAGCGATCTGAAATATGCTTTGTTGTTAAGATG   1047
p.cwp84His   CAAAATCATCGACAAATTCTATCCGAGCAGCGATCTGAAATATGCCTTTGTTGTTAAAGA   2880
Rev          ------------------------------------------------------------   1190

For          TAGCCAAGTCAAGCGATCTGAT---------GATGCTGGCAGTGTGCACTTGGGTGCCAA   1098
p.cwp84His   TGGTAGCAAAAGTCAGGGCGATCTGATTGATGGCCTGGCAGTTGGTGCACTGGGTGCCAA   2940
Rev          ------------------------------------------------------------   1190

For          CCGA---ATCTCGATTGTTCTGATTGATAACTGGATGAGC-------CGAAAACCGTGCT   1148
p.cwp84His   AACCGATTCTCCGGTTGTTCTGGTTGGTAATAAACTGGATGAAAGCCAGAAAAACGTGCT   3000
Rev          ------------------------------------------------------------   1190

For          TTGAAAGGCCA-----------------------------------------------   1159
p.cwp84His   GAAAAGCAAAAAAATCGAAACCCCGATTCGTGTTGGTGGCAATGGCAATGAAAGCGCATT   3060
Rev          ------------------------------------------------------------   1190

For          ------------------------------------------------------------   1159
p.cwp84His   TAATGAACTGAATACCCTGTTAGGTAAAGGCAGCACCACGGATCCGCTGGTGCCACGCGG   3120
Rev          ------------------------------------------------------------   1190
```

pTWIN1-CD630_cwp84.His (using Ssp intein for and His check seq Rev primers)

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Rev          ------------------------------------------------------------   0
p.cwp84His   GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGCGAAAATCATAA   780
For          GCCAGGACCACATAACTTTGTCGCGAATGACATCATTGTACACAACTGCGAAAATCATAA   93

Rev          ------------------------------------------------------------   0
p.cwp84His   AACCCTGGATGGTGTTGAAACCGCAGAATATAGCGAAAGCTATCTGCAGTATCTGGAAGA   840
For          AACCCTGGATGGTGTTGAAACCGCAGAATATAGCGAAAGCTATCTGCAGTATCTGGAAGA   153

Rev          ------------------------------------------------------------   0
p.cwp84His   TGTGAAAAATGGTGATACCGCCAAATATAACGGTGTTATTCCGTTTCCGCATGAAATGGA   900
For          TGTGAAAAATGGTGATACCGCCAAATATAACGGTGTTATTCCGTTTCCGCATGAAATGGA   213

Rev          ------------------------------------------------------------   0
p.cwp84His   AGGTACAACCCTGCGTAATAAAGGTCGTAGCAGCCTGCCGAGCGCATATAAAAGCAGCGT   960
For          AGGTACAACCCTGCGTAATAAAGGTCGTAGCAGCCTGCCGAGCGCATATAAAAGCAGCGT   273

Rev          ------------------------------------------------------------   0
p.cwp84His   TGCATATAATCCGATGGATCTGGGTCTGACCACACCGGCAAAAAATCAGGGTAGCCTGAA   1020
For          TGCATATAATCCGATGGATCTGGGTCTGACCACACCGGCAAAAAATCAGGGTAGCCTGAA   333

Rev          ------------------------------------------------------------   0
p.cwp84His   TACCGCATGGTCATTTAGCGGTATGAGCACCCTGGAAGCATATCTGAAACTGAAAGGTTA   1080
For          TACCGCATGGTCATTTAGCGGTATGAGCACCCTGGAAGCATATCTGAAACTGAAAGGTTA   393

Rev          ------------------------------------------------------------   0
p.cwp84His   TGGCACCTATGATCTGAGCGAAGAACATCTGCGTTGGTGGGCAACCGGTGGTAAATATGG   1140
For          TGGCACCTATGATCTGAGCGAAGAACATCTGCGTTGGTGGGCAACCGGTGGTAAATATGG   453

Rev          ------------------------------------------------------------   0
p.cwp84His   TTGGAATCTGGATGATATGAGCGGTAGCAGCAATGTTACCGCAATTGGTTATCTGACCGC   1200
For          TTGGAATCTGGATGATATGAGCGGTAGCAGCAATGTTACCGCAATTGGTTATCTGACCGC   513

Rev          ------------------------------------------------------------   0
p.cwp84His   ATGGGCAGGTCCGAAACTGGAAAAAGATATTCCGTATAACCTGAAAAGCGAAGCACAGGG   1260
For          ATGGGCAGGTCCGAAACTGGAAAAAGATATTCCGTATAACCTGAAAAGCGAAGCACAGGG   573

Rev          ------------------------------------------------------------   0
p.cwp84His   TGCAACCAAACCGAGCAATATGGATACCGCACCGACACAGTTTAATGTGACCGATGTTGT   1320
For          TGCAACCAAACCGAGCAATATGGATACCGCACCGACACAGTTTAATGTGACCGATGTTGT   633
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Rev          ------------------------------------------------------------   0
p.cwp84His   TCGTCTGAACAAGGATAAAGAAACCGTGAAAAACGCCATTATGCAGTATGGTAGCGTTAC   1380
For          TCGTCTGAACAAGGATAAAGAAACCGTGAAAAACGCCATTATGCAGTATGGTAGCGTTAC   693

Rev          ------------------------------------------------------------   0
p.cwp84His   CAGCGGTTATGCACATTATAGCACCTATTTCAACAAAGACGAAACCGCATATAACGCCAC   1440
For          CAGCGGTTATGCACATTATAGCACCTATTTCAACAAAGACGAAACCGCATATAACGCCAC   753

Rev          ------------------------------------------------------------   0
p.cwp84His   CAATAAACGTGCACCGCTGAATCATGCAGTTGCAATTGTTGGTTGGGATGATAACTATAG   1500
For          CAATAAACGTGCACCGCTGAATCATGCAGTTGCAATTGTTGGTTGGGATGATAACTATAG   813

Rev          ------------------------------------------------------------   0
p.cwp84His   CAAAGACAACT-TTGCCAGTGATGTTAAACCGGAAAGCAATGGTGCATGGCTGGTGAAAA   1559
For          CAAAGACAACTTTTGCCAGTGATGTTAAACCGGGAAGCAATGGTGCATGGCTGGTGAAAA   873

Rev          ------------------------------------------------------------   0
p.cwp84His   GCAGCTGGGGTGAATTTAACAGCATGAAAGGCTTTTTCTGGATCAGCTATGAAGATAAAA   1619
For          GCAGCTGGGGTGAATTTAACAGCATGAAAGGCTTTTTCTGGATCAGCTATGAAGATAAAA   933

Rev          ------------------------------------------------------------   0
p.cwp84His   CACTGCTGACCGATACCGATAACTATGCCATGAAAAGCGTTAGCAAACCGGATAGCGACA   1679
For          CACTGCTGACCGATACGGATAACTATGCCATGAAAAGCGTTAGCAAACCGGATAGCCGAC   993

Rev          ------------------------------------------------------------   0
p.cwp84His   AAAAAATGTATCAGCTGGAATATGCAGGCCTGAGCAAAATTATGAGCAATAAAGTTACCG   1739
For          AAAAATTGTATCAGCTGGAATATTGCAGGCCTGAGCCAAAATTATGAGCATAAGTTACGG   1053

Rev          ------------------------------------------------------------   0
p.cwp84His   CAGCCAACGTGTTTGATTTTAGCCGTGATAGCGAAAAACTGGATAGCGTTATGTTTGAAA   1799
For          CAGCCAACGTTGTTGATTTTAGCCTTGAATACCGAAACTTGGATAGGCGGTTATGTTTTG   1113

Rev          ------------------------------------------------------------   0
p.cwp84His   -------CCGATAGCGTTGGTGCCAAATATGAGGTTTATTATGCACCGGTTGTTAATGGT   1852
For          AACCCCGATATGCGTTTGGGTGCCAAATATGAGGTTTATTTAGAACCCGGTTTGTATAGG   1173

Rev          ------------------------------------------------------------   0
p.cwp84His   GTTCCGCAGAACAATAGCATGACCAAACTGGCAAGCGGCACCGTTAGCTATAGCGGTTAT   1912
For          GGGGTT--------------------------------------------------------   1179

Rev          ------------------------------------------------------------   0
p.cwp84His   ATCAATGTTCCGACCAATAGCTATAGTCTGCCGAAAGGTAAAGGTGCCATTGTTGTTGTT   1972
For          ------------------------------------------------------------   1179

Rev          ------------------------------------------------------------   0
p.cwp84His   ATTGACAATACCGCAAATCCGAATCGTGAAAAAAGTACCCTGGCATATGAAACCAACATC   2032
For          ------------------------------------------------------------   1179

Rev          ------------------------------------------------------------   0
p.cwp84His   GATGCCTATTATCTGTATGAAGCAAAAGCCAATCTGGGCGAAAGTTATATCCTGCAGAAC   2092
For          ------------------------------------------------------------   1179

Rev          ------------------ACCTAAGGGGAATTTCAGATCCCGGCCAACTTTTGGTATTAAA   43
p.cwp84His   AACAAATTCGAGGACATCAATACCTATAGCGAATTCAGTCCGGCAAACTTTGTGATTAAA   2152
For          ------------------------------------------------------------   1179

Rev          GCCATTACCCAAACCAGCAGGGGTCAGGCACCAAGCGGGGAAAGCCTGACCGGTGCAGAT   103
p.cwp84His   GCCATTACCAAAACCAGCAGCGGTCAGGCAACCAGCGGTGAAAGCCTGACCGGTGCAGAT   2212
For          ------------------------------------------------------------   1179

Rev          CGTTATGAAACAGCCAGTTAAGGTAGCCCAGAAGGTTGGACCAGCAGCCAGAATCCCGTC   163
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p.cwp84His   CGTTATGAAACAGCAGTTAAAGTTAGCCAGAAAGGTTGGACCAGCAGCCAGAATGCCGTT   2272
For          ------------------------------------------------------------   1179

Rev          TGGTGAATGGTGAATGCGATTGTTGATGCACTGACCGCAACACCTTTACAGCAGCAAATT   223
p.cwp84His   CTGGTGAATGGTGATGCGATTGTTGATGCACTGACCGCAACACCGTTTACAGCAGCAATT   2332
For          ------------------------------------------------------------   1179

Rev          GATAGCCCGAATTCTGCTGACAGGTAAAAAAATCTGAAAAGCAAAA-CCAAAGCAGACTG   282
p.cwp84His   GATAGCCCGATTCTGCTGACAGGTAAAGATAATCTGGATAGCAAAACCAAAGCAGAACTG   2392
For          ------------------------------------------------------------   1179

Rev          CAGCGTCTGGGCCCCAAAAAAGTTTATCTGATTGGTGGTGAAAACTCCCTGAGTAAAAAT   342
p.cwp84His   CAGCGTCTGGGCACCAAAAAAGTTTATCTGATTGGTGGTGAAAACTCCCTGAGTAAAAAT   2452
For          ------------------------------------------------------------   1179

Rev          GTGCAGACCCCAGCTGAGTAAATATGGGTATTAGCGTTGAACGTATTAGCGGTAGCGATC   402
p.cwp84His   GTGCAGACCCAGCT--GAGTAATATGGGTATTAGCGTTGAACGTATTAGCGGTAGCGATC   2510
For          ------------------------------------------------------------   1179

Rev          GGTATAAAACCCAGTATTAGCCTGGCACAGAAACTGAACAGCATTAAAAGCGTGAGCCAG   462
p.cwp84His   GGTATAAA-ACCAGTATTAGCCTGGCACAGAAACTGAACAGCATTAAAAGCGTGAGCCAG   2569
For          ------------------------------------------------------------   1179

Rev          GTTGCAGTTGCCAATGGTGTGAATGGTCTGGCAGATGCAATTAGCGTGGGTGCAGCAGCA   522
p.cwp84His   GTTGCAGTTGCCAATGGTGTGAATGGTCTGGCAGATGCAATTAGCGTGGGTGCAGCAGCA   2629
For          ------------------------------------------------------------   1179

Rev          GCCGATAATAACATGCCGATTATTCTGACCAACGAAAAAAGTGAACTGCAGGGTGCAGAT   582
p.cwp84His   GCCGATAATAACATGCCGATTATTCTGACCAACGAAAAAAGTGAACTGCAGGGTGCAGAT   2689
For          ------------------------------------------------------------   1179

Rev          GAATTTCTGAATAGCTCCAAAATCACCAAAAGCTATATTATCGGTGGTACAGCAACCCTG   642
p.cwp84His   GAATTTCTGAATAGCTCCAAAATCACCAAAAGCTATATTATCGGTGGTACAGCAACCCTG   2749
For          ------------------------------------------------------------   1179

Rev          AGCAGCAATCTGGAAAGTAAACTGAGCAATCCGACACGTCTGGCAGGTAGCAATCGTAAT   702
p.cwp84His   AGCAGCAATCTGGAAAGTAAACTGAGCAATCCGACACGTCTGGCAGGTAGCAATCGTAAT   2809
For          ------------------------------------------------------------   1179

Rev          GAAACGAATGCCAAAATCATCGACAAATTCTATCCGAGCAGCGATCTGAAATATGCCTTT   762
p.cwp84His   GAAACGAATGCCAAAATCATCGACAAATTCTATCCGAGCAGCGATCTGAAATATGCCTTT   2869
For          ------------------------------------------------------------   1179

Rev          GTTGTTAAAGATGGTAGCAAAAGTCAGGGCGATCTGATTGATGGCCTGGCAGTTGGTGCA   822
p.cwp84His   GTTGTTAAAGATGGTAGCAAAAGTCAGGGCGATCTGATTGATGGCCTGGCAGTTGGTGCA   2929
For          ------------------------------------------------------------   1179

Rev          CTGGGTGCCAAAACCGATTCTCCGGTTGTTCTGGTTGGTAATAAACTGGATGAAAGCCAG   882
p.cwp84His   CTGGGTGCCAAAACCGATTCTCCGGTTGTTCTGGTTGGTAATAAACTGGATGAAAGCCAG   2989
For          ------------------------------------------------------------   1179

Rev          AAAAACGTGCTGAAAAGCAAAAAAATCGAAACCCCGATTCGTGTTGGTGGCAATGGCAAT   942
p.cwp84His   AAAAACGTGCTGAAAAGCAAAAAAATCGAAACCCCGATTCGTGTTGGTGGCAATGGCAAT   3049
For          ------------------------------------------------------------   1179

Rev          GAAAGCGCATTTAATGAACTGAATACCCTGTTAGGTAAAGGCAGCACCGGGGATCCGCTG   1002
p.cwp84His   GAAAGCGCATTTAATGAACTGAATACCCTGTTAGGTAAAGGCAGCACCACGGATCCGCTG   3109
For          ------------------------------------------------------------   1179

Rev          GTGCCACGCGGTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAAGCTGAG   1062
p.cwp84His   GTGCCACGCGGTAGTTCCGCTCATCACCACCATCATCACCATCACCACCACTAAGCTGAG   3169
For          ------------------------------------------------------------   1179
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Rev          CAATAACTAGCATAACCCCTTGGGCCTCTAAAACCGGGTCTA------------------   1104
p.cwp84His   CAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAA   3229
For          ------------------------------------------------------------   1179
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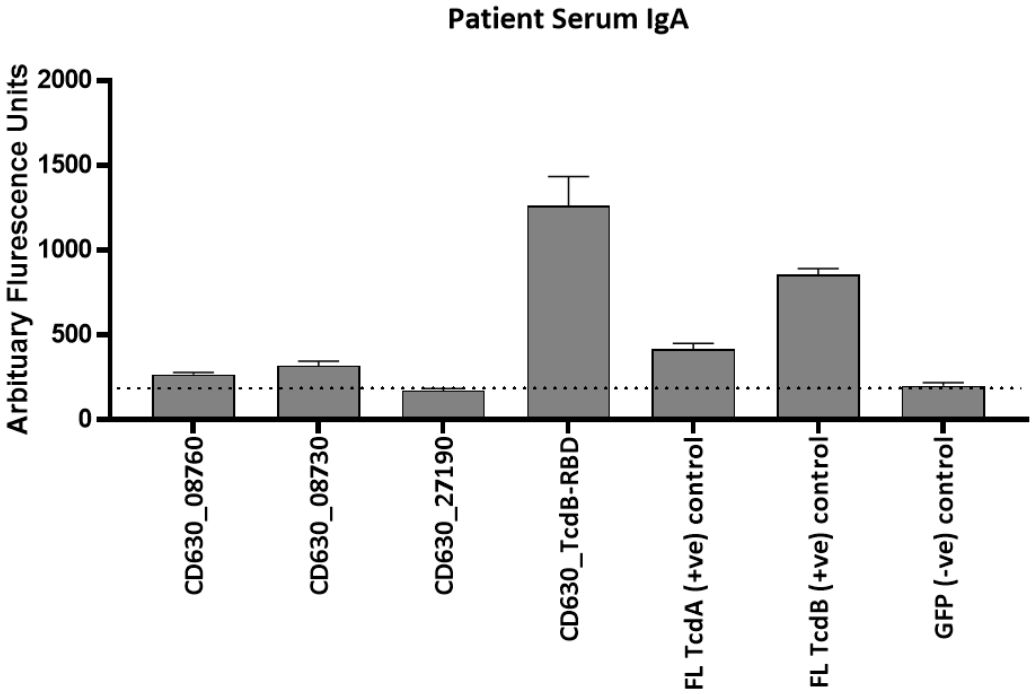

Figure A3- Sero-reactivity of purified protein to pooled *C. difficile* patient serum IgA using Microarray ELISA.

Microarray printing of antigens at 50 µg/ml in replicates of 6. CD630_08760, CD630_08730, CD630_27190, CD630_TcdB-RBD, full-length *C. difficile* VPI 1043 strain toxoids, TcdA and TcdB (FL TcdA and FL TcdB) (positive controls) (Public Health England) and GFP (negative control) were incubated with pooled serum (n=20) from *C. difficile* patients and tested for binding with Biotinylated goat anti-human IgA (1:1000) (Invitrogen™) followed by IRDye® 800CW Streptavidin (1:20 000) and Biotinylated goat anti-Streptavidin antibody (Vector Laboratories) (1:1000). Error bars indicating standard error of the mean (SEM). The dotted line represents the baseline detection using GFP as a negative control.

8.3 Supplementary material chapter 6

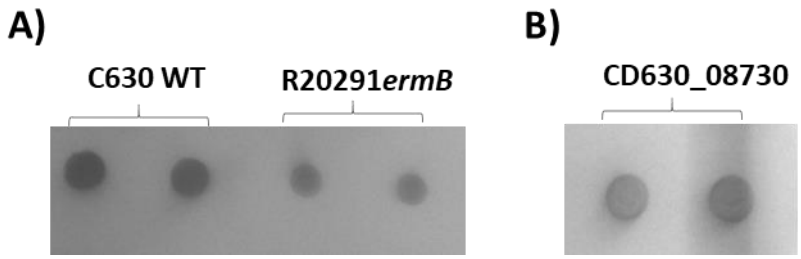

Figure A4- Immuno-dot blot performed on whole cell *C. difficile* to confirm surface exposure of CD630_08730 on the hypervirulent strain R20291*ermB* used for the challenge study probing with anti-CD0873 antibody.

A) *C. difficile* strain 630 wild type (WT) used as a positive control for known surface exposure (Kovacs-Simon *et al*., 2014) to compare with R20291*ermB*. Cells from each strain from an overnight culture was re-suspended in 100 µl PBS and 5 µl was spotted on to Nitrocellulose membrane in duplicate. B) Purified recombinant CD630_08730 was also included as a positive control and 5 µl at 2 ng/µl also spotted in duplicate. The membranes were blocked with 5% (w/v) dry-milk in TBST for 1 hour followed by an hour incubation with rabbit anti-CD0873 antibody (1: 5000) and then anti-rabbit IgG HRP antibody (1:1000) with 1% (w/v) dry-milk in TBST and visualised using 3, 3', 5, 5'-Tetramethylbenzidine (TMB).

Bibliography

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