EVALUATION OF PROPHYLACTIC THERAPIES FOR THE PREVENTION OF CAMPYLOBACTERIOSIS

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ABSTRACT

Campylobacter jejuni, a frequently isolated diarrhoeal pathogen [1], is encapsulated by a polysaccharide capsule. This can be exploited to develop capsule-based conjugate vaccines [2]. Before vaccine implementation, correlates of protection enable efficacy to be measured. This thesis describes the development of a Serum Bactericidal Assay (SBA) to evaluate the bactericidal activity of sera from New Zealand White Rabbits (NZWR) vaccinated with a GMP grade capsule-based conjugate *C. jejuni* vaccine 'CJCV1'; *Aotus nancymaae* Non-Human Primates (NHP), vaccinated with a research grade vaccine 'CCV'; and humans, vaccinated with 'CJCV1'. NZWR and NHP post-vaccination sera demonstrated an increase in serum titre achieving a bactericidal effect. Human sera did not demonstrate a consistent bactericidal effect. These experiments showed variability in readouts, suggesting that for the studies performed, the SBA may be of limited value in assessing vaccine efficacy. This thesis also presents results from a Phase II clinical trial assessing the efficacy of rifaximin in preventing campylobacteriosis in humans; whilst rifaximin prophylaxis does not prevent campylobacteriosis, it reduces the diarrhoeal burden. This study involved the development and use of a successful human challenge model, crucial for testing interventions against campylobacteriosis. Results from these studies suggest further development of interventions to control this highly problematic pathogen are required.

PUBLICATIONS

Work performed during this M.D., included or not included in this thesis, led to the following publications, the full texts and posters are attached as Appendices 1 - 4.

Rimmer, J.E., et Al., Rifaximin Fails to Prevent Campylobacteriosis in the Human Challenge Model: A Randomized, Double-Blind, Placebo-Controlled Trial. Clin Infect Dis, 2018. **66**(9): p. 1435-1441

Crofts A.A., et. Al., *Campylobacter jejuni* transcriptional and genetic adaptation during human infection. Nat Microbiol. 2018 Apr;3(4):494-502.

Harro C et. Al. Does Rifaximin Chemoprophylaxis Prevent Campylobacteriosis in the Human Challenge Model? A poster presentation at the Infectious Diseases Society of America Infectious Diseases Week, San Diego 2015.

Rimmer J.E et. Al., Development of a Serum Bactericidal Assay to determine functional antibody response following vaccination against *Campylobacter jejuni*. A poster presentation at the Vaccines for Enteric Diseases conference, Edinburgh 2015.

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Throughout the Phase II Clinical Trial the NMRC Clinical Trials Group; CAPT Mark Riddle (Head of the Enteric Disease Department), Dr Chad Porter, CAPT Ramiro Gutierrez, CDR Robert Gormley, Miss Kayla Jaep, Miss Ashley Acala and Mrs. Vicky Chapman, were unwavering in their support and gave me the opportunity to experience the complexities of planning a Phase II Inpatient Human Subject Trial, thank you. At the Johns Hopkins School of Public Health, I was welcomed and received very strong support from Dr David Sack, Dr Kawsar Talaat, Mrs Barbara DeNearing and, most of all, from Dr Clayton Harro, a pioneer in vaccine development.

The education that I have received from all members of the Enteric Disease Department within the NMRC has allowed me to not only develop a portfolio of laboratory skills, but also an understanding of the evolution of design and implementation of a human subject Phase II clinical trial, and the enormous importance, and relevance, of laboratory work to the planning of future clinical trials and advancement of medical knowledge.

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LIST OF ABBREVIATIONS

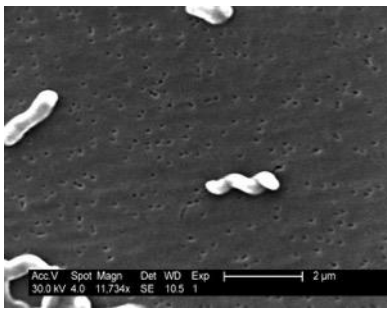
ASC	Antibody Secreting Cells
BAFF	B-cell activating factor
BCR	B-Cell Receptor
BRC	Baby Rabbit Complement
Ca^{2+}	Calcium
CaCl	Calcium chloride
cadF	*Campylobacter* adhesion to fibronectin
CapA	*Campylobacter* adhesion protein A
CCV	Capsule Conjugate Vaccine (research grade vaccine based on strain 81-176)
CDT	Cytolethal Distending Toxin
CFU	Colony Forming Units
cGMP	certified Good Medical Practice
CipA	Cell invasion protein A
CJCV1	*Campylobacter jejuni* Conjugate Vaccine 1 (cGMP grade vaccine based on strain 81-176)
CPS	Capsule Polysaccharide
DGV	Dextrose Gelatin Veronal
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ETEC	Enterotoxic *Escherichia coli*
Fab	Fragment antigen binding
Fc	Fragment crystallisable
FlaA	Major flagellin
FlaB	Minor flagellin
GBB	Glycerol Brucella Broth
GBS	Guillain Barré Syndrome
GI	Gastrointestinal
H_2O	Water
HS	Heat Stable
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IFNy	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LOS	Lipooligosaccharide

LT	heat-Labile Toxin
MEM	Minimum Essential Medium
MFS	Miller Fisher Syndrome
Mg^{2+}	Magnesium
$MgSO_4$	Magnesium Sulfate
MHB	Mueller Hinton Broth
MHC	Major Histocompatibility Complex
MHP	Mueller Hinton Plain
MOMP	Major Outer Membrane Protein
NHP	Non Human Primate
NaCl	Sodium chloride
Neu5Ac	N-acetylneuraminic acid
NK	Natural Killer cells
NMRC	Naval Medical Research Center
NZWR	New Zealand White Rabbit
OMV	Outer Membrane Vesicle
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PI-IBS	Post Infectious Irritable Bowel Syndrome
PS	Polysaccharide
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
ReA	Reactive Arthritis
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAE	Significant Adverse Event
SBA	Serum Bactericidal Assay
SD	Study Day
SEM	Standard Error of the Mean
SSP	Study Specific Procedure
T6SS	Type 6 Secretion System
TD	T-cell Dependent
T_{FH}	follicular helper T-cell
T_H	helper T-cell
TI	T-cell Independent
TLR	Toll like receptor
TLUS	Time to last unformed stool

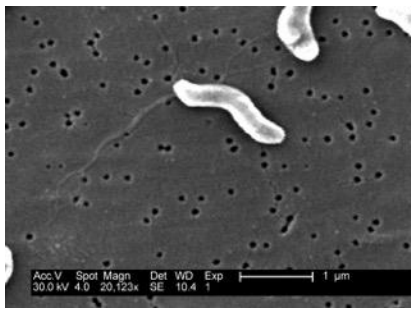
CHAPTER 1 INTRODUCTION

1.1 *CAMPYLOBACTER JEJUNI*

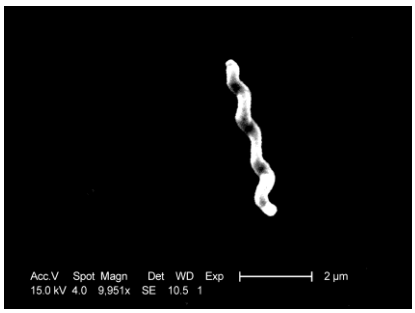
Campylobacter jejuni is an encapsulated, helical, gull-wing or S-shaped, often flagellated, gram-negative bacterium, Figures 1.1 (a)-(c). It is microaerophilic and naturally colonises species of animals and birds including poultry, wild birds, and cattle. A capsular polysaccharide (CPS) forms the outermost surface of most of the bacteria and it is this structure that determines not only its classification within the Penner Serotyping System [3], but also contributes to virulence, including adhesion to and invasion of epithelial cells [4-6]. The genome of the bacteria undergoes phase variation, when properties such as the expression of multiple surface structures including lipooligosaccharide (LOS), capsule, and flagellum, may change [7], therefore virulence potential may also change.



(a) PHIL Image 5778; Photo Provider CDC / Dr. Patricia Fields, Dr Colette Fitzgerald



(b) PHIL Image 5780; Photo Provider CDC / Dr. Patricia Fields, Dr Colette Fitzgerald



(c) PHIL Image 5781; Photo Provider CDC / Dr. Patricia Fields, Dr Colette Fitzgerald

Figure 1.1 Scanning Electron Micrographs images of *C. jejuni*

From the Center for Disease Control and Prevention Public Health Image Library, phil.cdc.gov. These scanning electron micrographs show the Gram-negative *Campylobacter jejuni* bacteria; (a) magnified 11,734x, (b) magnified 20,123x, (c) magnified 9,951x

1.1.1 Classification by the Penner Serotyping System

Development of the Penner Serotyping System, based on detection of heat-stable (HS) antigens, has allowed 80% of *C. jejuni* isolates to be typed, and has identified 47 serotypes [2]. The *C. jejuni* CPS is the primary serodeterminant for the Penner Serotyping System, Figure 1.2. Prototype vaccines for strains 81-176, (serotype HS23/36), and CG8486 (HS4 complex) have been synthesized [8]. Development of a multivalent vaccine to a collection of Penner system serotypes in order to optimize protection would be a useful strategy to control infection caused by multiple strains.

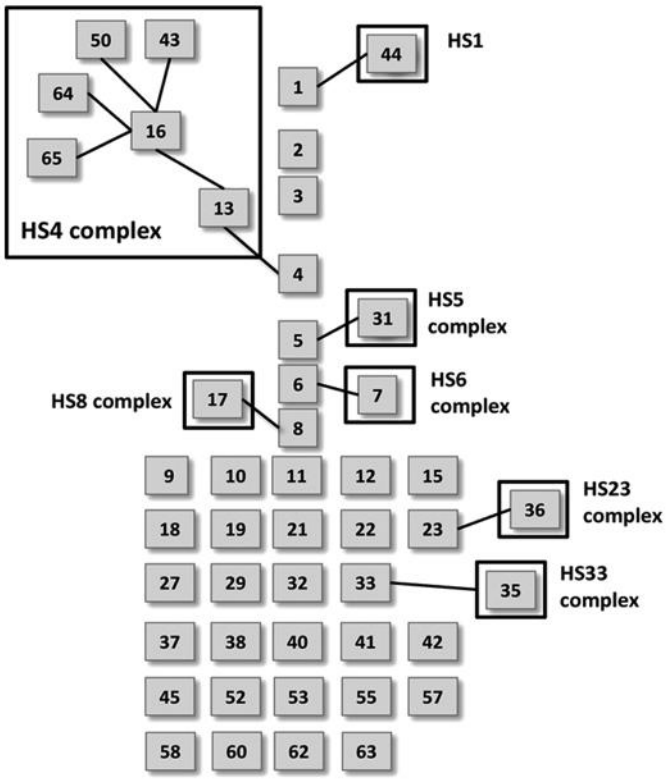


Figure 1.2 The Penner Serotyping System

Illustration of *C. jejuni* serotypes. In the cases of a serotype complex, such as the HS4 complex containing serotypes HS4, HS13, HS16, HS43, HS50, HS64 and HS65, or the HS1 complex containing serotypes HS1 and HS44, the serotypes within that complex are connected by lines. From: Maue, A.C., F Poly and P. Guerry. A capsule conjugate vaccine approach to prevent diarrhoeal disease caused by *Campylobacter jejuni.* Hum Vaccin Immunother. 2014. 10(6).

While geographical variation exists, it is estimated that eight serotypes account for more than 50% of all *C. jejuni* attributable diarrhoeal cases globally, and three serotypes; HS4 complex (HS4c), HS2 and HS1/44 are dominant inter-regionally as well as globally [3], Table 1.1.

Table 1.1 Comparison of HS Serotypes with proportional estimates by region

Serotypes or serotype complexes whose incidence proportion met or exceeded 2% are in bold and those that did not are in Italics. Pike BL, Guerry P, Poly F (2013) Global Distribution of *Campylobacter jejuni* Penner Serotypes: A Systemic Review. PLoS ONE 8(6):e6735. Doi:10.137/journal.pone.0067375

	Global %	Africa %	Asia %	Europe %	N. America %	Oceania %
	(n = 21,394)	(n = 566)	(n = 1,186)	(n = 18,184)	(n = 763)	(n = 695)
HS4c	**15.3**	**7.0**	**8.9**	**17.3**	**23.5**	**17.4**
HS2	**13.5**	**6.2**	**11.5**	**15.3**	**10.7**	**18.2**
HS1/44	**8.2**	**6.8**	**4.2**	**9.1**	**9.3**	**10.5**
HS11	**3.1**	*1.6*	*0.2*	**4.0**	**3.6**	*1.7*
HS5/31	**2.9**	**6.2**	*1.8*	**2.6**	**6.8**	*1.5*
HS8/17	**2.8**	**4.1**	**2.9**	**2.2**	**5.3**	**8.8**
HS6/7	**2.4**	*1.2*	*0.7*	**3.6**	**2.3**	*0.6*
HS3	**2.2**	**6.3**	**2.6**	*1.9*	**4.9**	*0.7*
HS37	*1.8*	*0.9*	**2.4**	*1.8*	**2.1**	*1.8*
HS23/36	*1.7*	**4.2**	**3.0**	*1.4*	*1.8*	**4.2**
HS21	*1.6*	*0.5*	*0.6*	*1.8*	**2.5**	*1.1*
HS19	*1.5*	**2.0**	**3.1**	*1.5*	*0.9*	*0.5*
HS12	*1.3*	*1.0*	*0.0*	**2.1**	*0.5*	*0.7*
HS58	*1.3*	*0.8*	*0.0*	**2.0**	*1.0*	*0.1*
HS15	*1.1*	*1.4*	**3.4**	*1.2*	*0.9*	*0.4*
HS18	*0.9*	*0.4*	*0.1*	*1.1*	**2.1**	*0.2*
HS53	*0.7*	**3.3**	*1.2*	*0.7*	*0.6*	*0.1*

1.1.2 Virulence of *C. jejuni*

There are many factors that affect the virulence of *C. jejuni*. These could be sub-categorized into those associated with the characteristics: adherence, invasion, motility, secretion systems and toxin production. As research continues however, and more is understood about this pathogen it is apparent that some of the bacteria's structures fulfil different roles, therefore the current understanding of the function of the different structures will be described instead.

1.1.2.1 Capsule

As described above *C. jejuni* is encapsulated and the CPS is the major serodeterminant for the Penner serotyping system. The genes encoding expression of the CPS undergo phase variation of two different forms; on/off expression, and modulation of the CPS structure [5], for example modulation of the level of phosphoramidate expression [5]. It is thought that this ability to modulate expression of the CPS infers that the CPS may generate a virulence advantage or disadvantage, dependant upon the environment and activity of the bacterium; for example invasion vs. colonisation of intestinal epithelial cells, and susceptibility or resistance to complement [5]. Therefore the CPS plays a role in bacterial survival within the environment and also in the face of the host immune responses. Regarding adherence, a non-encapsulated *C. jejuni* strain 81-176 mutant demonstrated 10-fold reduction in adherence to and invasion of human epithelial cells in vitro, as well as reduced virulence in a ferret challenge model [4].

1.1.2.2 Adherence factors

Adherence of *C. jejuni* to epithelial cells is dependent upon binding factors in order to facilitate an efficient interaction with the host epithelial cell [9]. These binding factors include, but are not limited to, *campylobacter* adhesion to fibronectin (CadF), the surface exposed adhesin lipoprotein JlpA, major outer membrane protein (MOMP), the periplasmic amino acid-binding protein PEB1 and *campylobacter* adhesion protein A (CapA) [9]. An important component of adhesion is the expression of the '*campylobacter* adhesion to fibronectin' (*cadF*) gene, this gene encodes the CadF adhesin, an outer membrane protein, which promotes the binding of bacteria to fibronectin in the extracellular matrix of, amongst others, intestinal epithelial cells [10]. The adhesin lipoprotein JlpA mediates adherence of the

C. jejuni bacterium to an epithelial cell, and is thought to be antigenic, triggering an inflammatory or immune response within the host cells following infection [9, 11]. MOMP, considered to be a porin and multifunctional surface protein of *C. jejuni*, and which is encoded for by the *porA* gene, binds to isolated membranes of intestinal cell membranes and mediates the attachment of the bacteria. When isolated under native conditions the MOMP partially inhibited the attachment of *C. jejuni* to the host cell, however, when isolated in the presence of sodium dodecyl sulphate this inhibitory ability was lost [12]. PEB1 is a binding protein of bacterial ATP-binding cassette transporters. Studies have shown that a disruption in the *peb1a* gene encoding PEB1 in *C. jejuni* strain 81-176 caused 50 – 100-fold less adherence to, and 15-fold less invasion of, epithelial cells in culture. Disruption of the *peb1a* gene also caused a significantly lower rate, and shorter duration, of intestinal colonization in a mouse model [13]. CapA is thought to function as an autotransporter protein, the absence of which, in the case of a *capA* mutant, has been shown to demonstrate a significant reduction in the bacteria's association with epithelial cells and cellular invasion when compared to the wild type [14].

1.1.2.3 Flagella

C. jejuni is highly motile with polar flagella, the flagella are required for the colonization of the mucus of the gastrointestinal tract and are also established as being crucial to pathogenicity [15]. The flagella are composed of two flagellin; FlaA (major flagellin, encoded by *flaA*) and FlaB (minor flagellin, encoded by *5lab*). Mutations in *flaA* result in a severe reduction in motility, which is not seen when *5lab* is mutated [15]. However, there is evidence to suggest that the flagella are important for more than just locomotion and

chemotaxis, specifically the secretion of non-flagella proteins that may modulate virulence; e.g. Cia proteins, FlaC proteins and FspA proteins [15].

1.1.2.4 Secretion systems

The use of secretion systems to export toxins into the surrounding environment, or directly through membranes into adjacent prokaryotic or eukaryotic cells, is a pathogenic property used by bacteria to influence the surrounding host cells or competing bacteria [16]. Many enteric pathogens depend upon type III secretion systems for virulence, however, genomic studies have indicated that strains of *C. jejuni* lack these systems [15]. The type VI secretion system (T6SS) forms a needle like structure, which facilitates this delivery of toxic effector molecules to neighbouring cells [17]. A functional T6SS has been reported in *C. jejuni* [17]; a gene cluster, comprising of 13 conserved T6SS genes including genes encoding Hcp and VgrG, typical T6SS proteins, has been found in 10% of *C. jejuni* strains [17]. This study also suggested that the activity of the T6SS was associated with haemolysis and bacteraemia, however T6SS activity was not observed in the presence of the *C. jejuni* CPS [17], demonstrating a possible consequence of the described intermittent nature of capsule expression by the bacteria. In an additional study the gene *hcp* was used as an indicator of the presence of T6SS in *C. jejuni* isolates from chicken meat in Northern Ireland, and, using a polymerase chain reaction (PCR) technique, was found to be present in 28.8% of these isolates [16]. These *hcp*$^{+}$ *C. jejuni* isolates possessed genetic and phenotypic properties (motility and *in vitro* invasiveness), associated with enhanced virulence [16].

1.1.2.5 Outer membrane vesicles

Outer membrane vesicles (OMV) potentially offer an alternative mechanism to typical secretion systems by which bacteria can modulate virulence. Analysis of *C. jejuni* OMV identified, amongst others, periplasmic and outer membrane associated proteins, as well as cytolethal distending toxin (CDT) subunits CdtA, CdtB and CdtC. These are established determinants for survival and pathogenicity [18], suggesting that the OMV has a role in virulence as OMV associated CDT produced by *C. jejuni* affects the host epithelial cell layer, interrupting cell division, and consequently leading to cell cycle arrest and cell death [19]. Phase variation of genes responsible for OMV function leads to alterations in virulence [20].

1.2 CLINICAL FEATURES OF *C. JEJUNI* INFECTION

1.2.1 Pathogenesis

First recognized as a cause of abortion in cattle, a finding published in 1909 [21], the small gram negative bacteria was not named *Campylobacter* until 1973 [22]. *C. jejuni* establishes a benign form of disease when it colonizes chickens, and is able to persistently exist in the poultry host without causing illness. In contrast, infected laboratory mice will not become colonized, instead they rapidly clear the bacteria. Rabbits, when infected, develop a diarrhoeal illness, indeed a disease model has been developed in infant rabbits, of relevance as rabbits are more closely related to primates than rodents in terms of phylogeny [23]. This disease model demonstrated inflamed intestinal mucosa in rabbits when infected with *C. jejuni* strain NCTC 11168 [23]. When *C. jejuni* infects humans it causes significant morbidity with inflammation of the gastrointestinal tract. This inflammation can lead to severe diarrhoea and dysentery.

1.2.2 Epidemiology

C. jejuni is one of the most frequently isolated diarrhoeal disease pathogens [1]. In humans it causes a spectrum of acute disease ranging from mild diarrhoeal episodes to dysentery, as well as being associated with the development of chronic intestinal and extra-intestinal disease, including irritable bowel syndrome (IBS) [24], inflammatory bowel disease (IBD) [25-27] reactive arthritis (ReA) [26] and Guillain-Barré Syndrome (GBS) [26, 28]. The incidence of campylobacteriosis varies widely, generally falling into one of three epidemiologic scenarios: 1) hyper-endemic levels (40,000/100,000 children < 5 years old) in developing regions, 2) endemic levels (20-100/100,000 population), occurring most commonly as sporadic disease in young adults and infants, in developed countries, and 3) travellers' diarrhoea in persons from industrialized countries visiting hyper-endemic regions [29-34]. Natural reservoirs for these bacteria include dairy cattle, poultry and wild birds [35, 36].

1.2.3 Chronic intestinal Sequelae

1.2.3.1 Irritable bowel syndrome

Infectious gastroenteritis is a well-characterized environmental risk factor for the development of persistent abdominal symptoms including IBS. Two published meta-analyses evaluating the incidence of IBS following an infectious intestinal illness were consistent in their suggestion of an increase of seven-fold and six-fold in the odds of developing IBS following an episode of acute infective gastroenteritis, [37, 38]. Specifically, studies have reported up to 14% of those with acute campylobacteriosis develop IBS [39]. Prophylaxis prior to inoculation in challenge studies may mitigate the development of IBS, as illustrated in animal models; a rat-model that challenged rats with *C. jejuni* strain 81-176, with or without

concurrent rifaximin prophylaxis, concluded that the use of rifaximin as a prophylactic agent mitigated the development of a long term alteration in stool form and bowel function [40].

1.2.3.2 Inflammatory bowel disease

IBD (Crohn's disease and ulcerative colitis) is a chronic inflammatory condition of the gastrointestinal tract, often with extra-intestinal manifestations, and can carry a significant level of morbidity and mortality. A Danish population based cohort study, with 15-year follow-up, demonstrated an increased risk of IBD, Hazard Ratio 3.1 (95% Confidence Interval 2.1-4.6), following a *campylobacter* infection [27].

1.2.3.3 Coeliac disease

Case studies exist implicating the development of coeliac disease after an episode of infectious diarrhoea, the attributable pathogen being *C. jejuni* [41], and a retrospective cohort study of US military personnel who had been diagnosed with *C. jejuni* (n=738), non-typhoid *Salmonella spp.* (n=624), *Shigella spp.* (n=378) or *Yersinia enterolitica* (n=17) found that those with a previous *Campylobacter*-related medical encounter had a 3.5-fold increase in the rate of coeliac disease diagnosis when compared with unexposed individuals, the median time from that encounter to the diagnosis of coeliac disease being made was 3.4 years [42]. Whilst the p-value for the fold-increase data was not significant, $p = 0.13$, there were no cases of coeliac disease diagnosed within this cohort following infection with the other pathogens, so it highlights that more work is needed in this area [42].

1.2.3.4 Reactive arthritis

ReA is an autoimmune condition that frequently follows an episode of enteric or genitourinary infection. A systematic review has determined that the incidence of *Campylobacter*-associated ReA ranged from 8% to 16% with a median of 8% among adults compared to 0% to 6%, with a median of 3% among children [43]. These percentages were similar for ReA associated with the other gastrointestinal pathogens *Shigella* and *Salmonella.*

1.2.3.5 Guillain-Barré Syndrome and ganglioside mimicry

GBS is an immune-regulated condition resulting in a progressive, usually symmetrical, ascending motor and/or sensory polyneuropathy, which may affect the peripheral nerves and bulbar nerves, or in the case of Miller Fischer Syndrome (MFS), the cranial and bulbar nerves. Since the reduction in incidence of polio, GBS has become the most common cause of acute flaccid paralysis worldwide [44] and usually occurs following a gastrointestinal infection. Clinical symptoms occur 1-3 weeks after a bacterial or viral infection, the most commonly identified trigger pathogen being *C. jejuni* (13-39% of cases), followed by cytomegalovirus (5-22%), Epstein-Barr virus (1-13%), and *Mycoplasma pneumonia* (5%) [45], all of which have carbohydrate sequences in common with peripheral nerve tissue [45]. The autoimmune nature of GBS arises on account of the immune system mistakenly attacking Schwann cells or axons following exposure to a pathogen, the surface of which contains polysaccharides that resemble glycoconjugates of the human nerve tissue [45]. This phenomenon, called ‘molecular mimicry’, is the dual recognition by a single B-cell or T-cell receptor of both a microbe’s structure, and a host antigen. If this occurs cross-reactive antibodies or T-cells are produced, i.e. autoantibodies and auto-reactive T-cells, therefore

triggering an autoimmune condition [45, 46]. Gangliosides are glycosphingolipids which contain sialic acid linked to the oligosaccharide core [47] and which are expressed throughout the body, but are highly concentrated in the nervous system. Glycosphingolipids are composed of a ceramide (N-acelated sphingosine) attached to one or more sugars (hexoses). The ceramide is hydrophobic and as a consequence is immersed in the lipid membrane, the sugar carbohydrate is hydrophilic, and when exposed extracellularly is a target for autoantibodies [47]. Four gangliosides are recognized; GM1, GD1a, GT1a, GQ1b and the clinical pattern of disease depends upon the type of anti-glycoside autoantibody generated. *C. jejuni* infection is associated with axonal rather than demyelinating GBS, as well as the MFS variant. *C. jejuni* carrying GM1-like or GD1a-like lipooligosaccharides will induce anti-GM1 and anti-GD1b autoantibodies, as these gangliosides are expressed on the motor nerves of the limbs, the axonal form of GBS will ensue [44]. *C. jejuni* carrying GQ1b-like lipooligosaccharides will induce anti-GQ1b autoantibodies, these bind to GQ1b expressed on the occulomotor nerves and muscle spindles, therefore potentially causing MFS [44]. It is estimated that approximately one in 1000 cases of *C. jejuni* infection will result in GBS [48]. Strains of *C. jejuni* expressing sialylated lipooligosaccharide are frequently isolated in stool from subjects whose enteritis has not been complicated by GBS [48]. This raised the query of whether additional pathogenic factors were implicated in the development of GBS. Capsular genotyping of *C. jejuni* isolates has established that Penner serotypes types HS1/44 complex, HS2, HS4 complex, HS19, HS23/36 complex and HS41 are markers for GBS and may contribute to GBS susceptibility [48].

1.3 IMMUNOLOGICAL RESPONSE TO *C. JEJUNI*

In order for a pathogen to cause disease it must make contact with the host then establish a focus of infection. The human immune response to invading organisms proceeds from immediate innate defences, to induced innate defences, and finally to adaptive immunity [49].

1.3.1 The innate immune response to *C. jejuni*

The innate immune system provides the crucial first line of defence, independent of B and T-cells, against potential pathogens, including gram-negative bacteria such as *C. jejuni*. Innate immune defences exist as barriers; physical barriers in the form of epithelial cell surface junctions, mucus production and native microbiota; chemical barriers in the form of low gastric pH [50, 51], antimicrobial enzymes and peptides; and humoral barriers demonstrated by the complement system and cytokines. The innate immune system also includes phagocytes, including macrophages and dendritic cells.

1.3.1.1 Toll-like receptors

The innate immune system is not completely non-specific, as it has the ability to differentiate between self and a variety of pathogens (microbial non-self). The innate immune system recognizes microorganisms via a limited number of host germline-encoded pattern-recognition receptors (PRR) [52]. Toll-like receptors (TLRs) are a form of PRR. TLRs exist as dimeric proteins, either as heterodimers or homodimers [53], expressed on human epithelial cell lines [54]. They recognize microbial components, referred to as pathogen-associated molecular patterns (PAMPs), which are often essential for the survival of the microorganism and are therefore difficult for the microorganism to alter. TLRs can be specific to types of pathogen, for example Gram-negative bacteria, where the LPS is recognised by TLR4. Upon

recognition of microbial factors such as LPS, TLRs induce epithelial responses including epithelial cell proliferation, secretion of Immunoglobulin A (IgA) into the lumen and production of mucins and antimicrobial peptides. These responses promote the intestinal barrier function [55]. PRRs have been shown to contribute to the identification of, and subsequent response to, *C. jejuni*. *C. jejuni* lipooligosaccharide (LOS) is a potent activator of TLR4-mediated innate immunity, and the degree of sialylation, phosphorylation, and abundance of ester linkages in the *C. jejuni* LOS affects TLR4 signalling and the subsequent innate immune response [56]. Another TLR that may have been considered to be relevant to the recognition of *C. jejuni* is TLR5, which identifies flagellin. Interestingly however *C. jejuni* produce flagellins that lack proinflammatory properties and therefore escape the TLR5 flagellin-specific host immune response [57].

1.3.1.2 Complement activation

Complement is a major component of the innate immune system [58]. It comprises of a collection of more than 30 soluble, heat labile proteins found in blood and other fluids [49, 59]. Complement facilitates antibody mediated killing of pathogens by mechanisms that include cell-independent bactericidal activity by the formation of the membrane attack complex, and opsonisation for uptake and killing by phagocytic cells [59] as part of the innate immune response. Humans with defects in the complement cascade can show a variably increased risk of infection. Defects in the synthesis or activity of core components of the cascade (e.g. C3) are associated with an increased risk to infection and poor outcome, whereas individuals with a loss of other components, such as C9, often are healthy, but can have an increased risk to some infections, such as those caused by *Neisseria meningitidis*. Complement can not only kill bacteria, including *C. jejuni*, in the absence of antibodies, as

part of the innate immune system, but also augment the opsonization and killing of bacteria by antibodies.

There are three pathways by which complement augments the work of antibodies, the Classical, Alternative, and Lectin Pathways [49], Figure 1.3. Each pathway is a cascade of proteolytic cleavage reactions activating complement proteins to form proteases and therefore activating the next enzyme in the series [49]. The Classical pathway, activated by antibodies, is thought to be the pathway that contributes to the in-vitro killing of *C. jejuni* [60]. The specific involvement of this pathway has been illustrated by adding Ethylene Glycol Tetraacetic Acid (EGTA), a chelator highly selective for Ca^{2+}, to a serum killing assay. The Classical pathway is dependent upon the presence of Ca^{2+}, therefore by adding EGTA to a serum killing assay and chelating the Ca^{2+} present, the Classical pathway is inhibited. Research has demonstrated that by chelating Ca^{2+} less bacterial killing is seen, thus supporting the involvement of the Classical pathway [60].

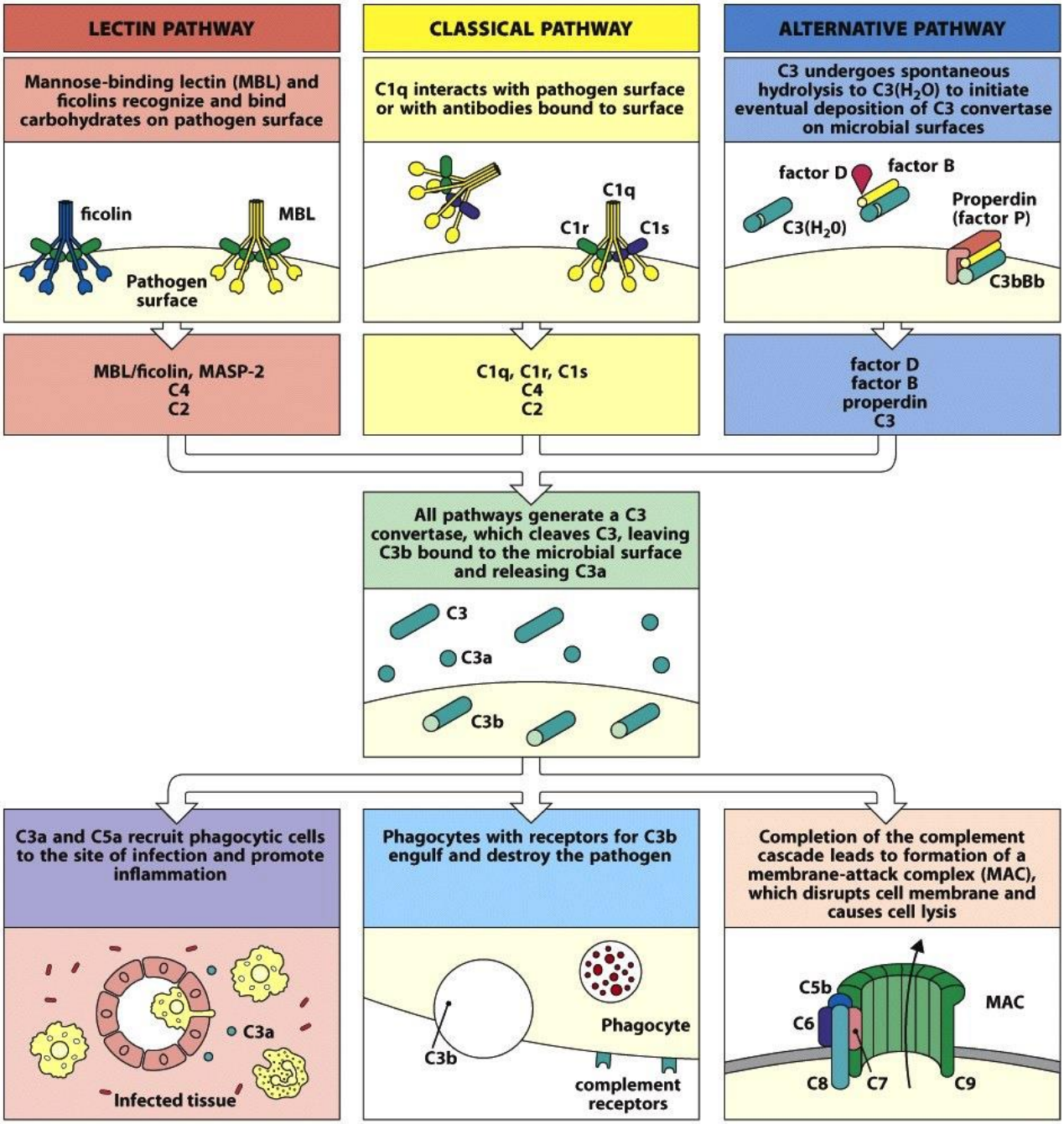


Figure 1.3 Illustration of the Lectin, Classical and Alternative pathways for Complement Activation Illustration taken from Janeways Immunobiology, 8ed. Copyright Garland Science 2012

1.3.1.3 Immune cells involved in the innate immune response

Pluripotent haematopoetic stem cells divide to produce common lymphoid and common myeloid progenitor cells, Figure 1.4. Hematopoietic cells involved in the innate immune response include the granulocytes: neutrophils, eosinophils and basophils; mast cells; monocytes which mature into macrophages after migration into tissues; dendritic cells and natural killer (NK) cells [53]. The three types of phagocytes of the innate immune system are monocytes and macrophages, granulocytes, and dendritic cells. The mammalian innate immune system employs macrophages, monocytes and neutrophils to phagocytose and expose microbes to harmful compounds such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [61]. The robust inflammatory innate immune response that accompanies human *C. jejuni* infection can cause a severe colitis, characterised by an influx of neutrophils and macrophages. The direct antimicrobial activity of phagocytes during *C. jejuni* infection is attributable to the production of antimicrobial peptides and proteins, an oxygen independent process, and the oxygen dependant release of ROS into the neutrophil phagosome for pathogen killing [62, 63]. The success of phagocytosis of *Campylobacter* is variable, and is likely to be due to variations in strain as well as phase variation of the bacterium. As described above, phagocytosis is often enhanced by opsonins such as complement [64]. Dendritic cells produce pro-inflammatory cytokines which activate macrophages and T-cells, and upon interaction with bacteria and LPS they develop into antigen presenting cells, therefore are involved in both the innate and adaptive immune responses [62]. Dendritic cells exist in the intestinal lamina propria with their dendrites penetrating the epithelial layer in order to directly sample the luminal contents of the intestine [65], they are therefore ideally placed to encounter both invasive and non-invasive bacteria. It has been demonstrated that the presence or absence of *C. jejuni* capsule is an important mediator of cytokine production

by dendritic cells, minor changes in capsule composition such as the lack of O-methyl phosphoramidate can lead to increased cytokine production [66].

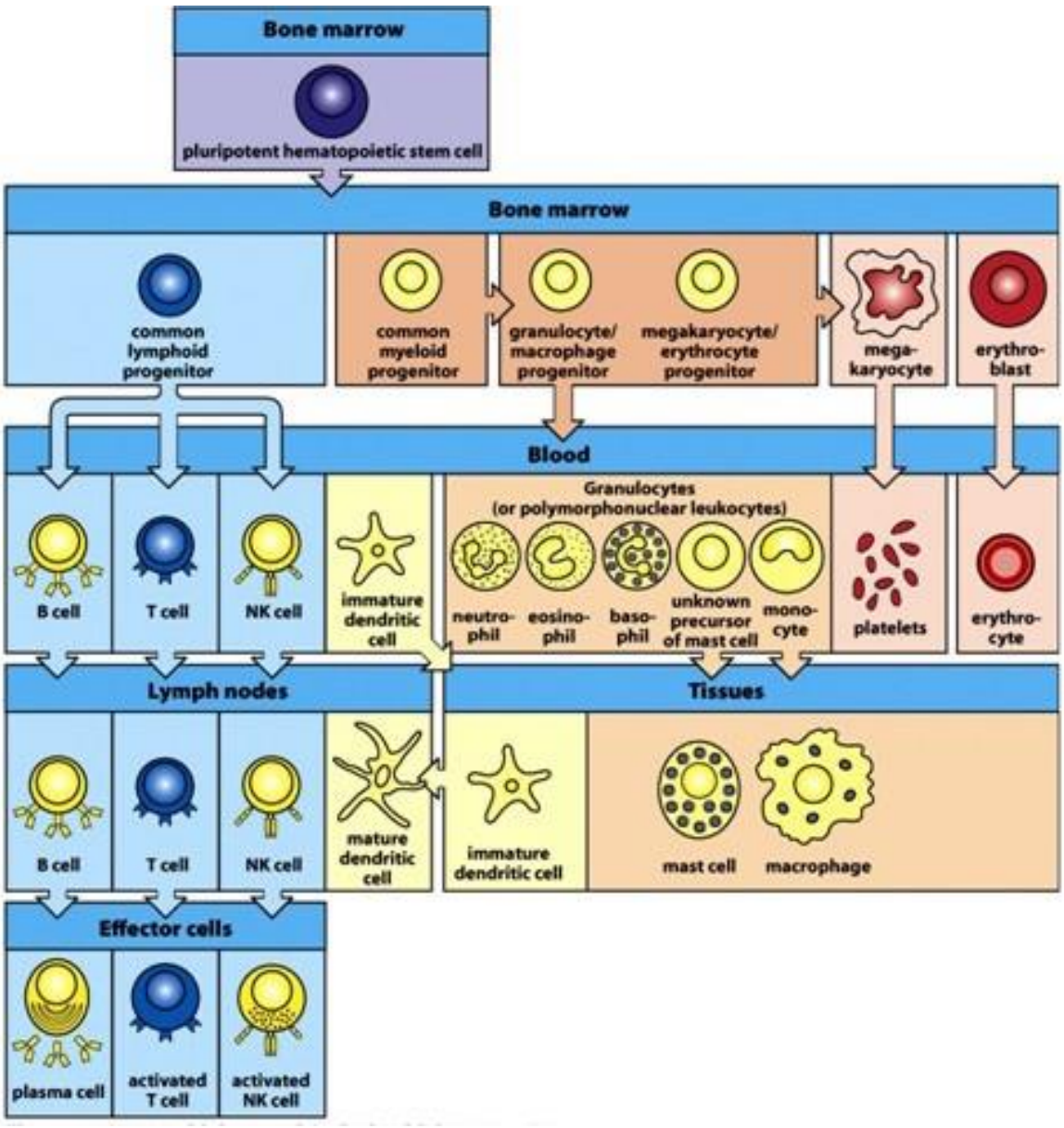


Figure 1.4 An overview of haematopoiesis.

Myeloid stem cells give rise to leukocytes, erythrocytes and megakaryocytes (producers of platelets). The leukocytes include neutrophils, eosinophils, basophils, mast cells and macrophages. Lymphoid stem cells give rise to T and B cells. NK cells also originate from the lymphoid progenitor but lack antigen specificity, which is a key feature of adaptive immunity. Illustration taken from Janeways Immunobiology, 8ed. Copyright Garland Science 2012

1.3.2 The Adaptive Immune response to C. jejuni

When the innate immune response is unsuccessful in preventing an infection, and the cellular environment has been altered as a consequence of the innate immune response, the adaptive immune response is triggered. Mature T-cells circulate and re-circulate between the blood stream and peripheral lymphoid tissue, where dendritic cells present antigens. Prior to encountering their antigen they are referred to as naïve T-cells. Upon antigen recognition the naïve T-cell will proliferate, differentiate into effector cells, and then leave the lymphoid tissue to re-enter the bloodstream [49]. Naïve CD8 T-cells, which recognize pathogenic peptides presented by major histocompatibility complex (MHC) Class I molecules, differentiate into cytotoxic effector T-cells, capable of recognising and killing infected cells. Naïve CD4 T-cells, having recognized pathogenic peptides presented in a MHC Class II molecule, can differentiate into effector subsets, including: T_H1, T_H2, T_H17 and T_{FH} (T follicular helper cell). These effector T-cells activate their target cells; T_H1 activating macrophages by cytokine release, T_H2 activating eosinophils, mast cells and basophils by IL-4, IL-5 and IL-6 release, T_H17 activating neutrophils through IL-17 mediated activation of stromal cells to produce chemokines, and T_{FH} activating B-cells and plasma cells [49]. CD4 T-cells also differentiate into regulatory subsets that limit immune activity. A *C. jejuni* ex-vivo human gut model of infection, where healthy paediatric terminal ileal or colonic mucosal biopsies were incubated with *C. jejuni* for 8-12 hours, examined the adaptive T-cell response. Using flow cytometry and ELISA the study observed a significant increase in Th-17, Th-1 and Th-17/Th-1 double-positive cells and corresponding cytokines, suggesting that the host immune system activates a CD4 T-cell response in the presence of *C. jejuni* [67].

1.3.2.1 Antibodies

Antibodies, also called immunoglobulins, are Y-shaped proteins employed by the immune system to destroy pathogens. Antibodies are produced by plasma cells following B-cell activation, discussed below. Each antibody is composed of 2 identical heavy chains and 2 identical light chains. A portion of the heavy chain is referred to as the 'Fragment crystallizable' (Fc); it determines the class of antibody and is involved in the method of elimination of a bound antigen. The remaining portion of heavy chains, and the light chains, are referred to as the 'Fragment antigen-binding' (Fab). Within this section the heavy and light chains have a 'constant' region and a 'variable' region, the variable region is the antigen-binding site, it is this region that makes the antibody specific to a particular antigen.

The function of antibodies is to destroy the pathogen and prevent further spread of infection. Antibodies achieve this through three processes; neutralization, opsonization or by activating the classical pathway of the complement system to facilitate phagocytosis. Neutralization involves the antibody binding to the pathogen, which prevents the pathogen from binding to the cell surface of a target host cell, and therefore prevents the pathogen from entering the host cell, or releasing toxins that enter the host cell. Opsonization involves the antibody binding to the surface of the pathogen and therefore coating it, facilitating phagocytosis as the antibody will be recognized by a phagocytic cell.

There are 5 main classes of human immunoglobulins (Ig), IgM, IgD, IgG (with subclasses IgG 1-4), IgE and IgA (with subclasses IgA1 and IgA2), all of which can exist as trans-

membrane antigen receptors, or be secreted as antibodies [49]. It has been demonstrated that, following an episode of *C. jejuni* associated enteritis, levels of serum immunoglobulins IgA, IgG, and IgM become elevated during the second week after a *C. jejuni* infection; and IgG and IgM elevations persist longer than IgA [68]. These three immunoglobulin classes will be further discussed.

1.3.2.1.1 IgA

IgA has a short half-life of 6 days. It is found not only in the blood stream but also on mucosal membranes. IgA is found in the lamina propria, where it is synthesized, and can be transported across the epithelium to, for example, the lumen of the gastrointestinal tract, allowing faecal IgA to be measured. *C. jejuni* is known to elicit both a serum and faecal IgA response. An experimental *C. jejuni* human challenge model has demonstrated that following infection with *C. jejuni*, serum IgA, faecal IgA and IgA antibody secreting cells (ASC) increased from baseline, however the levels fell back toward baseline in the following 3 months [69], and upon subsequent re-challenge there was only minimal increase, suggesting a lack of immune memory. A human challenge study investigating the protective efficacy of rifaximin vs. placebo in preventing campylobacteriosis found raised serum and faecal IgA levels in both groups, levels peaked at day 9 for both serum and faecal IgA [70]. IgA is a weak activator of complement and whilst it has opsonin properties, these are less potent than those of IgG; complement and phagocytes are not usually present on the epithelial surfaces where IgA is located. However IgA has neutralising properties, and therefore is able to use this method to destroy pathogens encountered on epithelial surfaces in locations such as the lumen of the gastrointestinal tract.

1.3.2.1.2 IgM

IgM is the first antibody to be produced following exposure to a pathogen, it is produced prior to B-cells undergoing somatic hypermutation and so the momomer form of the immunoglobulin has a low affinity for antigens [49]. However, IgM exists as a pentamer, and has 10 antigen-binding sites, and therefore has a high avidity for antigens, especially multivalent pathogens such as bacterial CPS. As a pentamer it has a high molecular weight (960 kDa), and therefore is referred to as a macroglobulin (and so given the letter M – IgM). Whilst frequently found in the bloodstream it is less commonly seen in tissues, due to the high molecular weight. IgM activates the Classical complement pathway. The half-life of IgM in serum is 10 days. Detection of *C. jejuni* IgM antibody infers recent infection, a study carried out in Brazil used ELISA to test the serum of 63 patients diagnosed with Guillain-Barr**é** Syndrome, and identified *C. jejuni* IgM in 17% of the samples [71].

1.3.2.1.3 IgG

IgG antibodies is the principle class of antibodies found in blood and extracellular fluid. IgG is an efficient opsonin, and subtypes IgG1-3 activate the classical complement pathway. Following infection in two *C. jejuni* human challenge models IgG responses were demonstrated at 182 and 84 days post infection [69, 70].

1.3.3 Evidence that antibodies are important

Antibodies have been proven to be very important for host protection against diarrhoeal pathogens such as *V. Cholerae*. Vaccination and challenge models are ideal for demonstrating this. Subjects who received a single dose of the oral cholera vaccine CVD 103-HgRag, and were subsequently challenged with *V. cholera* 01 El Tor demonstrated a

strong correlation between serum vibriocidal antibody seroconversion and protection against moderate to severe cholera [72]. The development of a vaccine for *C. jejuni* has not progressed to a vaccination and challenge study yet, however a *C. jejuni* strain CG8421 challenge and 3-month re-challenge study demonstrated a lack of protection at the re-challenge point. Following primary infection subjects demonstrated immunological responsiveness to primary infection, with increases in serum IgG and IgA, faecal IgA, IgA ASC, and *C. jejuni*–specific IFN-γ, from baseline. However, other than IgG, these responses fell to almost baseline in the three months between doses, and all subjects who received the second challenge subsequently developed campylobacteriosis. It is possible that the immune response stimulated by infection is not as robust as that induced by vaccination, especially if the vaccine is given in the presence of an adjuvant [69].

1.3.4 Antibody production

Antibody production is a key part of the adaptive immune response. B-cells, which have been exposed to the pathogenic antigen, subsequently undergo proliferation then differentiation into antibody secreting plasma cells. This process is initiated when a pathogenic antigen binds to the antigen-receptor of the B-cell, the B-cell receptor (BCR). The BCR sends a signal into the interior of the B-cell, and also internalizes the antigen to be degraded into peptides, which are presented on the B-cell surface, bound to MHC class II molecules. These two processes activate helper T-cells. Helper T-cells signal to the B-cell, via the CD40 ligand and release of cytokines IL-4, IL-5 and IL-6, inducing B-cell proliferation and differentiation into plasma cells capable of producing antibodies to this specific antigen. The humoral response described above is illustrated in Figure 1.5.

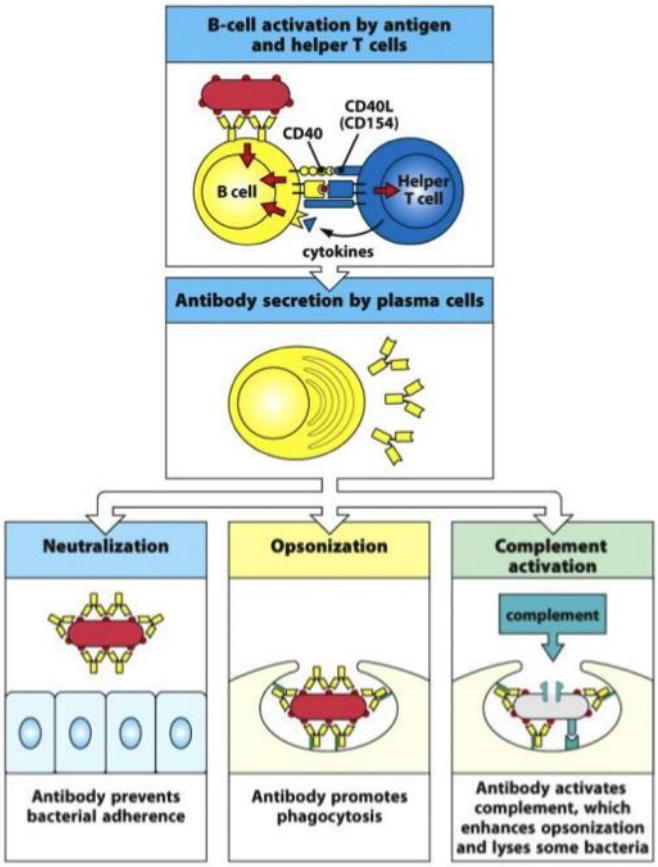


Figure 1.5 B-cells are activated by antigens and helper T-cells to produce antibodies, which facilitate pathogen killing by neutralization, opsonization and complement activation.

Illustration taken from Janeways Immunobiology, 8ed. Copyright Garland Science 2012

1.3.4.1 Thymus independent antibody production

T-cell independent generation of antibodies is possible, and naïve B-cells are stimulated by some polysaccharides, polymeric proteins and lipopolysaccharides in the absence of T-cell help. These antigens are referred to as thymus-independent (TI) antigens [49]. Bacterial capsular polysaccharides, such as those encapsulating *C. jejuni* bacteria, are thymus independent type 2 (TI-2) antigens. TI-2 antigens are only able to activate mature B-cells, therefore often do not elicit a robust antibody response in the under 5 year old population.

This is why many polysaccharide-based vaccines are conjugated to a protein, as this will generate a helper T-cell response, giving a more robust immune response. B-cell activation by TI-2 antigens involves crosslinking at multiple points between the antigen and BCR and leads to production of IgM antibodies. The presence of dendritic cells can enhance this as dendritic cells release cytokines, such as B-cell Activating Factor (BAFF), that augment the production of antibodies and induce class switching to allow production of IgG antibodies, Figure 1.6.

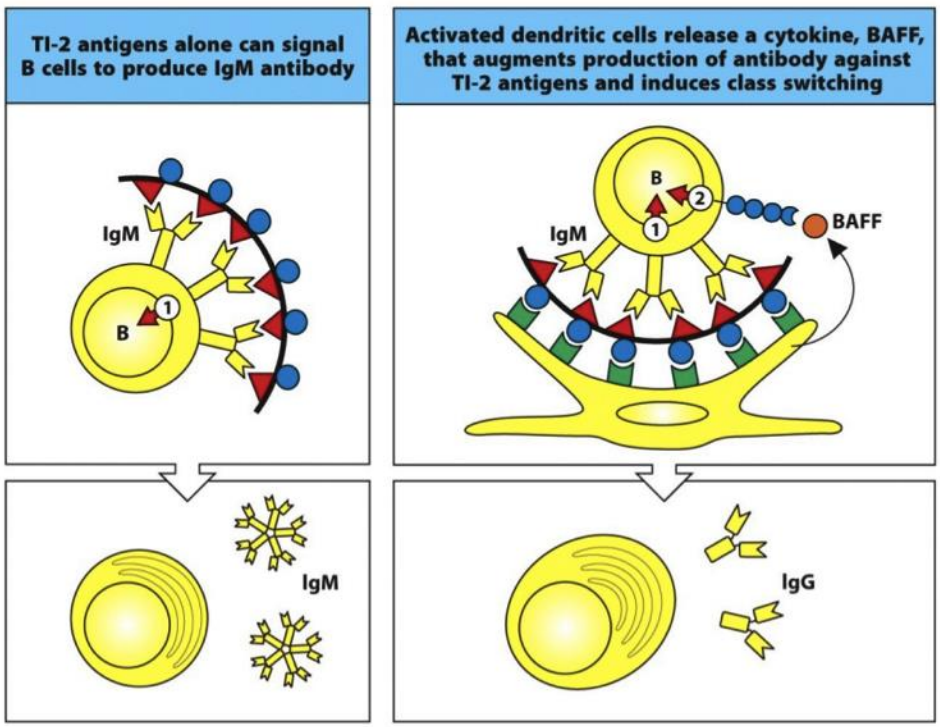


Figure 1.6 TI-2 activation of B-cells is enhanced by the presence of dendritic cells

TI-2 activation of B-cells can be caused by bacterial capsular polysaccharide, such as that of *C. jejuni* bacteria, and can stimulate IgM and IgG production. Illustration taken from Janeways Immunobiology, 8ed. Copyright Garland Science 2012

1.3.5 Approaches to *C. jejuni* vaccine development

Currently there is no licensed vaccine for *C. jejuni*. Whilst different approaches have been attempted, as described below, the antigenic diversity, as well as a limited understanding of the bacteria's pathogenicity, complicates vaccine development. *C. jejuni* vaccine development has not advanced beyond Phase I testing in humans [73]. This is a field in which work is on going. In order for a vaccine to be efficacious against an enteric pathogen, it is widely regarded that intestinal immunity must be stimulated. With this in mind, the oral route was often chosen for the administration of vaccines against enteric pathogens, and was the route of choice for the first candidate *Campylobacter* vaccine, a killed whole cell vaccine [74], more recently the intramuscular route has been used in clinical trials.

1.3.5.1 Live attenuated vaccines

Live attenuated vaccines against human infections have been successful medical interventions; smallpox was declared eradicated in 1980; poliomyelitis is nearing global eradication and measles has been controlled in most parts of the world [75]. Live attenuated vaccines are created by reducing the virulence of a pathogen whilst still keeping it viable. Successful examples of this include the oral polio vaccine, attenuated by serial passage; the Ty2lA vaccine strain of *Salmonella enterica* serovar Typhi, attenuated by chemical mutagenesis; and the cholera toxin A subunit-deleted cholera vaccine CVD 103HgR, attenuated by targeted deletion of genes encoding for virulence factors [74]. With regard to using a live attenuated approach for the development of a vaccine for *C. jejuni*, it has been proven by challenge studies that the degree of immunity provided by infection is variable, dependent upon the strain; infection with the wild-type strain 81-176 provides protective immunity when re-challenged 3 months later [76], however infection with strain CG8421

does not provide protective immunity upon re-challenge at 3 months [69]. The ability to induce protective immunity following natural infection suggests that a live attenuated vaccine approach may be successful for some strains, however, given the risk of C. *jejuni* infection precipitating GBS, the development of a safe live attenuated vaccine is difficult and whilst it is possible to select a strain without the genetic capability to achieve the molecular mimicry associated with development of GBS this is not a route that is being pursued currently. A mouse model has been used to test the efficacy of an attenuated *Salmonella enterica* serovar Typhimurium vaccine expressing *Campylobacter* antigens, however protection (judged by colonization after oral challenge) was not demonstrated [77].

1.3.5.2 Killed whole cell vaccines

In contrast with live attenuated vaccines, killed whole cell vaccines consist of components of pathogens that have been cultured then killed. The inactivated microorganisms offer advantages as they may possess many antigens that are important to immune protection, and, similar to the influenza vaccine where the prevalent serotypes are selected, multiple key antigens could be included. This approach has been used successfully for the oral inactivated cholera vaccine composed of heat- and formalin-killed *Vibrio cholera* whole cells [74, 78, 79]. With regard to killed whole cell vaccination for *C. jejuni*, this approach was tested in the early days of *C. jejuni* vaccine development using a mouse model; an oral vaccine combining heat- and formalin-killed *C. jejuni* 81-176 with the effective mucosal adjuvant *E. coli* heat-labile toxin (LT) demonstrated a protective efficacy of 87% when measured as clearance of challenge inoculum in faecal pellets at day 4 post challenge [80]. This method of oral killed whole cell vaccination combined with LT has also been tested in rhesus monkeys, and following vaccination *Campylobacter*-specific IgA and IgG ASC were detected in the sera of

the monkeys [81]. Both models found that the addition of LT enhanced the mucosal and serum response, the disadvantage of the addition of LT however is enterotoxicity, this can by mitigated by the use of a specific *E. coli* LT; LT(R192G) which has a mutation in the A subunit cleavage site, rendering it less toxic in mice and humans [74]. The risk of inducing GBS is also of concern in using a whole cell approach, and this route has not been pursued.

1.3.5.3 Subunit vaccines

Subunit vaccines present antigens, often consisting of a recombinant protein, to the host immune system, without introducing pathogens, either whole or parts thereof. These proteins may be given alone or in combination with an adjuvant [74]. The benefit of this is that they can be used in most individuals including those where live attenuated vaccines are contraindicated due to immunocompromise. *C. jejuni* subunits identified as possible antigens for vaccine development are the antigens flagellin and PEB1 [74]. Intranasal vaccination with the recombinant flagellin protein rFla-MBP provided 60% protective efficacy against diarrhoeal illness in a ferret challenge model, however a human challenge study has not been carried out as Phase 1 trials revealed low to moderate immune responses in terms of serology, ASC and mucosal IgA [74]. ACE Biosciences developed a protein subunit vaccine for *C. jejuni,* ACE 393, however it was not efficacious in Phase 2B trials with *C. jejuni* strain GC8421 [2].

1.3.5.4 Capsule-based conjugate vaccines

Since capsule-based conjugate vaccines are acellular, they are therefore generally safer than whole cell, live attenuated or killed vaccines. This is especially true in the case of *C. jejuni* where, in addition to pathogenicity, the whole cell vaccine may induce GBS due to

gangliocide mimicry as described above. Whilst CPS vaccines may exist in a purified form, they are often covalently coupled to a conjugate carrier protein in order to enhance immunogenicity. Capsules usually act to protect the organism against opsonization by phagocytes, however if a vaccine is generated from isolated polysaccharide capsule then antibodies specific to the polysaccharides will be elicited and will be protective. This process is illustrated in Figure 1.7, using an example of a polysaccharide epitope conjugated to a tetanus toxoid protein.

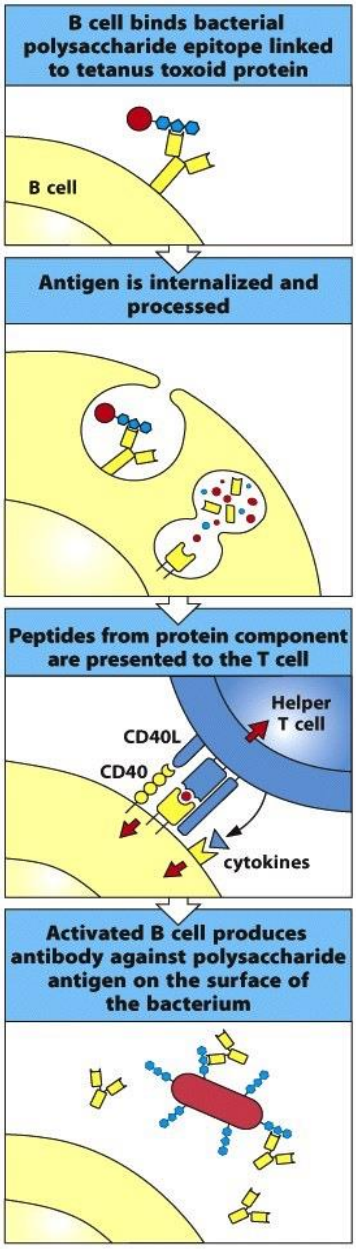


Figure 1.7 Antibody production as a consequence of vaccination with a capsule conjugate vaccine

This illustration is taken from Janeways Immunobiology, 8ed. Copyright Garland Science 2012

Following vaccination, a B cell receptor (BCR) will bind to an epitope of the CPS, the B cell will then internalize both the polysaccharide and the conjugated protein into an endosome, the endosome will fuse with an endocytic vesicle containing proteolytic enzymes at a low pH, and the protein will be broken down into peptides [49]. A MHC class II molecule will be

released from the endoplasmic reticulum and transported to this low pH endosomal compartment containing the antigenic, including peptide, particles, and peptide loading onto the MHC class II molecule will occur [49]. The MHC class II molecule will migrate to the surface of the B cell where it will be expressed, presenting the peptide. Effector $CD4^+$ T-cells will recognize MHC class II molecules with bound peptide, but MHC class II molecules will not process polysaccharides [82], hence the need for a carrier protein. Through mechanisms including, but not limited to, the release of cytokines and binding of CD40 by a CD40 ligand, the T-helper cell will activate the B-cell to proliferate and differentiate into ASC and memory B cells, and produce anti-polysaccharide or anti-CRM antibodies [49]. Capsule-based conjugate vaccines typically provide protection by eliciting antibodies with an opsinophagocytic or bactericidal function.

This polysaccharide conjugate approach has been proven to be safe and efficacious against encapsulated invasive mucosal pathogens such as *Streptococcus pneumoniae* [83], *Neisseria meningitidis* [84], and *Haemophilus influenzae* [85], as well as enteric pathogens. A polysaccharide conjugate vaccine is being investigated for *Shigella flexneri* 2a; a bioconjugate vaccine made from the polysaccharide component of the *S. flexneri* 2a O-antigen, conjugated to the exotoxin protein A of *Pseudomonas aeruginosa* has demonstrated a robust humoral response, as well as producing functional antibodies as determined by SBA, in humans [86].

1.3.6 Development of a capsule-based conjugate vaccine to *C. jejuni*

Capsule-based conjugate vaccines typically elicit an IgG antibody response. As *C. jejuni* has a polysaccharide capsule, a capsule-conjugate vaccine approach for this pathogen is being

evaluated [2]. CPS is conjugated to the diphtheria toxin mutant CRM_{197} by reductive amination to form CPS-CRM_{197}. The conjugation process is shown in Figure 1.8. A challenge in the development of a *C. jejuni* vaccine is the association with ganglioside mimicry due to the *N*-acetyl neuraminic acid (Neu5Ac) on the LOS of the bacteria [87]. However, as yet, unlike LOS, there has been no ganglioside mimicry associated with a *C. jejuni* CPS. Polysaccharides are TI antigens that generally do not elicit robust immune responses. In order to elicit a more potent and longer lasting B-cell response against a TI antigen, the polysaccharide must be conjugated to an antigen such as a protein, and the TI response converted to a helper T-cell mediated response, as discussed in Section 1.3.4.1.

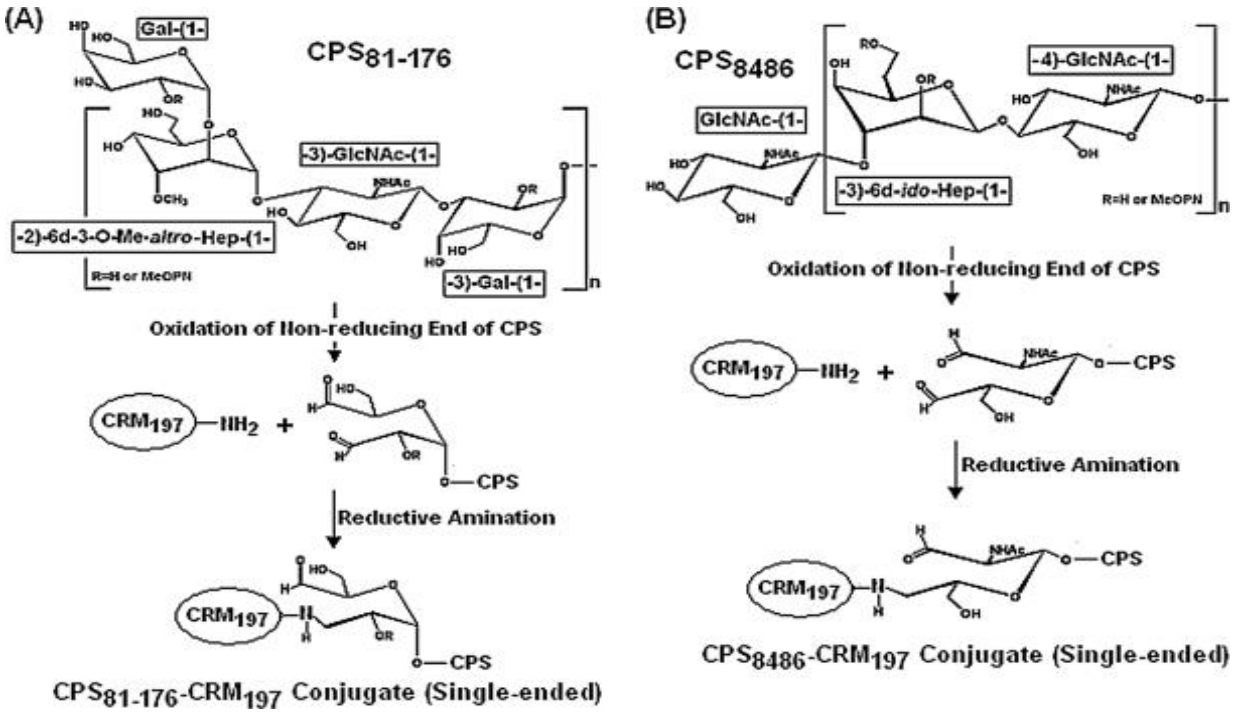


Figure 1.8 Chemical structures and conjugation of CRM_{197} to *C. jejuni* strain 81-176 CPS (A) and *C. jejuni* strain 8486 CPS (B)

This Figure illustrates how, through the processes of oxidation and reductive amination, conjugation of CPS to CRM_{197} is achieved. Monteiro MA, Baqar S, Hall ER, Chen cGMP, Porter CK, Bentzel DE, Applebee L, Guerry P. Capsule polysaccharide conjugate vaccine against diarrhoeal disease caused by *C. jejuni.* Infect Immun. 2009 Mar;77(3):1128-36.

The US Navy *Campylobacter* Working Group has carried out extensive work developing a vaccine for *C. jejuni.* This work has resulted in the development of different versions of the capsule-based conjugate vaccines, which have subsequently been used in safety and immunogenicity studies in NZWR, challenge studies in NHP, and Phase 1 studies in humans. This work has been the prelude to this thesis; the sera from these studies has been stored and has been available for the development of a SBA as part of this M.D.

1.3.7 Correlates as substitute endpoints to demonstrate protection after vaccination

The ability to demonstrate protective efficacy of a vaccine, without having to test the clinical outcome of each individual vaccinated following exposure to the pathogen, can be achieved by demonstrating substitute endpoints. These endpoints are often referred to as 'surrogates' or 'correlates' for protection. The WHO have clearly defined this terminology; to illustrate this Figure 1.9 is a simplified model of the induction of protective immunity following vaccination [88]. In Fig 1.9 Immune Marker 1 is a measurable entity that lies directly on the pathway involved in the development of protective immunity, this is a surrogate. Immune Marker 2 does not lie on that direct pathway and whilst it will occur as a consequence of the vaccination, it is not directly involved in the process that results in protective immunity, this marker is a correlate. Immune markers that occur as a consequence of vaccination are correlates, therefore all surrogates are considered correlates, but only those directly involved in the pathway achieving protective immunity are considered surrogates.

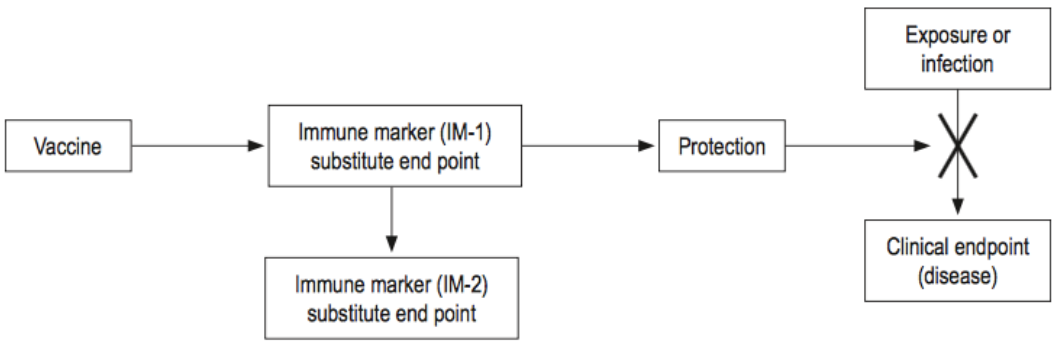


Figure 1.9 An illustration of correlates vs. surrogates

This illustration is taken from the WHO Initiative for Vaccine Research (IVR), Department of Immunization, Vaccines and Biologicals publication: Correlates of vaccine-induced protection: methods and implications, May 2013

The fact that a vaccine produces a specific immune response, which may be a substitute endpoint, does not prove that the vaccine is protective, or that the substitute endpoint can infer protection. In order to prove this, certain criteria should be met. Prentice described an approach to the validation of substitute endpoints [89], the 'Prentice Criteria', and the WHO considers that these can be adapted for vaccine trials, illustrated by Figure 1.10 [88].

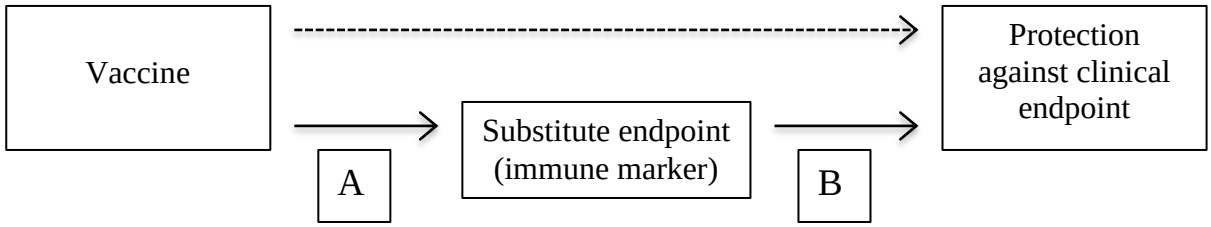


Figure 1.10 The simplest possible relationship between vaccine, substitute endpoint and clinical endpoint.

This illustration is taken from the WHO Initiative for Vaccine Research (IVR), Department of Immunization, Vaccines and Biologicals publication: Correlates of vaccine-induced protection: methods and implications, May 2013, and is used to illustrate the Prentice Criteria, listed below.

Prentice Criteria to validate substitute endpoints in clinical trials:

1. Protection against the clinical endpoint is significantly related to having received the vaccine (the dotted line in Fig 1.10)
2. The substitute endpoint is significantly related to the vaccination status (step A in Fig 1.10);
3. The substitute endpoint is significantly related to protection against the clinical endpoint (step B in Fig 1.10)
4. The full effect of the vaccine on the frequency of the clinical endpoint is explained by the substitute endpoint, as it lies on the sole causal pathway.

An established means of validating protective efficacy following vaccination is by demonstrating a specific bactericidal titre, or fold-increase in titre, capable of >50% killing, as determined by a SBA. This has been used for the gram-negative encapsulated organism, *Neisseria meningitidis.* Those working with the meningococcal serogroup C conjugate (MCC) vaccine use a 50% killing bactericidal titre ≥4 as a 'gold standard' correlate for protection [90], this titre is applicable when human complement is used in the SBA. Rabbit complement has also been used and it has been determined that, using rabbit complement, the protective 50% killing titre following vaccination is 128. However, for those subjects with a titre result, when tested using rabbit complement of between 8 and 128, if the fold-increase in 50% killing titre is ≥4-fold, then that is also a correlate for protection [90]. These values, conferring protection, have also been applied to the meningococcal A conjugate vaccine MenAfriVac [91].

1.3.8 Serum bactericidal assays

Serum bactericidal assays (SBA) are used to measure the antibody mediated, complement-dependent, cell-independent, killing of bacteria following vaccination, and therefore measure the functionality of the antibodies in the serum of vaccine recipients. For many vaccines, it is considered the "gold standard" for measuring the ability of vaccine-induced antibodies to kill bacteria, including for organisms such as *Neisseria meningitidis* [92]. Although the model used varies depending upon the nature of the organism and the vaccine, for many the functional bactericidal titre is considered to be the highest titre that achieves a killing percentage of >50% of the bacterial cells when compared to the designated control. The titre is reported as the reciprocal of that serum dilution. The assay is dependent upon a source of immunoglobulin, a source of complement (often baby rabbit complement) and bacteria [59].

Variations in results will exist reflecting alterations in method such as the source/species of complement used, the percentage of complement used, the number of bacterial cells added to the assay, and different diluents used. These all need to be optimized, then standardized if the assay is to be used by different laboratories. As an example, the defined and validated conditions for the SBA used for vaccines against *Neisseria meningitides* was first determined by a panel of experts convened by the WHO [93].

1.4 PREVIOUS IN-VIVO STUDIES WITH SPECIFIC RELEVANCE TO SBA DEVELOPMENT

This thesis explores the development of an SBA to determine a correlate or surrogate for protection against campylobacteriosis. The sera used are archived specimens from studies carried out by the Naval Medical Research Centre, Enteric Disease Department. The studies, and consequent sources of sera, are detailed below.

1.4.1 New Zealand White Rabbits – cGMP Grade Vaccine CJCV1 Safety Study

The safety study for the CJCV1 cGMP grade vaccine used New Zealand White Rabbits (NZWR). The purpose of this study was to evaluate the safety and immunogenicity of (CPS-CRM_{197}) when administered to NZWR by the intramuscular route on Study Days (SD) 1, 29, and 57. The rabbits were administered three doses of the vaccine CPS-CRM_{197} alone or in combination with Alhydrogel by the intramuscular route. Upon completion of the vaccination period, designated main-phase animals were sacrificed two days after the final vaccination. Recovery-phase rabbits were held for a further 14-day period (to Day-71) in order to evaluate the persistence, delayed onset, or reversibility of any effect of the vaccine.

The safety study commenced in April 2013 titled:

Campylobacter Capsule Conjugate Vaccine: A 71-Day Repeat Dose Intramuscular Toxicity Study in the New Zealand White Rabbit. AVANZA Study No. 2056-11033

The intramuscular route was selected because it was the intended route of administration for humans. Table 1.2 details the group designation and doses. The dose of 10 μg of CPS was selected to equal the maximum anticipated clinical dose in humans.

Table 1.2 A 71-Day Repeat Dose Intramuscular Safety Study in the New Zealand White Rabbit; Group Designation and Dosage Levels

Group	Vaccination Days	Treatment	CPS antigen / adjuvant (μg) per dose	Number in Group
1	1, 29, 57	Control Article PBS	0/0	20
2		CJCV1 vaccine: CPS-CRM_{197}/ Alhydrogel	10/125	20
3		CJCV1 vaccine: CPS-CRM_{197}	10/0	20

Blood was collected prior to each dose of the vaccine, and on study days 3 and 59. Blood was also collected from each animal by cardiac puncture prior to its sacrifice on study day 71. For the purpose of the SBA, serum from study days 0 (pre-vaccination), 56, (following 2 vaccinations), and the final bleed on Day-71, (following 3 vaccinations), was tested.

1.4.2 *A. nancymaae* NHP – research grade CCV Vaccination and Challenge

A model of *Campylobacter*-induced diarrhoeal disease that mimics aspects of human illness has been developed in the NHP *A. nancymaae* [94]. To determine if vaccination with CPS-CRM_{197} would protect against diarrhoeal disease, and also to review immunogenicity, the US Naval Medical Research Unit in Lima, Peru, performed a dose escalation and immunogenicity study of the research grade CCV vaccine using the NHP *A. nancymaae.* The animals were immunized subcutaneously at 6-week intervals with three doses of either PBS (control) or escalating doses (0.1, 0.5 and 2.5 μg by conjugated polysaccharide) of CCV with Alhydrogel [8]. There were 5 animals in each group. Table 1.3 shows the vaccination groups.

Table 1.3 Vaccination groups for the NHP vaccination and challenge study

Group	Vaccination Schedule	Treatment	CPS antigen (μg) per dose	Number in Group
1	3 Doses of vaccine at 6-week intervals.	CCV vaccine: CPS-CRM_{197} with alhydrogel	0.1	5
2			0.5	5
3			2.5	5
4		PBS	0	5

Nine weeks (63 days) after the final vaccination the animals were orally challenged with ~ 10^{11} CFU of *C. jejuni* 81-176. The attack rate for those that received the PBS control was 60% (3/5 animals), the attack rates for the 0.1 μg, 0.5 μg and 2.5 μg conjugated CPS groups were 40% (2/5), 20% (1/5) and 0% (0/5) respectively, the highest dose therefore giving 100% protection [8]. Those in Group 2 demonstrated significantly raised levels of plasma anti-CPS IgG (by ELISA) after the third dose, and Group 3 after the second dose, compared with baseline [8]. Whilst production of sustained levels of IgG has been proven, as well as

protective efficacy following challenge in NHP, serum bactericidal killing activity and development of a surrogate or correlate for protection is required. Serum from this study was also available for SBA development. The challenge aspect of this study meant that a correlation between SBA titre and protection could be investigated.

1.4.3 Human CJCV1 vaccination – Phase 1 Clinical Trial

Serum collected during the study: *Safety Study of a Capsule-Conjugate Vaccine to Prevent Campylobacter-Caused Diarrhea (CJCV1-01),* ClinicalTrials.gov Identifier: NCT02067676, was available for SBA analysis. This was an open-label, dose-escalation study in which a total of 48 healthy volunteers received 2 vaccinations (one on Day 0 and one on Day 28 ± 2 days). There were 3 groups, each group receiving a different intramuscular dose of the vaccine; 2 μg, 5 μg, or 10 μg of CPS in the vaccine CJCV1. The groups were split into sub-groups, with 2 sub-groups of 8 subjects in each group, Table 1.4. The 2 sub-groups allowed direct comparison in immunological response of administration of the vaccine with or without 125 μg Alhydrogel.

Table 1.4 Vaccination groups for the Human Phase 1 Clinical Trial

Group	Vaccination Days	Vaccination	CPS antigen / adjuvant (μg) per dose	Number in Group
1A	0, 28	CPS-CRM_{197}/ Alhydrogel	2/0	8
1B			2/125	8
2A			5/0	8
2B			5/125	8
3A			10/0	8
3B			10/125	8

1.5 ANTIMICROBIAL THERAPY FOR DIARRHOEAL ILLNESS

Diarrhoeal disease caused by bacterial pathogens may be self-limiting, however, for a subset of patients whose symptoms persist, who have severe disease, who are immunocompromised or who are at high risk of transmitting the pathogenic bacteria to others, antimicrobial therapy may be prescribed. In a small subset of patients, such as travellers and those in small Military Units, prophylaxis against diarrhoeal disease may be advantageous for the individual or for the successful completion of an Operational Mission.

1.5.1 Antimicrobial therapy for treatment of diarrhoeal disease due to *C. jejuni*

Macrolides such as azithromycin are considered first line therapy for campylobacteriosis, with fluoroquinolones such as ciprofloxacin employed as an alternative. Whilst there is emerging resistance to both of these antimicrobials, fluoroquinolone resistance is more widespread. Azithromycin interferes with protein synthesis; the drug binds to the 23S recombinant RNA nucleotides 2,058 and 2,059 in the 50S ribosomal subunit, and this causes the blockage of the translocation step of protein synthesis, thereby preventing release of tRNA after peptide bond formation and resulting in the termination of peptide chain elongation [95]. In contrast, fluoroquinolones target DNA gyrase and topoisomerase IV, enzymes essential for DNA replication, transcription, recombination, and repair [95].

1.5.2 Antibiotic chemoprophylaxis for travellers' diarrhoea

Non-absorbable oral antibiotic therapy may be efficacious for the prevention as well as treatment of bacterial diarrhoeal disease. As these drugs are not systemically absorbed, but rather remain within the lumen of the gastrointestinal tract, they have the potential to exert their effects on bacteria within this location, whilst having little impact on bacteria residing on

or within tissues outside of the gastrointestinal tract, such as skin, respiratory or genito-urinary systems. Whilst this lumen-only feature has the potential to reduce efficacy against diarrhoea caused by some invasive bacterial infections such as strains of *Shigella* and *Campylobacter*, which invade the colonic mucosa, if it is able to exert an effect on these types of pathogens prior to mucosal invasion, this may not be such an issue. Doxycycline, an antimicrobial agent in the tetracycline group, was one of the first antibiotics to be tested as a prophylactic agent against diarrhoea, and showed some degree of efficacy in preventing travellers' diarrhoea (TD) in the field [96-99]. However, antimicrobial resistance to tetracyclines increased to the point that doxycycline could no longer be recommended for prophylaxis. Fluoroquinolone antibiotics replaced doxycycline as the agent of choice for the prevention of TD [100-102]. Now, with the advent of simple and highly effective therapies, treatment rather than prophylaxis has become the preferred course for travellers visiting high-endemic settings.

Whilst systemic antibiotics have been proven to be efficacious in the prophylaxis of TD, this is not generally encouraged due to the risk of developing antibiotic resistance. The ideal prophylactic antibiotic candidate would have broad spectrum efficacy against enteric pathogens, would not be associated with widespread bacterial resistance or be prone to inducing persistent resistance, would be safe in all persons requiring prophylaxis, and would be available in formulations suitable for administration to all age groups. Non-absorbable oral antibiotic therapies such as rifaximin fulfil many of these characteristics. Rifaximin, a non-absorbed antibiotic, has already been proven to be an efficacious prophylactic medication against the development of diarrhoeal disease when tested in a double blind, placebo controlled, inpatient clinical trial against *Shigella* [103], however its efficacy against the

specific pathogen *C. jejuni* has not as yet been similarly examined. A double blind, placebo controlled, clinical trial investigating the protective efficacy of rifaximin in preventing campylobacteriosis was performed. The results of this study are discussed in Chapter 4 and address the second objective of this thesis.

1.6 RIFAXIMIN

Rifaximin is a non-aminoglycoside semi-synthetic, non-systemic antibiotic derived from rifamycin and is a structural analogue of rifampin. It exerts its bactericidal activity by binding to the β-subunit of bacterial DNA-dependent RNA polymerase, this results in inhibition of bacterial RNA synthesis [104]. The antimicrobial spectrum (*in vitro*) includes most gram-positive and gram-negative bacteria and both aerobes and anaerobes.

In May 2004 the US Food and Drug Administration (FDA) approved the use of rifaximin for treatment of patients (≥ 12 years of age) who were suffering from TD caused by non-invasive strains of ETEC. In March 2010, rifaximin was also given FDA approval for the treatment of Hepatic Encephalopathy (HE). Salix Pharmaceuticals, Inc. [104], Morrisville, North Carolina, US, hold a US Patent for rifaximin and markets the drug under the name XIFAXAN®, available in tablets of 200 mg (treatment of TD caused by ETEC, 200 mg three times daily) and 550 mg (treatment of HE). It is also an off-license treatment option for pouchitis and small bowel bacterial overgrowth. The drug has been shown to be beneficial in trials investigating its use in patients with diarrhoea predominant IBS [105], the mechanisms for which are under investigation in mouse models [106]. The side effect profile of the drug patent describes no significant side effects [107].

The chemical name for rifaximin is (2*S*,16*Z*,18*E*,20*S*,21*S*,22*R*,23*R*,24*R*,25*S*,26*S*,27*S*,28*E*)-5,6,21,23,25-pentahydroxy-27-methoxy-2,4,11,16,20,22,24,26-octamethyl-2,7-(epoxypentadeca-[1,11,13]trienimino)benzofuro[4,5-e]pyrido[1,2-á]-benzimidazole-1,15(2*H*)-dione,25-acetate. The empirical formula is $C_{43}H_{51}N_3O_{11}$ and its molecular weight is 785.9 [104], a 2D structure is shown at Figure 1.11. Each 550 mg tablet contains the inactive ingredients colloidal silicon dioxide, glycerol palmitostearate, microcrystalline cellulose, polyethylene glycol/macrogol, polyvinyl alcohol, red iron oxide, sodium starch glycolate, talc, and titanium dioxide [104]. Rifaximin is a microcrystalline, red-orange powder that is odourless.

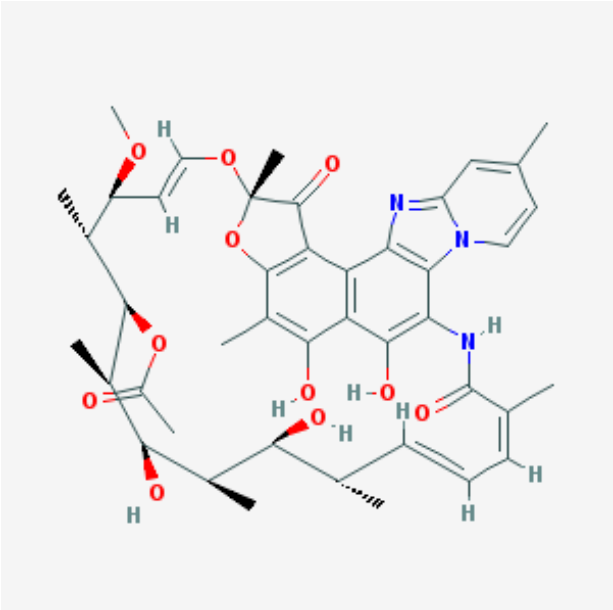


Figure 1.11 The 2D Structure of Rifaximin

Downloaded from Pubchem Open Chemistry Database; Compound Summary for CID 6436173, https://pubchem.ncbi.nlm.nih.gov/compound/Rifaximin

1.6.1.1 Human Pharmacokinetics

Rifaximin is poorly absorbed following oral administration [108], more than 95% of the drug is excreted in the faeces as unchanged drug. This was established by a mass balance study; after oral administration of 400 mg of carbon labelled ^{14}C-rifaximin to healthy volunteers, 96.94% of the total amount administered was recovered, and of that amount recovered, 96.62% of radioactivity was recovered in faeces almost exclusively as the unchanged drug, 0.32% was recovered in urine mostly as metabolites with 0.03% as the unchanged drug [104]. This suggests that the absorbed rifaximin undergoes metabolism with minimal renal excretion of the unchanged drug [104]. The presence of inflamed colonic mucosa does not alter the degree of absorption. Plasma levels of rifaximin were measured in a Shigellosis human challenge model, which had been successful in inducing diarrhoeal disease, the results showed a lack of cumulative systemic absorption of rifaximin in individuals with mucosal inflammation of the colon, consistent with published reports demonstrating minimal rifaximin plasma concentrations and a lack of drug accumulation [109].

Following a three-day dosing regime of rifaximin 400 mg twice daily, analysis of faecal material demonstrated a mean rifaximin concentration of 7,961 μg/g of faeces on day one post treatment, 7,425 μg/g on day two, 3,266 μg/g on day five and 154 μg of rifaximin/g of faeces on the sixth day post treatment [110]. Assuming equivalent densities of stool (1 g/mL) this is equivalent to 8,000 μg rifaximin/mL of stool at the therapeutic site of action [110]. The same study determined the MIC_{50} and MIC_{90} of rifaximin for bacterial isolates from subjects who developed TD whilst in Guadalajara, Mexico, during the summers of 1997 and 1998. The study tested the following numbers of bacterial strains: 120 strains of ETEC, 17 strains of *Shigella* and 8 strains of *Salmonella*. Rifaximin was shown to have a MIC_{50} of 12.5 μg/mL,

and MIC_{90} of 50 µg/mL for the 145 isolates tested [110]. Therefore whilst the MIC for *C. jejuni* was not tested in this study, it demonstrated that a rifaximin concentration at the site of action, estimated to be approximately 8000 µg/mL following the dosing regime above, is much higher than the MIC required for other invasive pathogens, therefore suggesting that the host may be protected from susceptible invasive pathogens prior to invasion. The MIC_{50} and MIC_{90} for *C. jejuni* were tested by Sierra et al. in Barcelona, and they found the MIC_{50} of rifaximin for *C. jejuni* to be 256 µg/mL, and the MIC_{90} to be 512 µg/mL [111]. In contrast, the *in vitro* data for rifaximin's MIC_{90} has been reported to be as low as 32 µg/mL using *C. jejuni* pathogens recovered from individuals with TD from Kenya [112]. Both of these studies demonstrate MICs lower than the anticipated concentration of rifaximin that would be present in the lumen of the bowel following a twice daily dose of 400 mg of rifaximin. This suggests that the anticipated faecal concentrations should prevent campylobacteriosis.

1.6.1.2 Efficacy of Rifaximin against TD and *C. jejuni*

As a minimally-absorbed antibiotic, rifaximin's bactericidal activity appears to be limited to the lumen of the gastrointestinal tract. As a result, rifaximin may to be less effective in treating bacterial pathogens that have successfully penetrated the bowel epithelial layer. In a clinical trial investigating treatment for travellers' diarrhoea, rifaximin was less effective than ciprofloxacin in reducing the time to last unformed stool (TLUS) and in providing clinical resolution, when the causative agent was an enteric pathogen capable of crossing the bowel epithelium. Such pathogens include *Shigella* species, *C. jejuni*, and *Salmonella* species [113].

Rifaximin has been shown to be effective in treating TD in studies carried out in Mexico, Guatemala, Jamaica, and Kenya and was equivalent to ciprofloxacin in shortening the

duration of diarrhoea in students from the US and in international travellers to Mexico and Jamaica. The safety profile equivalent of rifaximin in these studies was equivalent to placebo [103, 114-116]. In addition, more recently, a study carried out by the US and UK Military in Afghanistan, Honduras, Kenya and Djibouti showed that single-dose azithromycin, levofloxacin, and rifaximin with loperamide were comparable for treatment of acute watery diarrhoea [117].

When considering efficacy as a prophylactic agent, a Phase 3 randomized, double-blind, placebo controlled study carried out in Mexico showed that when rifaximin was given prophylactically at a dose of 600 mg/day for 14 days, the incidence of TD in adults was significantly reduced, ($p< 0.0001$), in this study the cumulative incidence of TD over 14 days in those taking rifaximin was 15% vs. 47% in the placebo group [118]. Furthermore, it was found that rifaximin 200 mg three times daily for three days was completely effective in preventing experimental shigellosis, an invasive pathogen, in a human challenge model [103]. This suggests that rifaximin may indeed be effective in preventing infection with invasive pathogens by eradicating them before they colonize and/or invade the colonic epithelial cells.

Within the Military population, in 2005-2006, a study to evaluate rifaximin for prevention of TD amongst a deployed US military population was conducted. This randomized, double-blind, placebo-controlled clinical trial enrolled 100 subjects deployed to Incirlik Air Base, Turkey. On arrival they received rifaximin 1100 mg, or placebo, once daily for 2 weeks [119]. Participants were reviewed daily for 2 weeks. 6.3% (3/48) of participants taking rifaximin developed TD, compared with 19.1% (9/47) of those taking placebo, $p = 0.051$ [119].

Within a civilian population, between 2009 and 2012 in Tübingen, Germany, a double-blind, placebo controlled trial of rifaximin vs. placebo for individuals travelling to south and south-east Asia was carried out. Study participants had to be in the destination country for 6-28 days, the study found that 48/117 participants (41%) in the placebo group and 30/122 participants (25%) in the rifaximin group reported episodes of TD, they concluded that in this area of the world rifaximin offered moderate protection [120].

Therefore, multiple studies have suggested that rifaximin may be a prophylactic option amongst travellers and Military personnel on deployment for prevention of diarrhoeal disease. Most recently, within the US and UK military, a field study is taking place to assess rifaximin as a chemoprophylaxis option to prevent TD and therefore increase force health protection; PREVENT TD, ClinicalTrials.gov Identifier: NCT02498301. This is a randomized, placebo-controlled, double-blind, clinical trial evaluating two dose regimens of rifaximin (550mg daily or 550mg twice daily) for chemoprophylaxis against TD among deployed U.S. and British Military personnel.

1.7 AIMS AND OBJECTIVES

This aim of this thesis is to examine two different forms of prophylaxis against campylobacteriosis. The first form of prophylaxis is an experimental capsule-based conjugate vaccine. Prior to my involvement with the NMRC *Campylobacter* Working Group the Group had developed a vaccine and carried out studies as described above, section 1.4. A correlate for protection was yet to be established. The starting hypothesis is that vaccination induces an antibody response with a functional bactericidal capability, demonstrable by a SBA, the titre of which could represent a correlate for protection. The second form of prophylaxis is the

minimally-absorbed antibiotic rifaximin, the starting hypothesis is that rifaximin protects against campylobacteriosis in a human *C. jejuni* challenge model.

The work carried out for this M.D. has two objectives:

Objective 1: To develop a serum bactericidal assay capable of demonstrating, by titre, the functional bactericidal effect of antibodies following *C. jejuni* vaccination.

Objective 2: To demonstrate the protective efficacy of rifaximin vs. placebo in a *C. jejuni* CG8421 human challenge model.

CHAPTER 2 MATERIALS AND METHODS

2.1 INTRODUCTION

In order for an SBA for *C. jejuni* to be carried out the following key reagents are required; growth media, diluent, gas to ensure a microaerobic environment, serum, complement, and bacteria.

2.1.1 Growth Media, Diluents and *Campylobacter* gas

The media used for culturing, diluting and storing *C. jejuni* are described below in Table 2.1. As the development of the assay evolved, different diluents were used.

2.1.1.1 Ensuring optimal levels of Mg^{2+} and Ca^{2+} in the Diluent

Complement pathways are dependent upon sufficient amounts of calcium (Ca^{2+}) and magnesium (Mg^{2+}), the concentrations of these within reagents was reviewed. Alternative diluents were made to replace minimum essential medium (MEM), thus ensuring high Ca^{2+} and Mg^{2+} concentrations. The data from replicate assays carried out using these diluents was not consistent; therefore the details of these diluents are not included in the report. Upon further literature searches into diluents for complement mediated assays Dextrose Gelatin Veronal (DGV) was found [121], and was subsequently used for suspending *C. jejuni* cells and as a diluent for the SBAs.

Table 2.1 Growth Media and Diluents; Description of Use and suppliers

<table>
<tr><th>Diluent or Media</th><th>Description of Use</th><th>Supplier</th></tr>
<tr><td>15% Glycerol Brucella Broth (GBB)</td><td>Suspending C. jejuni cells for freezing</td><td>Glycerol: Fisher Bioreagents BP229-1
Brucella Broth: Becton Dickinson and Co. ref 211088</td></tr>
<tr><td>Muller Hinton Plain agar plates (MHP)</td><td>Growth and expansion of C. jejuni, plating for CFU counts</td><td>Difco - Made into solution and poured on site</td></tr>
<tr><td>Mueller Hinton Broth (MHB)
pH 7.3 (+/- 1)</td><td>Suspending C. jejuni cells for SBAs initially</td><td>Difco - Made into solution on site</td></tr>
<tr><td>Minimum Essential Medium (1X) (MEM), no glutamine</td><td>SBAs, multi-well plate diluent</td><td>Gibco
Ref: 11090-081</td></tr>
<tr><td>Phosphate Buffer Solution (PBS) powder pH 7.4</td><td>SBA diluent</td><td>Sigma
lot SLBJ9934V</td></tr>
<tr><td>Dextrose Gelatin Veronal (DGV) pH 7.42, formulation below, from Lonza product information:
<table>
<tr><td>$CaCl_2$</td><td>20 mg/L</td></tr>
<tr><td>MgSO4·7H2O</td><td>120 mg/L</td></tr>
<tr><td>NaCl</td><td>8,500 mg/L</td></tr>
<tr><td>Gelatin</td><td>600 mg/L</td></tr>
<tr><td>Glucose</td><td>10,000 mg/L</td></tr>
<tr><td>Sodium Veronal</td><td>380 mg/L</td></tr>
<tr><td>Veronal</td><td>580 mg/L</td></tr>
</table></td><td>SBA diluent</td><td>Lonza
Catalog No. 10-539B</td></tr>
<tr><td>Triton X 100</td><td>Detergent to add to diluents, as 0.05% final concentration</td><td>Sigma T9284-100 mL
Lot 100M01281V</td></tr>
</table>

2.1.2 Microaerobic *Campylobacter* Gas

C. jejuni requires a microaerobic environment in which to survive, therefore all incubation periods must be carried out in this environment. Multi-well plates and MHP agar plates were placed in bags which were filled with *Campylobacter* gas then secured using elastic bands. The *Campylobacter* gas used by the *Campylobacter* Working Group is sourced from Airgas; reference number 90-116200870-3. The composition of this gas is shown below in Figure 2.1.

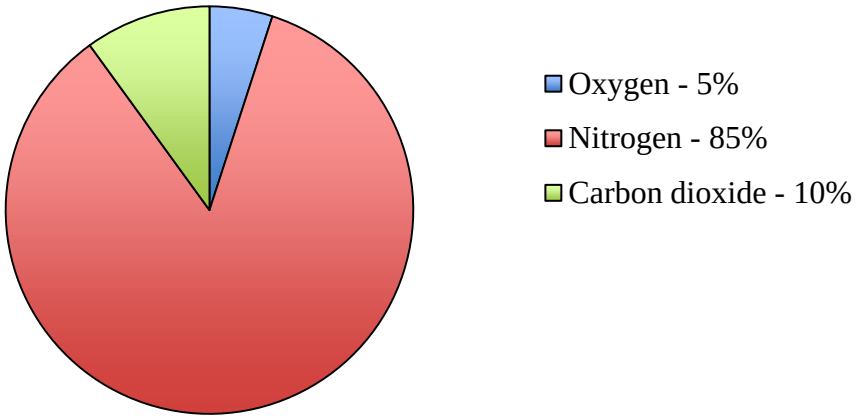


Figure 2.1 Microaerobic gas, by percentage composition
The pie-chart shows the percentage composition of each component that makes up *Campylobacter* gas.

2.2 SOURCES OF SERA

During the initial development of the SBA the serum from a hyper-immune NZWR vaccinated with the vaccine CJCV1 was used. When the method had been established the SBA was used to assess the response to vaccination over different time points in multiple subjects, thus looking for a titre change that may represent a correlate for protection. Three different types of sera were tested for a response; sera from NZWR enrolled in a CJCV1

safety study, sera from NHP *A. nancymaae* vaccinated with the vaccine CCV and human sera from subjects enrolled in a CJCV1 Phase 1 clinical trial.

2.2.1 Hyper-immune NZWR CJCV1 serum

This serum, stored at NMRC Enteric Diseases Department, Immunology Department, was obtained from the final bleed of a hyper-immune New Zealand White Rabbit, rabbit number 1905, following vaccination at the Harlan Laboratories, Maryland USA. The rabbit was vaccinated with the cGMP grade *C. jejuni* capsule-based conjugate vaccine CJCV1. This rabbit was deemed to be hyper-immune as it had demonstrated a strong response to vaccination as determined by ELISA. Having demonstrated a robust immune response it was considered an appropriate candidate to provide serum for initial assay development. As the final bleed was used there was sufficient serum to test different SBA conditions.

2.2.2 New Zealand Rabbit CJCV1 Safety Study Serum

This serum, from 60 NZWR, stored at NMRC Enteric Diseases Department, Immunology Department, was obtained on study days 0, 56 and 71 during the CJCV1 safety study, detailed above in section 1.4.1. This study sampled serum at three time points, over the course of which it was hoped a titre increase could be observed. Whole blood samples were centrifuged (1300 rpm for 10 minutes) within 1 hour of collection of each blood sample. The serum was stored under conditions set to maintain -75°C ± 15°C until shipped to the NMRC Immunology Laboratory where they were stored at -80°C until they were tested.

2.2.3 NHP A. nancymaae Serum

This serum, from 20 NHP, stored at NMRC Enteric Diseases Department, Immunology Department, was obtained during the *A. nancymaae* CCV dose escalation study, detailed in section 1.4.2. During this study blood was collected on days 0, 21, 42, 63, 84, 105, 125, 140, 147, 154, 161, and 168, centrifuged and stored as serum. For the purposes of SBAs the serum tested was collected on study days 0, 63 and 125, as these time points were anticipated to demonstrate titre increases.

2.2.4 Human serum, CJCV1 phase 1 study

This serum, from 48 study subjects, stored at NMRC Enteric Diseases Department, Immunology Department, was obtained during the clinical trial detailed in section 1.4.3. As part of the immunogenicity testing, the functional bactericidal titre was tested by SBA, and for the purpose for the SBA, serum collected on days 0 (pre-vaccination), 28 and 56 was used.

2.3 PREPARATION OF SERA

In order for functional activity to be present, a bacteriostatic or bactericidal element is required; antibodies or complement in the serum, or both, could achieve this effect. As the aim of the SBA was to determine the specific bactericidal activity of antibodies produced as a result of vaccination, the effect of endogenous complement had to be removed. Therefore, when the stock sample was thawed, prior to being tested in an SBA, a 100 µL aliquot was placed in a microfuge tube, then immersed in a 56°C water-bath for 30 minutes thus heat inactivating the endogenous complement.

To determine a change in functional bactericidal activity over the course of a vaccination schedule, serum from different time points can be tested for that individual subject. It is important that the same bacterial preparation is used for each time point, i.e. the serum drawn from each time point must be tested in the same SBA. This is important to limit variations in results due to alterations in behaviour of *C. jejuni* due to phase variation, as described above.

2.4 COMPLEMENT

For the purpose of a SBA, the source of complement needs to be exogenous and ideally naïve to exposure to *C. jejuni*. With this in mind, complement from 3-week old rabbits, baby rabbit complement (BRC), was the source chosen for the SBA. Studies evaluating the bactericidal activity of antibodies induced by meningococcal vaccines have shown that higher killing titres are achieved using rabbit complement rather than human complement, and the correlates for protection are interpreted according to the source of complement used [90]. A recognised variable in bactericidal function is the relative resistance or sensitivity of the organism to complement mediated killing, and this is difficult to control as it may be affected by, amongst other things, the phase of growth of the organism as well as composition and density of the capsule [122]. This may explain the different values for complement-only killing seen in replicates of a specific assay. Different sources and volumes of complement were tested prior to the purchase of a stock of Baby Rabbit complement (BRC). Different lots from Cedarlane (Lot Numbers 12057640, 11117628, 12067642, 09107594) and Pel-freez Biologicals (Lot Numbers 03935EL, 13035EL) were tested to find the source with the lowest non-specific complement killing effect. The source chosen was Cedarlane BRC, Lot Number 12067642. During the SBA development high levels of complement-only killing were observed, calculated as a percentage of *C. jejuni* CFU killed when compared to the control well

containing just *C. jejuni* in suspension, therefore further sources of human, rabbit and guinea pig complement were purchased, Table 2.2. Cedarlane BRC, Lot Number 12067642 remained the source with the lowest level of complement-only killing.

Table 2.2 Alternative complement sources tested:

Human	**Rabbit**	**Guinea Pig**
SIGMA Lyophilized complement Sera Human S1764- 1mL Lot: 100M01281V	CEDARLANE Low Tox Rabbit complement Lot Number 4547	SIGMA, Product Number S1639, Batch Number SLBG4298V
		CEDARLANE Hemo-lo Guinea Pig complement Lot Number 6166
Complement TECH INC Human complement as Normal Human Serum, Lot: 31	**CEDARLANE BRC Lot Number 12067642 – as used throughout SBA development**	Complement TECH INC, as Normal Guinea Pig Serum, Lot Number 4
		CEDARLANE Low Tox Guinea Pig complement Lot Number 2140

2.5 *C. JEJUNI* STRAINS AND PREPARATION

Three different strains of *C. jejuni* were used in this thesis, summarized in Table 2.3.

Table 2.3 *C. jejuni* Strains

***C. jejuni* Strain**	**Description**
81-176	Wild type, penner serotype HS23/36. This strain was used for the development of the SBA. The NMRC *Campylobacter* Research Group maintains stock vials of *C. jejuni* 81-176, these vials are maintained at -80°C in 15% Glycerol Brucella Broth (GBB) and the bacteria checked regularly for viability and resistance to Normal Human Sera. From each stock vial a high volume batch of cells can be grown then aliquotted and refrozen at -80°C for use in subsequent assays.
2875 Unflagellated	This strain was used specifically to test whether the presence of flagella affected the results. The strain is held by the NMRC *Campylobacter* Working Group.
CG8421	This strain was isolated from a 29 year-old male U.S. soldier deployed for training exercises in May 1999 in Nakhon Ratchisima (Khorat), Thailand. This is the strain used as the challenge inoculum for the rifaximin vs. placebo clinical trial. The strain is susceptible to fluoroquinolones and macrolides, and is probe-negative for NeuNAc genes, therefore unable to induce GBS through ganglioside mimicry. This *C. jejuni* challenge strain underwent cGMP production at Charles River Laboratories (358 Technology Drive, Malvern, PA 19355) in October 2006. A Master Cell Bank was prepared from the research seed. The cGMP cell bank was released after viability and purity checks and stored as 1 mL aliquots in 2 mL cryostorage tubes held – 80°C ± 10°C at the Walter Reed Army Institute of Research Pilot Bioproduction Facility.

The strain used for SBA development and evolution of the methodology was *C. jejuni* 81-176. Section 2.5.1 below describes the method of preparation of bacteria from a stock vial. This was necessary due to the limited number of stock vials available.

2.5.1 Method for the preparation of *C. jejuni* 81-176 from a stock vial

At room temperature defrost a *C. jejuni 81-176* stock vial of approximately 1 mL, distribute the bacteria equally between three Muller Hinton Plain (MHP) agar plates. Incubate the plates at 37^{o}C for 20 hours (+/- 1hour) under microaerobic conditions in *Campylobacter* gas. Using a sterile cotton swab, pass the growth from the three plates to nine plates, (three new plates for each original plate). Again, incubate at 37^{o}C for 20 hours (+/- 1hour) under microaerobic conditions. After the second period of incubation, using a sterile cotton swab, scrape the plates into 15% GBB. Using 15% GBB set the suspended cell solution to an optical density of 1.0, OD_{600} = 1.0 (1.00 – 1.09). Pipette 1 mL aliquots of the *C. jejuni* into freezer tubes and store at -80°C until use. For each SBA a new 1 mL vial of frozen *C. jejuni* was used. The method of preparation of *C. jejuni* for an SBA is detailed below, in Section 2.5.2.

2.5.2 Preparation of *C. jejuni* for an SBA; Initial Method

At room temperature defrost a vial of *C. jejuni* and distribute the bacterial solution between three MHP agar plates. Incubate the plates at 37^{o}C for 20 hours (+/- 1hour) under microaerobic conditions. Using a sterile cotton swab, scrape the growth into a 6 mL volume of Muller Hinton Broth (MHB) and check the OD_{600}, this reading will be the value of Z in the calculation below. Pipette the calculated volume of *C. jejuni* into a shaker flask containing 30

mL of MHB. The volume of *C. jejuni* to be inoculated into the 30 mL of MHB is determined using the calculation below:

$$X = \frac{30 \times Y}{Z}$$

Where:

X = the volume of *C. jejuni* to be inoculated into the flask of 30 mL MHB.

Y = the desired OD_{600} starting point. For an end OD_{600} of 1.0 the Y value is 0.03. This is a pre-established value.

Z = the OD_{600} reading of the *C. jejuni* solution.

Having added the bacteria to the MHB, loosely tape the lid of the shaker flask to the body of the shaker flask and seal in a microaerobic environment. Shake at 200 rpm, at 37ºC for 20 hours (+/- 1 hour). After shaking, remove 5 mL of the solution and transfer it to a 10 mL tube. Check the OD_{600} and if it is not at the required OD_{600} of 1.0 (1.00 – 1.09) then set it to this range using MHB. An OD_{600} of 1.0 gives ~ 2.7×10^9 organisms/mL. The required OD_{600} changed as the development of the assay evolved, the OD value used was either 1.0 or 0.1 as will be described.

During the development of the SBA the method of preparation for *C. jejuni* was revised with the intention of making the assay simpler and quicker, and therefore more likely to be performed on a wider scale. One of the refinements was removing the second 20-hour incubation period described above. The time 0 and 60 minute CFU counts of both preparation methods were comparable. The revised method is described below in Section 2.5.3

2.5.3 Preparation of *C. jejuni* for a SBA; Revised Method

At room temperature defrost a vial of *C. jejuni* and distribute the bacterial solution between three MHP agar plates. Incubate the plates at 37^{o}C for 20 hours (+/- 1hour) under microaerobic conditions. Scrape one of the plates into a 6 mL volume of MHB and check the OD_{600}. Using MHB and the remaining plates set the OD_{600} to the required value.

2.6 EVOLUTION OF SBA METHODS

Throughout the development of the SBA different method versions were examined, and the method evolved. Whilst the general format of the SBA method remained constant, in order to compare different conditions and optimize the method, various alterations were made such as diluent, number of bacteria, and percentage by volume of complement. However, alterations in conditions were not only limited to reagents, in order to streamline the assay with a view to making it more efficient, and therefore more likely to be used on a wider, or commercial, scale, elements such as cell culture and incubation were also modified. In the first version of the method the bacteria and serum were incubated together for 30 minutes prior to adding to complement. It was thought that this pre-exposure period was necessary however as the method evolved this step was removed. The details of these alterations are summarized in Table 2.4. The following sections detail the methods used during the evolution of the SBA.

2.6.1 Controls for determining CFU counts and bactericidal effect

For all SBA method versions ‘well-1’ in the multi-well plate is the bacteria-only control well, it contains *C. jejuni* bacteria without complement or serum. This is the control used to determine the complement-only killing effect. The second control well is ‘well-2’ which

contains *C. jejuni* with complement but no serum. This, with well-1, enables the complement-only killing effect to be established. Thereafter well-2 was the control for serum-mediated bactericidal killing due to vaccination.

2.6.2 Analysis of hyper-immune NZWR CJCV1 serum, SBA method version 1

C. jejuni 81-176 cells were prepared as per the initial method, section 2.5.2, setting the OD_{600} to 1.0. In 1.5 mL microfuge tubes, the following were incubated at room temperature for 30 minutes, this was considered to be 'pre-exposure' (total volume 300 μL);

Tube A: 30 μL *C. jejuni* 81-176 from an OD_{600} of 1.0; 270 μL MHB – this will be used for the control wells 1 and 2.

Additional Tubes: heat-treated serum - (the volume of serum will vary according to the volume to be tested); 30 μL *C. jejuni* 81-176 from an OD_{600} of 1.0 and MHB to make the volume up to 300 μL.

A 24 well plate was prepared, (total volume per well is 1000 μL). Well-1 did not contain serum or complement as it was the *C. jejuni* only control, well-2 did not contain serum as it was the control well for complement-only killing. To prepare the 24-well plate, wells contained MEM at room temperature, the volumes were 900 μl of MEM if complement was not to be added, or 850 μl if a 1:20 complement concentration was required, as 50 μl complement was added. Complement was defrosted and incubated on ice. When testing different concentrations of complement the volume of MEM was altered accordingly to ensure that the final volume remained 1000 μL. Lastly, *C. jejuni* +/- serum from the pre-exposure tubes was added, Tube A was for control wells 1 and 2. Before adding the bacteria, the pre-exposure microfuge tubes were vortexed and 100 μL removed, then added to the wells

Each well will have had a different volume of serum added to assess bactericidal effect at different volumes. The 24-well plate was shaken on a mechanical plate shaker at 2.5 rpm for 1 minute. 100 μL of the assay solution was removed from well-1, the *C. jejuni* only well, serially diluted 1:10 in MHB and plated in duplicate on MHP agar at chosen dilutions in order to determine time = 0 CFU counts. These MHP agar plates were incubated at 37°C for 48 hours under microaerobic conditions. The 24-well plate was incubated at 37°C under microaerobic conditions for set time point(s); 60 vs. 120 minutes incubation time was examined, a SBA determined 60 minutes was sufficient. After the 60 minute incubation period serial dilutions using 100 μL from each well were performed, and the chosen dilution plated in duplicate onto MHP agar plates then incubated at 37°C for 48 hours under microaerobic conditions. CFU on the plates were counted. The complement-only percentage killing was determined as well the serum bactericidal percentage killing.

2.6.3 Analysis of hyper-immune NZWR CJCV1 serum, SBA method version 2

C. jejuni 81-176 cells were prepared as per the initial method, section 2.5.2, setting the OD_{600} to 1.0. In a 96-well plate 30 μL *C. jejuni* was mixed with the volume of CJCV1 serum to be examined, the volume of complement appropriate to achieve the complement concentration being examined, and MEM. Well-1 contained MEM and *C. jejuni* only, well-2 contained MEM, *C. jejuni* and complement, and the other wells contained MEM, *C. jejuni*, complement and serum, however the volume of serum and complement concentration was altered according to the specific SBA conditions. To facilitate this the volume of MEM was altered to ensure the final volume remained 300 μL. Using a multi-channel pipette each well was mixed then. 100 μL was removed from well-1, the *C. jejuni* only well, and serially diluted 1:10 in MHB then plated in duplicate on MHP agar at chosen dilutions in order to determine

time = 0 CFU counts, the two MHP plates were incubated at 37°C for 48 hours under microaerobic conditions. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes. After the 60 minute incubation period 100 μL of each well was serially diluted and plated in duplicate onto MHP agar plates, then incubated at 37°C for 48 hours under microaerobic conditions. CFU on the plates were counted. The complement-only percentage killing was determined as well the serum bactericidal percentage killing.

2.6.4 Analysis of hyper-immune NZWR CJCV1 serum, SBA method version 2.1

This method differed from method version 2 only in the size of the plate; a 24-well plate was used.

2.6.5 Analysis of hyper-immune NZWR CJCV1 serum, SBA method version 3

This method differed from method version 2 only in the number of *C. jejuni* bacteria used. *C. jejuni* 81-176 cells were prepared as per the initial method, section 2.5.2, setting the OD_{600} to 1.0 then the *C. jejuni* suspension was serially diluted 1:10 (100 μL cells into 900 μL MHB) 4 times. Either the 3rd or 4th dilutions were added to the wells in the 96-well plate, the volume added was 30 μL.

2.6.6 Analysis of hyper-immune NZWR CJCV1 serum, SBA method version 4

This method differed from method version 2 in the following ways: size of the plate; preparation of *C. jejuni* and type of diluent. A 48-well plate was used to allow 300 μL to easily fit in the well whilst still using the 2.5 rpm shaker, which is not used for the 96-well plate. The revised method of *C. jejuni* preparation was used, section 2.5.3, and the diluent used was DGV.

C. jejuni 81-176 cells were prepared as per the revised method, section 2.5.3, setting the OD_{600} to 1.0. In a 48-well plate 30 μL *C. jejuni* was mixed with the volume of CJCV1 serum to be examined, the volume of complement to achieve the complement concentration being examined, and DGV. Well-1 contained DGV and *C. jejuni* only, well-2 contained DGV, *C. jejuni* and complement, and the other wells contained DGV, *C. jejuni*, complement and serum, however the volume of serum and complement concentration was altered according to the SBA conditions. To facilitate this the volume of DGV was altered to ensure the final volume was 300 μL. Using a multi-channel pipette, each well was mixed. 100 μL was removed from well-1, the *C. jejuni* only well, serially diluted 1:10 in DGV and plated in duplicate on MHP agar at chosen dilutions in order to determine time = 0 CFU counts, then the two MHP plates were incubated at 37°C for 48 hours under microaerobic conditions. The 48-well plate was incubated at 37°C under microaerobic conditions for 60 minutes. After the 60 minute incubation period 100μL of each well was serially diluted, and plated in duplicate onto MHP agar plates, then incubated at 37°C for 48 hours under microaerobic conditions. CFU on the plates were counted. The complement-only percentage killing was determined as well the serum bactericidal percentage killing.

2.6.7 Statistical Analysis

For many of the development steps of the SBA replicates were not possible due to limited resources, therefore whilst trends could be observed, formal statistics were not determined. Where replicates were performed error bars are shown which illustrate standard error of the mean. Prism software was used to determine a p-value. For comparison of two groups, if the data was considered parametric then either a paired t-test or unpaired t-test was used, dependent upon the data. If the data was considered non-parametric then a Mann-Whitney

test was used. For comparison of more than one group, if the data was considered parametric then a one-way ANOVA was used, and if considered to be non-parametric then a Kruskal-Wallis test was used. This was also the case for the NZWR, NHP and human SBA data.

Table 2.4 Summary of alterations in conditions between method versions

	Version 1	Version 2	Version 2.1	Version 3	Version 4
Multi-well plate size (24/48/96)	24, final volume 1000 μL	96, final volume 300 μL	24, final volume 300 μL	96, final volume 300 μL	48 or 96, final volume 300 μL
C. jejuni preparation	Initial method section 2.5.2	Initial method	Initial method	Initial method	Revised method Section 2.5.3
30 minute Pre –exposure period	Yes	No	No	No	No
Diluent	MEM or PBS	MEM or PBS	MEM or PBS	MEM	DGV
Number of *C. jejuni* cells used	1 x 10^8 into the pre-incubation tube, 3 x 10^7 into the 24 well plate	1 x 10^7	1 x 10^7	9 x 10^4 or 9 x 10^3 into the wells of the plate	1 x 10^7 When specified in the results section, 1 x 10^5 (3^{rd} dilution) and 1 x 10^4 (4^{th} dilution) were used

2.7 SBA TO DETERMINE THE 50% KILLING TITRE, NZWR CJCV1 SAFETY STUDY

Having developed a SBA method, serum from the New Zealand Rabbit CJCV1 safety study could be tested.

The *C. jejuni* was prepared as per Section 2.5.3. The OD_{600} of the *C. jejuni* solution was set to 0.1 (0.1 – 0.109) in DGV. The serum to be tested was immersed in a water bath at 56^{o}C for 30 minutes, then incubated on ice. The complement was defrosted and incubated on ice. The serum was diluted making 3-fold serum dilutions; starting with 30 μL of serum in 60 μL DGV, then serially diluted 30 μL into 60 μL, giving a pre-bleed dilution range from 1:30 to 1:810 (reciprocal titres 30 – 810) and a post-vaccination dilution range from 1:30 to 1:2,430 (reciprocal titre 30 – 2,430). For identification purposes the pre-vaccination serum dilutions were labelled 1-4, the Day-56 post-vaccination serum dilutions were labelled A-E, and the Day-71 post-vaccination serum dilutions were labelled AA-EE, Table 2.5. A 96-well plate was prepared using the template shown in Table 2.6.

In order to check the Time = 0 status of the *C. jejuni,* the solution in well-1 was used. The contents of the well were pipetted to mix, then 100 μL from well-1 was removed and added to a microfuge tube containing 900 μL PBS with 0.05% Triton X 100, this was serially diluted at 1:10 dilutions 4 times. 100 μL of the 3rd and 4th dilutions of well-1 was plated onto MHP agar plates in duplicate at T=0; the plates were incubated for 48 hours at 37^{o}C, under microaerobic conditions, then the CFU were counted. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes. It was then pipetted up and down to mix, and 100 μL

from wells 1-16 was removed and added to a microfuge tube containing 900 μL PBS with 0.05% Triton X 100. This was serially diluted at 1:10 dilutions 4 times. In duplicate, 100 μL of microfuge tubes at dilutions #2, #3, #4 was plated onto MHP agar plates, incubated for 48 hours microaerobically at 37ºC, and the CFU counted to calculate the % killing for the complement-only well as well as each serum titre, and the highest titre to achieve 50% killing was established.

Table 2.5 NZWR serial serum dilutions and titres

Pre-vaccination Dilutions	**Day 56 Dilutions**	**Day 71 Dilutions**	**Volume of Sera in SBA after Dilution**	**Titre**
1	A	AA	10 μL	30
2	B	BB	3.3 μL	90
3	C	CC	1.1 μL	270
4	D	DD	0.37 μL	810
-	E	EE	0.12 μL	2,430

Table 2.6 96-well plate template used to test the NZWR Safety Study Sera

Well number:	1	2	3	4	5	6	7	8
Description of well	81-176 only	Complement-only	pre-vaccination Titre 30	pre-vaccination Titre 90	pre-vaccination Titre 270	pre-vaccination Titre 810	Day-56 post-vaccination Titre 30	Day-56 post-vaccination Titre 90
DGV (µL)	270	215	185	185	185	185	185	185
Complement (18%) (µL)	-	55	55	55	55	55	55	55
Serum: 30 µL of the diluted serum solution	-	-	30 µL of 1	30 µL of 2	30 µL of 3	30 µL of 4	30 µL of A	30 µL of B
81-176 (µL), OD= 0.1	30	30	30	30	30	30	30	30

Well number:	9	10	11	12	13	14	15	16
Description of well	Day-56 post-vaccination Titre 270	Day-56 post-vaccination Titre 810	Day-56 post-vaccination Titre 2430	Day-71 post-vaccination Titre 30	Day-71 post-vaccination Titre 90	Day-71 post-vaccination Titre 270	Day-71 post-vaccination Titre 810	Day-71 post-vaccination Titre 2430
DGV (µL)	185	185	185	185	185	185	185	185
Complement (18%) (µL)	55	55	55	55	55	55	55	55
Serum – 30 µL of the diluted serum solution	30 µL of C	30 µL of D	30 µL of E	30 µL of AA	30 µL of BB	30 µL of CC	30 µL of DD	30 µL of EE
81-176 (µL), OD= 0.1	30	30	30	30	30	30	30	30

2.8 SBA TO DETERMINE THE 50% KILLING TITRE, *A. NANCYMAAE* AND HUMAN SERUM

The SBA developed for the NZWR sera was very labour and material intensive, so given the rather limited volumes of *A. nancymaae* and human serum available, further refinement was necessary. The method was therefore revised and is described below.

C. jejuni was prepared as per Section 2.5.3, the revised method. Serum was immersed in a water bath at 56ºC for 30 minutes and then incubated on ice. Complement was defrosted and incubated on ice. A DGV/complement mix of 2700 µL of DGV and 800 µL of complement was prepared in a 10 mL tube, 70 µL of this mix in a total volume of 100 µL will give a 16% complement concentration. 90 µL DGV was added to well-1 and 70 µL of the DVG/complement mix was added to the other wells of the 96-well plate. Serial 2-fold serum dilutions for each of the three serum time points were prepared, giving a titre range of 1:10 – 1:640 for the pre-vaccination samples, and 1:10 – 1:20480 for the post-vaccination serum samples. 20 µL of each serum dilution was added into its specific well, in well-2 that did not contain serum, an additional 20 µL of DGV was added, Table 2.7.

Using a sterile cotton swab, *C. jejuni* 81-176 cells were scraped into 5 mL of DGV and the $OD_{600,}$ was set to OD_{600} of 0.1 (0.095 – 0.105). 100 µL of *C. jejuni* cells (at OD_{600} 0.1) were serially diluted into 900 µL of DGV 5 times. 10 µL of the 3rd *C. jejuni* dilution was added to the wells and pipetted to mix. 25 µL of the 4th and 5th dilutions of *C. jejuni* were plated onto MHP agar plates in duplicate for T=0 time-point checks then incubated at 37°C under microaerobic conditions for 48 hours. The 96-well plate was incubated at 37ºC under

microaerobic conditions for 1 hour. 25 µL from each well was plated in duplicate onto MHP agar plates, which were incubated at 37ºC under microaerobic conditions for 48 hours. The CFUs were counted and the % killing calculated using the complement-only well as the control. In addition the complement-only killing effect was calculated by comparing the complement-only well with the *C. jejuni*-only negative control well.

In addition to using this method for the NHP and human serum, some New Zealand White Rabbit CJCV1 safety study sera were re-tested using this method.

Table 2.7 96-well plate template used to test the NHP and Human Sera

Well number:	1	2	3 – 10	11 – 20	21 – 30
Description of well	81-176 only	Complement-only	Pre-vaccination Titres 20 – 1280	Day-63 for NHP Day-28 for human Titres 40 – 20480	Day-125 for NHP Day-56 for human Titres 40 – 20480
DGV (μL)	90	20	0	0	0
DGV/Complement mix (μL)	-	70	70	70	70
Serum (μL)	-	-	20	20	20
81-176 (μL) set to OD= 0.1, add bacteria diluted to 10^{-3}	10	10	10	10	10

2.9 CLINICAL TRIAL 'RIFAXIMIN VS. PLACEBO FOR THE PREVENTION OF CAMPYLOBACTERIOSIS'

With regard to the materials and methods used in the Clinical Trial 'Rifaximin vs. placebo for the prevention of Campylobacteriosis', the Study Specific Procedures (SSP) detailing the following methodology are detailed as appendices;

1. The preparation of the challenge strain inoculum – Appendix 6
2. The processing of stool samples – Appendix 7

For this trial all growth media, diluents and equipment were provided on a commercial contract by The Johns Hopkins School of Public Health who facilitated the preparation of the challenge inoculum, as well as the majority of the stool processing, and therefore further information regarding sourcing is not detailed in this thesis. The source of the challenge inoculum bacteria, *C. jejuni* strain CG8421, is detailed in section 2.5.

CHAPTER 3 RESULTS FOR OBJECTIVE 1 – TO DEVELOP A SERUM BACTERICIDAL ASSAY CAPABLE OF DEMONSTRATING, BY TITRE, THE FUNCTIONAL BACTERICIDAL EFFECT OF ANTIBODIES FOLLOWING *C. JEJUNI* VACCINATION

3.1 INTRODUCTION

The first objective of this thesis is to develop a SBA capable of demonstrating, by titre, the functional bactericidal effect of antibodies following *C. jejuni* vaccination. The SBA is required to test the functional bactericidal activity of individual serum samples at different time-points and so determine whether or not functional activity increases over the course of a vaccination schedule. Demonstration of a functional bactericidal effect may lead to establishing a correlate for protective immunity as discussed in Chapter 1. The sera from the vaccinated NZWR, *A. nancymaae* NHP and human subjects, described in the previous chapters, was tested. Whilst enzyme-linked immunosorbant assay (ELISA) results for CPS IgG had previously been established for the serum samples as part of the vaccination studies, these results do not necessarily infer a level of functional bactericidal activity. In addition, for the NHP that were vaccinated then challenged, it was hoped that there would be a significant correlation between the post vaccination titre, either as a titre or fold-increase in titre when compared to the baseline titre, and whether or not the animal was protected when challenged.

The functional bactericidal activity will be determined by the highest serum titre capable of demonstrating a killing effect of ≥50% using the SBA method. Changes in 50% killing titre after each vaccination may be observed. The titre recorded is the reciprocal titre of the highest serum dilution that achieves ≥50% killing, for example, if the highest serum dilution to achieve ≥50% killing is a 1:810 serum dilution, then the titre will be recorded as 810.

Only a small volume of sera was available for each subject in the NZWR safety study, NHP study and human study. Rather than use this serum in the development of an assay, it was reserved, to be tested only when the optimal SBA conditions had been established. Therefore, for the initial development and evolution of the optimal SBA conditions, and whilst establishing the method for the SBA, serum from a NZWR that had also been immunized using the cGMP grade vaccine CJCV1 was used. As the serum from this rabbit was from a final bleed, there was an adequate supply to facilitate testing different SBA conditions, however not enough to perform replicates of all the assays. This rabbit had demonstrated a robust immune response by ELISA, and was referred to as 'hyper-immune'.

3.2 INITIAL DEVELOPMENT STAGES OF THE SBA

The serum available from the hyper-immune NZWR was from the final bleed, serum from serial pre- and post-vaccination time points was not available. However, as this serum was to be used solely to develop a robust SBA method, serial time points were not required at this stage. Therefore with the method development assays, changes in functional bactericidal activity over time are not examined. As described above, serum quantity was limited, so in many cases replicates for each assay were not performed, and therefore for these assays error bars are not shown. Whilst statistical data analysis cannot be performed in these instances, trends can be

observed. This is discussed further in the limitations section. Towards the end of the SBA method development, when a successful method had been established, replicates were performed, and error bars showing the standard error of the mean (SEM) are shown for these replicates. Whilst it would have been best practice to perform replicates of assays for each stage of the method development, with the limited resources available this was not feasible.

3.2.1 Establishing an incubation time

In order for an SBA to facilitate killing, the reagents must be incubated together. The purpose of this assay was to examine whether both a 60-minute and 120-minute incubation period would facilitate killing. The relevance of this is that if the SBA is to be used on a large scale then it must be as time and resource efficient as possible. The assay used method version 1, as detailed in Section 2.6.2. For this assay a 5% complement concentration was used, i.e. 5% of the total well volume was complement. 10 μl of serum was added to the pre-exposure tube. Results are shown in Figure 3.1. The incubation periods demonstrated different % killing effects, but both incubation periods facilitated bactericidal killing. The complement-only killing effects were 39% following a 60-minute incubation period and 23% following a 120-minute incubation period. With the addition of serum, both incubation periods demonstrated bactericidal activity, 70% and 82% respectively. Error bars are not shown as replicates were not performed. Having established that the incubation time of 60 minutes was sufficient to facilitate a bactericidal effect, other conditions were investigated.

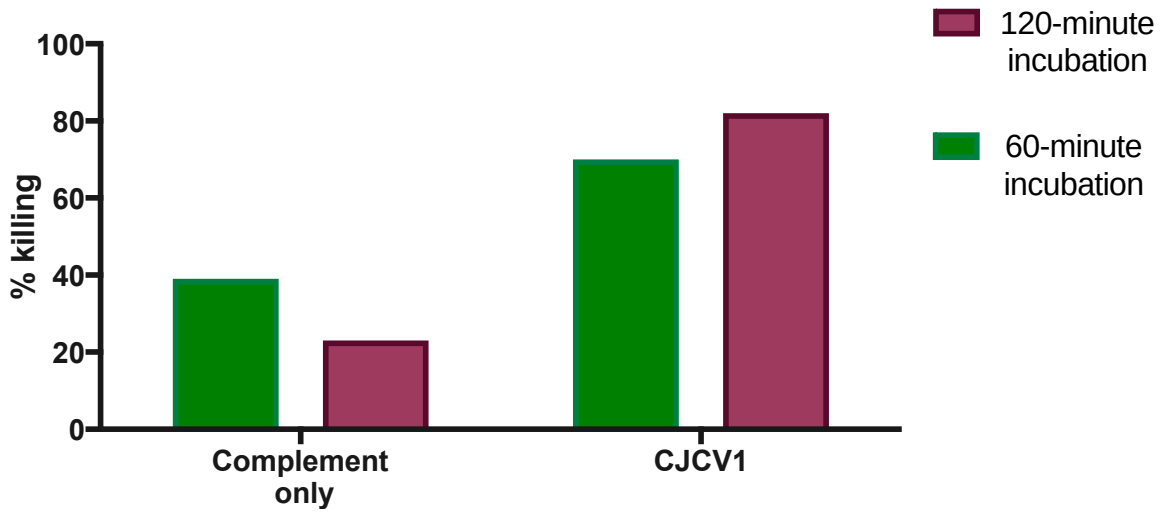


Figure 3.1 Bactericidal activity was achieved following both 60 and 120-minute incubation periods.

C. jejuni, 1 x 10^8 CFU, was incubated with, or without, 10 µl of serum in MHB for 30 minutes in pre-exposure tubes. From the pre-exposure tubes 100 µl was added to complement in MEM giving a final complement concentration of 5% in the 24-well plate (method version 1 described in table 2.4). Well-2 of the 24-well plate contained *C. jejuni* without serum. The 24-well plate was incubated at 37°C under microaerobic conditions, both 60-minute and 120-minute incubation periods were examined. The percentage killing was established after plating and 48 hour incubation at 37°C under microaerobic conditions.

3.2.2 Establishing the serum bactericidal activity in the presence of 2.5% - 1.25% complement concentrations

Having examined the killing effect of 5% complement in the above assay, the SBA was repeated using lower concentrations of complement, by percentage of final well volume (1000 µl); 2.5% (25 µl); 2% (20 µl) and 1.25% (12.5 µl). The method used was version 1 and 10 µL of CJCV1 serum was used, with an incubation period of 60 minutes. The SBA was repeated giving two replicates, and therefore error bars showing the standard error of the mean are included in the results, Figure 3.2.

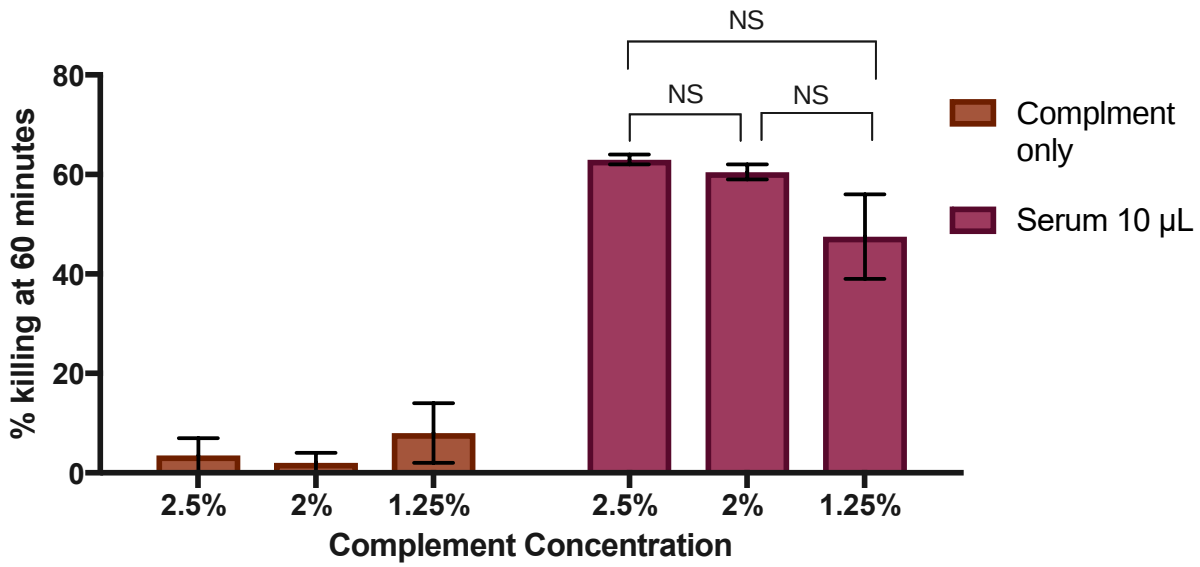


Figure 3.2 Reducing the complement concentration from 2.5% to 1.25% does not significantly reduce serum bactericidal activity

C. jejuni, 1 x 10^8 CFU, was incubated with, and without, 10 µl of serum in MHB for 30 minutes in pre-exposure tubes. From the pre-exposure tubes 100 µl was added to 2.5%, 2% and 1.25% complement concentrations in MEM in the 24-well plate (method version 1 described in table 2.4). The 24-well plate was incubated at 37°C under microaerobic conditions. Complement only killing at each complement concentration as well as bactericidal activity was calculated.

These results illustrate the observation that as the concentration of complement reduces, the serum bactericidal killing also reduces. However the decrease in serum mediated bactericidal killing in the presence of these different complement concentrations did not reach statistical significance, at 2.5% vs. 2% complement (paired t-test p = 0.50), 2% vs. 1.25% complement (paired t-test p = 0.42) or 2.5% vs. 1.25% complement (paired t-test p = 0.29). This may be due to insufficient numbers of replicates, or the low complement concentrations. Error bars show the SEM.

This observation, albeit not reaching statistical significance, is not unexpected as bactericidal function is complement mediated, and therefore a higher concentration of complement should induce a higher percentage of complement-mediated killing. This observation suggests that in

order for a SBA to be used for determination of a correlate or surrogate for protection, the concentration of complement used must be standardized.

3.2.3 The effect of 10-fold increases in serum volume upon bactericidal activity

Ultimately serum was to be tested at different volumes, as titres. Therefore 10-fold increases in serum volumes were tested; 0.1 μL, 1.0 μL and 10 μL in the 1 mL 30 minute pre-exposure tube. In addition, different complement concentrations were tested; 20% (200 μL), 10% (100 μL), 5% (50 μL) and 2.5% (25 μL), the volume in brackets indicating the volume of complement added to the well. This facilitated not only the comparison of different serum volumes with varying complement concentrations, Figures 3.3 and 3.4, but also the complement-only killing effect of different complement concentrations, Figure 3.5. Replicates were not performed therefore error bars are not shown. This assay demonstrates, in Figure 3.3, the trend that as complement concentrations reduce, so too does the % killing effect of serum, and this trend remains, despite 10-fold differences in serum volume. The same data presented slightly differently in Figure 3.4 illustrates that as the volume of serum is reduced, the percentage killing effect also decreases. This suggests that the conditions of the SBA at this stage of development are able to discern a difference in the bactericidal effect of different volumes of serum, suggesting a difference between titres could be observed. Figure 3.5 illustrates a high level of complement-only killing when using a 20% complement concentration. This killing effect halves when the complement concentration is 10%, and suggests that the optimal complement concentration should be <20% of the final volume.

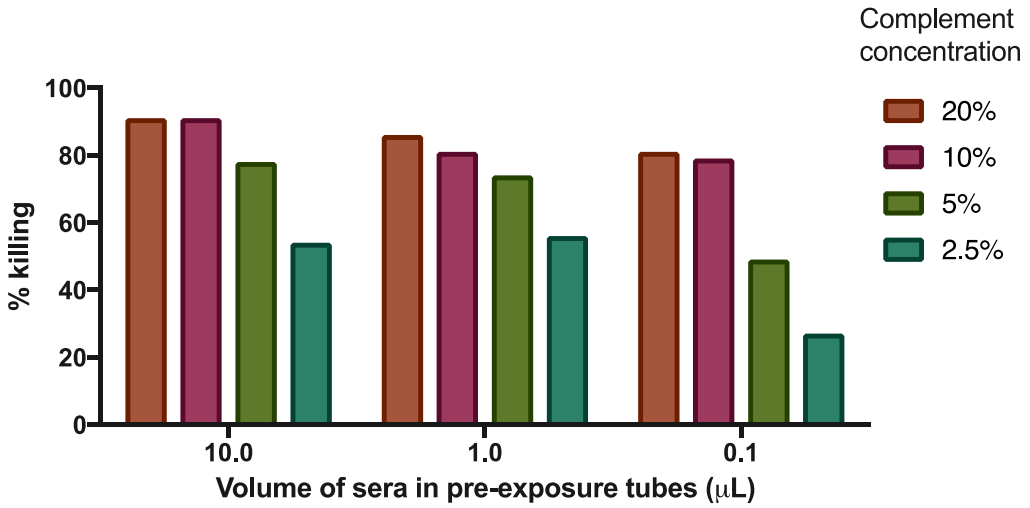


Figure 3.3 Despite 10-fold differences in serum volume, similar trends in % killing using different complement concentrations are observed

C. jejuni, 1 x 10^8 CFU, was incubated with, and without, serum. The volumes of serum added to the pre-exposure tubes were 0.1, 1.0 and 10 µl. From the pre-exposure tubes 100 µl was added to complement in MEM, giving final complement concentrations of 20%, 10%, 5% or 2.5% in the 24-well plate (method version 1 described in table 2.4). The 24-well plate was incubated at 37°C under microaerobic conditions then plated. Percentage killing at each complement concentration and serum volume was calculated. This Figure groups data by serum volume.

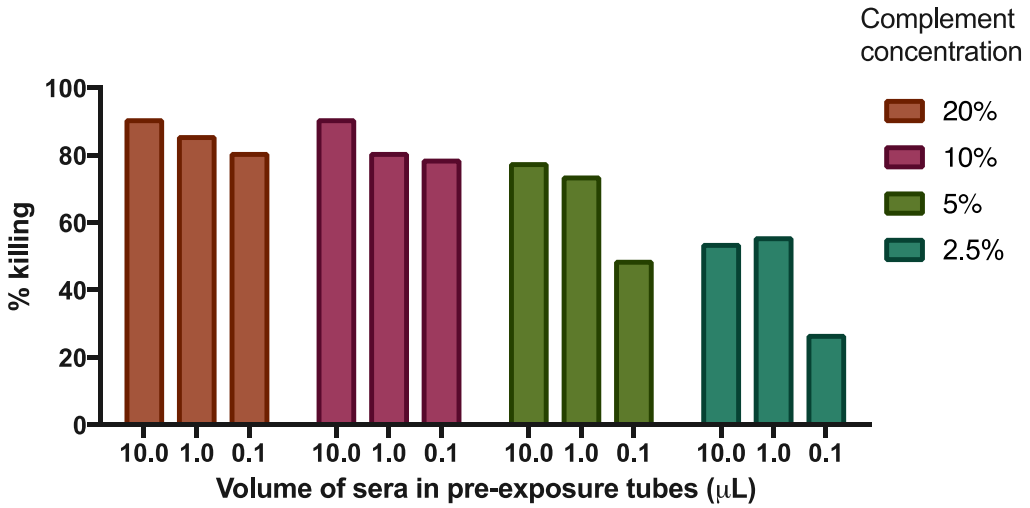


Figure 3.4 Percentage killing decreases as serum volume decreases

C. jejuni, 1 x 10^8 CFU, was incubated with, and without, serum. The volumes of serum added to the pre-exposure tubes were 0.1, 1.0 and 10 µl. From the pre-exposure tubes 100 µl was added to complement in MEM, giving final complement concentrations of 20%, 10%, 5% or 2.5% in the 24-well plate (method version 1 described in Table 2.4). The 24-well plate was incubated at 37°C under microaerobic conditions then plated. Percentage killing at each complement concentration and serum volume was calculated. This Figure groups data by complement concentration.

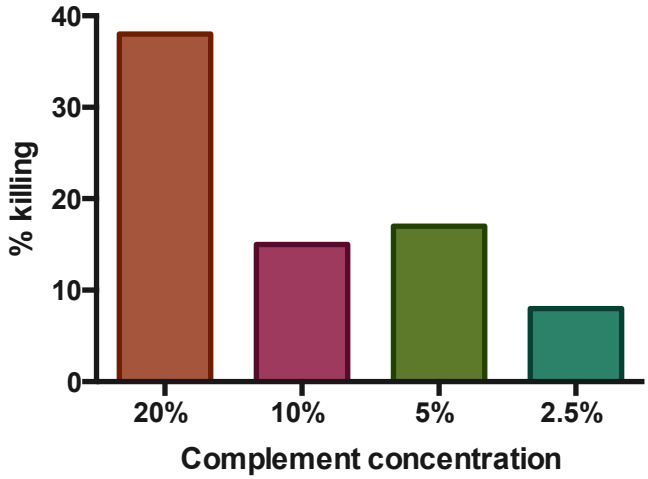


Figure 3.5 The complement-only killing effect reduces with a reduction in the complement concentration below 20%

C. jejuni, 1 x 10^8 CFU, was incubated with, and without, serum. The volumes of serum added to the pre-exposure tubes were 0.1, 1.0 and 10 µl. From the pre-exposure tubes 100 µl was added to complement in MEM, giving final complement concentrations of 20%, 10%, 5% or 2.5% in the 24-well plate (method version 1 described in table 2.4). The 24-well plate was incubated at 37°C under microaerobic conditions then plated. Percentage killing at each complement concentration was calculated.

Using the data above, when comparing the bactericidal function of the three different serum volumes, 0.1, 1.0 and 10 µL in the presence of a 20% and 10% complement concentration, the bactericidal effect (% killing) with a 20% complement is similar to that when using 10% complement, Figures 3.3 and 3.4. This is despite the complement-only killing effect being much higher at a 20% rather than a 10% complement concentration, Figure 3.5. This suggests that for these volumes of serum, bactericidal function is maintained despite the drop from 20% to 10% complement concentration. The optimal complement concentration for evolution of the assay, to allow for a robust bactericidal effect and without a high complement-only killing effect was considered to be <20% concentration.

The aspiration for future assays was to use a 96-well plate rather than a 24-well plate. This was to allow:

- A reduction in the volume of reagents required
- The ability to perform multiple time points, each with serially diluted titres, at each iteration of the assay

Therefore the method was altered, to version 2. There are notable differences in method between method versions 1 and 2; summarized in Table 3.1.

Table 3.1 Comparison of method differences; version 1 vs. version 2

Detail of method difference	Version 1	Version 2
Final Volume in well, μL	1000	300
Size of plate	24	96
Volume of CJCV1 Serum, μL (% of final volume)	10 in the pre-exposure tube, 3.3 (0.33) in the well	10 (3.33) in the well
Volume of Complement, μL (% of final volume)	100 (10)	30 (10)
Number of *C. jejuni* CFU	1×10^8 into the pre-exposure tube, 3×10^7 into the well	1×10^7 in the well
30 minute incubation period – serum and *C. jejuni*	Yes	No

In version 1 an equivalent of 3.3 μL of serum was added to 100 μL of complement (1:30 ratio) in each well of the plate, the serum had been incubated with the *C. jejuni* for 30 minutes prior to adding the complement, whereas in version 2, 10 μL of serum is added to 30 μL of complement (1:3 ratio), with no early incubation, pre-exposure, period.

The alterations were made as it was anticipated that they would make the assay not only more efficient in terms of materials and time, but also simpler to perform. It was also hoped that by removing the early incubation period and combining all the reagents together from the start, potential technical discrepancies when pipetting and vortexing could be avoided. In an attempt validate this change an assay comparing the different method versions was carried out. Given the alterations that had been made it was expected that there would be significant variation in results.

3.2.4 Comparison of the SBA method, version 1 vs. version 2

This assay was performed in order to establish whether the results of the two method versions were comparable. For each SBA 10 μL of CJCV1 serum and a 10% complement concentration were used, the following aspects were compared.

- Method version 1 vs. version 2 (differences detailed in Table 3.1).
- The assays were performed using MEM and also PBS as diluents.

Irrespective of the type of diluent used, the % killing effect was higher using method version 1. It could be that the 30 minute early incubation period in method version 1 resulted in a higher killing effect, or that it was due to the higher ratio of complement to serum in this method, despite the concentration of complement in the well being 10% for both versions.

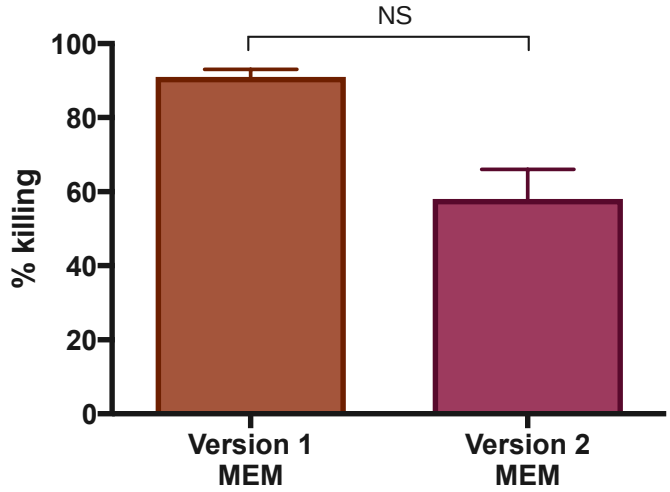


Figure 3.6 No significant difference in % killing is demonstrated between method versions 1 and 2 when using the diluent MEM

Method versions 1 and 2 were compared. Version 1 - *C. jejuni*, 1 x 10^8 CFU, was incubated with, and without, 10 µl of serum. From the pre-exposure tubes 100 µl was added to MEM containing complement, the final volume giving a 10% complement concentrations. The 24-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then plated and incubated for a further 48 hours at 37°C under microaerobic conditions. Version 2 – In a 96-well plate *C. jejuni*, 1 x 10^7 was added to MEM and complement, the final volume giving a 10% complement concentration, and the non-control wells contained 10 µl of serum. Incubation times and conditions were the same.

The difference in percentage killing between the method versions 1 and 2, using MEM, is not significant (unpaired t-test, p = 0.057). Two replicates were performed; error bars show the standard error around the mean. This result may not have reached statistical significance as only 2 replicates were performed, therefore a type 2 error may exist, and this is discussed in the limitations section.

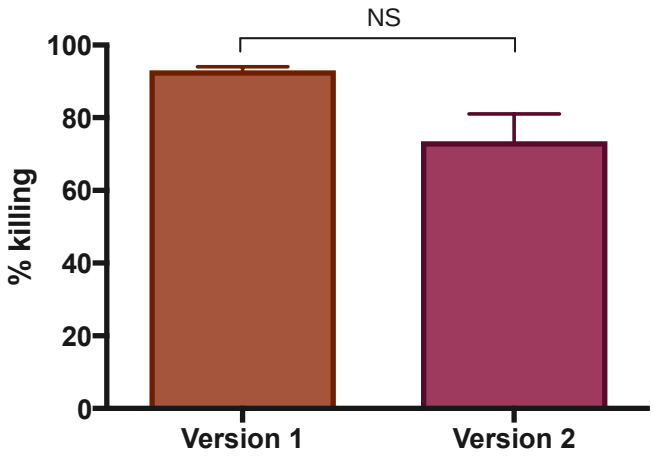


Figure 3.7 No significant difference in % killing is demonstrated between method versions 1 and 2 when using the diluent PBS

Method versions 1 and 2 were compared. The conditions were identical to that of Figure 3.6, however PBS was used instead of MEM as a diluent.

The difference in percentage killing between the method versions 1 and 2, using PBS, is not significant (unpaired t-test, $p = 0.123$). Two replicates were performed; error bars show the standard error around the mean. As with Figure 3.6, this result may not have reached statistical significance as only 2 replicates were performed, therefore a type 2 error may exist, and this is discussed in the limitations section.

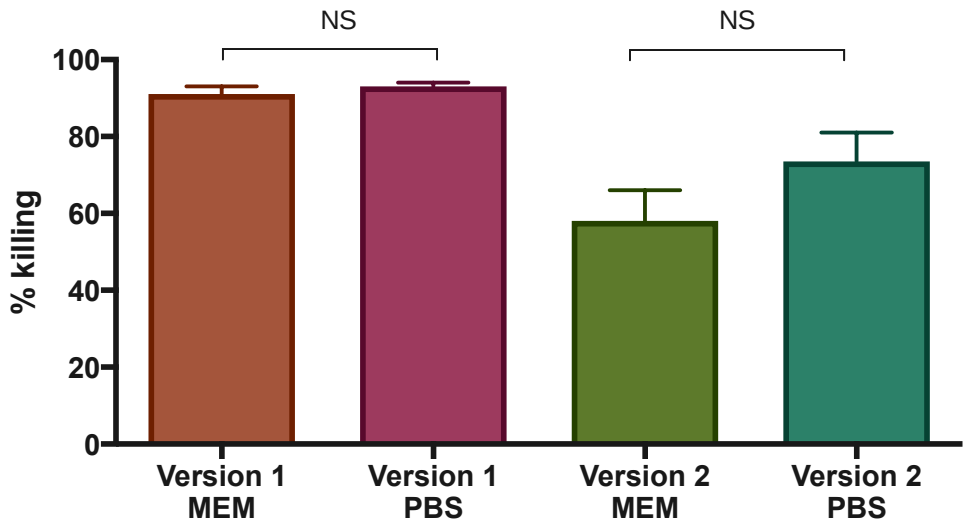


Figure 3.8 No significant difference in % killing using diluents MEM vs. PBS

The data from figures 3.6 and 3.7 are combined in this Figure to illustrate the differences in serum bactericidal effect using different method versions and specifically the different diluents MEM and PBS.

There is no significant difference in the % killing for method version 1 when using the different diluents MEM vs. PBS, unpaired t-test p = 0.47. Similarly there is no significant difference in the % killing for method version 2 when using the different diluents MEM vs. PBS, unpaired t-test p = 0.29. Two replicates were performed; error bars show the standard error around the mean.

There are observable differences between method versions 1 and 2, however a statistically significant difference was not achieved. This is possibly due to limited numbers of replicates. As the aspiration was that the assay would be carried out in a 96-well plate in order to facilitate testing many different samples, and titre levels, method version 2 was pursued, as when using the 96-well method, method version 2, there was still a demonstrable functional killing effect. Having decided to evolve to method version 2, it was required to re-establish the optimal complement concentration capable of facilitating high specific killing (serum dependent) with low non-specific killing (complement-only). Therefore a comparison of different complement concentrations was performed, testing the following concentrations; 1:5 (20%), 1:10 (10%), 1:20 (5%) and 1:40

(2.5%), in the presence of 10 µL of serum, Figure 3.9. Replicates were not performed therefore there are no error bars.

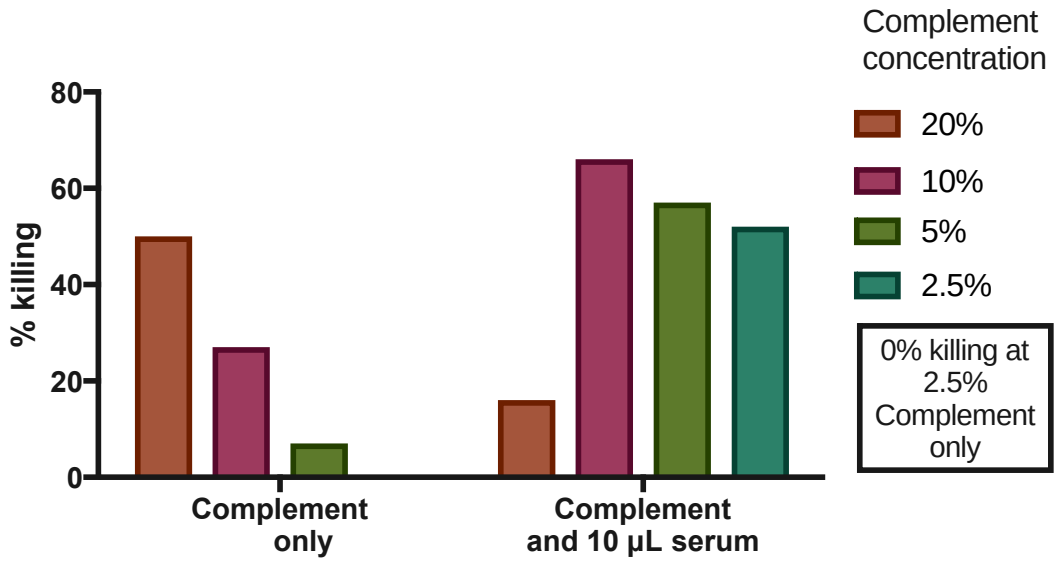


Figure 3.9 Method version 2, 10% complement concentration appears optimal
In a 96-well plate *C. jejuni*, 1 x 10^7 CFU was added to MEM and complement, the final volume giving different complement concentrations; 20%, 10%, 5% and 2.5% To all wells, other than the control wells, 10 µl of serum was added. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

At a 20% complement concentration, there is a high complement-only killing effect. The low % killing seen with 20% complement and serum was unexpected. A 10% complement concentration allows a high serum bactericidal effect whilst keeping the complement-only killing effect low, similar to Figures 3.3 – 3.5, which is the desired outcome.

Method version 2 demonstrated a bactericidal effect, with trends similar to the trends seen with method version 1. Therefore development of method version 2 continued, as it was anticipated that future SBAs would use a 96-well plate method.

3.2.5 Comparison of % killing of different serum volumes at different complement concentrations

Using method version 2 this assay tested serum volumes of 1, 10, 30 and 60 µL in the presence of a 10% or 20% complement concentration. The assay was performed using the diluents MEM and PBS. The results, Figure 3.10, were unexpected; there was an inverse % killing effect; as the volume of serum decreased the % killing increased. Replicates were not performed therefore error bars are not shown.

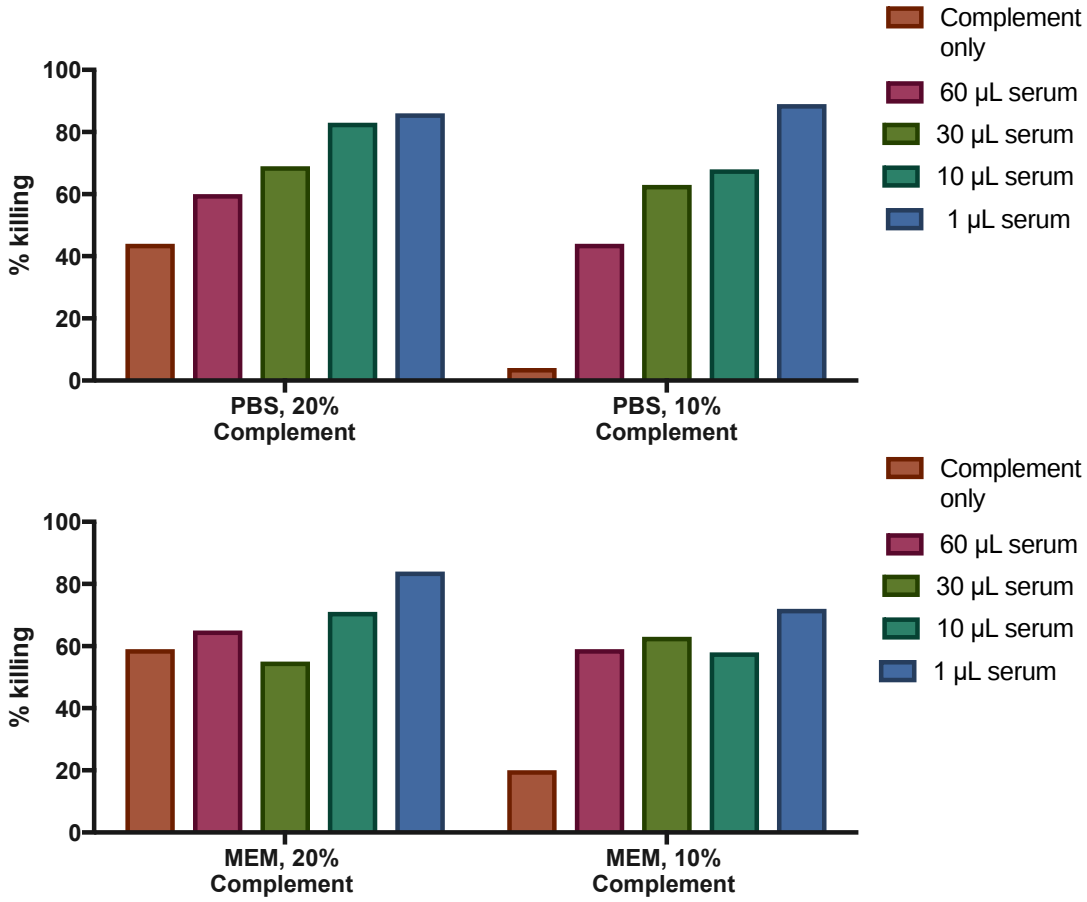


Figure 3.10 Decreasing serum volumes result in a trend of increasing % killing

In a 96-well plate *C. jejuni,* 1 x 10^7 CFU was added to MEM or PBS and complement, the final volume giving a complement concentrations of 20% or 10%. Different volumes of serum were added to the non-control wells; 1, 10, 30 or 60 µL. The plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents were plated in duplicate.

This assay demonstrated an inverse trend to that expected, as the serum volume decreased, the % killing increased. This was the first occasion during this SBA development that such an inverse trend was seen, of note this assay was also the first SBA that used such high volumes of serum, whilst this phenomenon had not previously been observed, higher volumes of sera were used in this assay than had been tested in previous assays; Fig 3.4 did not demonstrate this trend however the serum volumes were lower in that assay (0.1-10 μL). This trend was more pronounced using PBS as a diluent however was observed to a degree with the diluent MEM. The trend was observed at both 20% and 10% complement concentrations. The complement-only killing was again higher at 20% than at 10% complement, when using both diluents. The concern was that the inverse % killing trend was due to the volume ratios of reagents in the 96-well plate.

Given the unexpected result in Figure 3.10, the assay was repeated comparing method version 1 and with method version 2, and with an additional method, version 2.1 as described in Table 2.4. Method version 2.1 was a very slight modification of version 2, the only change was that the assay was carried out in a 24-well rather than a 96-well plate, therefore the wells were a larger size, this modification was introduced in an attempt to see if the well size, rather than differences in reagent ratios, contributed to these unexpected, inverse trend results, the results of this comparison assay are shown in Figure 3.11. The volumes of serum tested were 1, 10, 30 and 60 μL, with a 10% complement concentration. The diluent was MEM. Replicates were not performed.

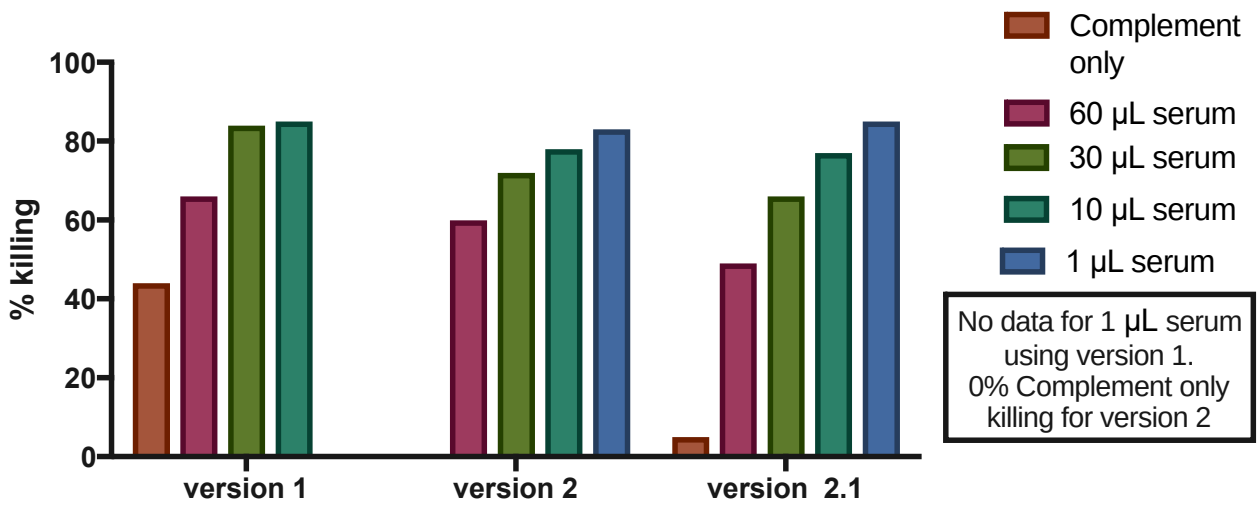


Figure 3.11 Decreasing serum volumes result in a trend of increasing % killing irrespective of method version

Method versions 1, 2 and 2.1 were compared. Version 1 - *C. jejuni*, 1 x 10^8 CFU, was incubated with, or without, serum (60, 30, 10 or 1 µL). From the pre-exposure tubes 100 µl was added to MEM containing complement, the final volume giving a 10% complement concentrations. The 24-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then plated and incubated for a further 48 hours at 37°C under microaerobic conditions. Version 2 – In a 96-well plate *C. jejuni*, 1 x 10^7 was added to MEM and complement, the final volume giving a 10% complement concentration, and the non-control wells contained different volumes of serum (60, 30, 10 or 1 µL). Version 2.1 - In a 24-well plate *C. jejuni*, 1 x 10^7 was added to MEM and complement, the final volume giving a 10% complement concentration, and the non-control wells contained different volumes of serum (60, 30, 10 or 1 µL). Subsequent incubation times and conditions were the same as version 1.

The inverse % killing trend remained, irrespective of the method version; for each method version; a decreasing volume of serum resulted in an increasing % killing. This therefore suggests that the inverse trend seen was not due to the total assay volume, inclusion of a pre-exposure period, type of plate, or size of the well being used in the assay, and rather suggested that it may be due to the high volumes of serum being tested, higher than previously investigated, and raised the question of whether it was simply the volume of serum, or rather the volume of serum when compared to the number of bacterial cells used in the assay, that caused this effect.

3.2.6 Examining the effect of reducing the number of *C. jejuni* in the assay

In order to establish whether the inverse % killing trend was due to an excess of bacteria, the number of *C. jejuni* cells added to the assay was scaled down; for this method version 3, as summarized initially in Table 2.4, was developed. In this evolution to method version 3, either 9 x 10^4 or 9 x 10^3 *C. jejuni* were added to the wells of the 96-well plate, rather than 3 x 10^7 as was the case in method versions 1 and 2. Table 3.2 details the differences between method versions 1 and 3.

Table 3.2 Comparison of method version 1 with method version 3

	Version 1	Version 3
Final Volume in well, μL	1000	300
Size of plate	24	96
Volume of CJCV1 Serum, μL	Equivalent of: 0.33, 3.33, 10, 20	1, 10, 30, 60
Volume of Complement, μL (% of final volume)	100 (10)	30 (10)
Number of *C. jejuni* CFU	1 x 10^8 into the 30 minute pre exposure tube, 3 x 10^7 into the well	9 x 10^4 and 9 x 10^3 into the well
Pre-treatment incubation period	Yes	No

For this SBA method versions 1 and 3 were examined, using a 10% complement concentration and serum volumes of 1, 10, 30 and 60 μL. The results are shown in Figure 3.12. Replicates were not performed therefore error bars are not shown.

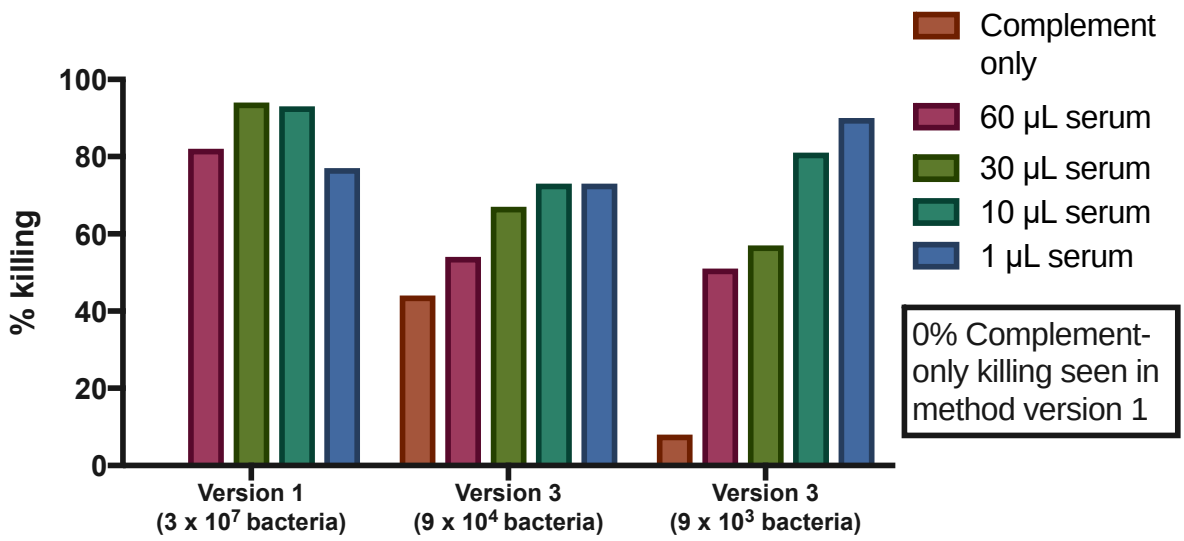


Figure 3.12 Reduced numbers of bacteria do not affect the inverse killing trend
Method versions 1, and 3 were compared. Version 1 - *C. jejuni*, 1 x 10^8 CFU, was incubated with, or without, serum (60, 30, 10 or 1 µL). From the pre-exposure tubes 100 µl was added to MEM containing complement, the final volume giving a 10% complement concentrations. The 24-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then plated and incubated for a further 48 hours at 37°C under microaerobic conditions. Version 3 – In a 96-well plate *C. jejuni*, 9 x 10^4 or 9 x 10^3 was added to MEM and complement, the final volume giving a 10% complement concentration, and the non-control wells contained different volumes of serum (60, 30, 10 or 1 µL). Subsequent incubation times and conditions were the same as version 1.

A decrease in serum volume causes the percentage killing to increase despite a reduction in the number of bacteria. Therefore the phenomenon is unlikely to be due to bacterial numbers in the assay. In version 3, which uses lower numbers of bacteria, as the volume of serum decreases, the % killing increases. This trend is similar to the phenomenon seen previously using method version 2, where 1 x 10^7 bacteria were used. The effect is not so pronounced in the results for method version 1. With the volumes of serum exceeding 10 µl the results showed a consistently inverse trend in % killing to that expected, despite the method or amount of bacteria used in the assay. This was possibly due to the **'pro-zone'** effect, where higher volumes of serum resulted in a lower % killing effect. This will be expanded upon in the discussion section.

In an attempt to reduce this inverse killing effect, and also in anticipation of the serum titration process that was planned for the intended ultimate use of the assay; establishing the highest titre still capable of achieving a 50% killing effect, the assay was modified to use much lower volumes of serum. As discussed in Chapter 2, another candidate to be examined for use as a diluent was Dextrose Gelatin Veronal (DGV). As described in Section 2.1.1.1, in order for a complement mediated SBA to be effective, a diluent with optimal Ca^{2+} and Mg^{2+} concentrations is desirable. The diluent DGV contains both Ca^{2+} and Mg^{2+} and therefore was tested for use in subsequent SBAs as a medium in which to suspend the *C. jejuni* cells, and also as a diluent for the assay. In addition, at this stage of assay evolution, the method by which the *C. jejuni* cells were prepared was also modified, from the initial method (section 2.5.2) to the revised method (section 2.5.3). The difference is that rather than a 2-stage growth period of 20 hours each, the first on solid MHP agar, the second in liquid MHB, the cells were now grown for 20 hours on MHP agar then used directly from the plate. These changes evolved the method, to method version 4.

3.2.7 Using the revised method of *C. jejuni* preparation, and the diluent DVG

When the initial and revised methods of *C. jejuni* growth were compared, by carrying out the growth process then plating on MHP agar plates, the CFU counts at time point 0 and 60 minutes for each method were comparable, therefore the revised, shorter, method was used for subsequent SBAs. By removing steps deemed unlikely to affect the results, the assay becomes more likely to be used in future as time and resources are saved. Table 3.3 details the differences between method versions 2 and 4.

Table 3.3 Comparison of method version 2 with method version 4

Detail of method difference	Version 2	Version 4
Final Volume in well, μL	300	300
Size of plate	96	96 or 48
Volume of Serum, μL (% of final volume)	10 (3.33)	Different titres
Volume of Complement, μL (% of final volume)	30 (10)	Variable
Number of *C. jejuni* CFU	1×10^7	1×10^7 For Figure 3.13, 1×10^5 (3^{rd} dilution) and 1×10^4 (4^{th} dilution) were used
C. jejuni preparation method	Initial	Revised
30 minute incubation period – serum and *C. jejuni*	No	No
Diluent	MEM or PBS	DGV

Figure 3.13 shows results using method version 4. The SBA used scaled down numbers of bacteria, as well as lower volumes of CJCV1 sera: 0.05, 0.1, 10 and 30 μL, and a complement concentration of 10%. Replicates were not performed therefore error bars are not shown.

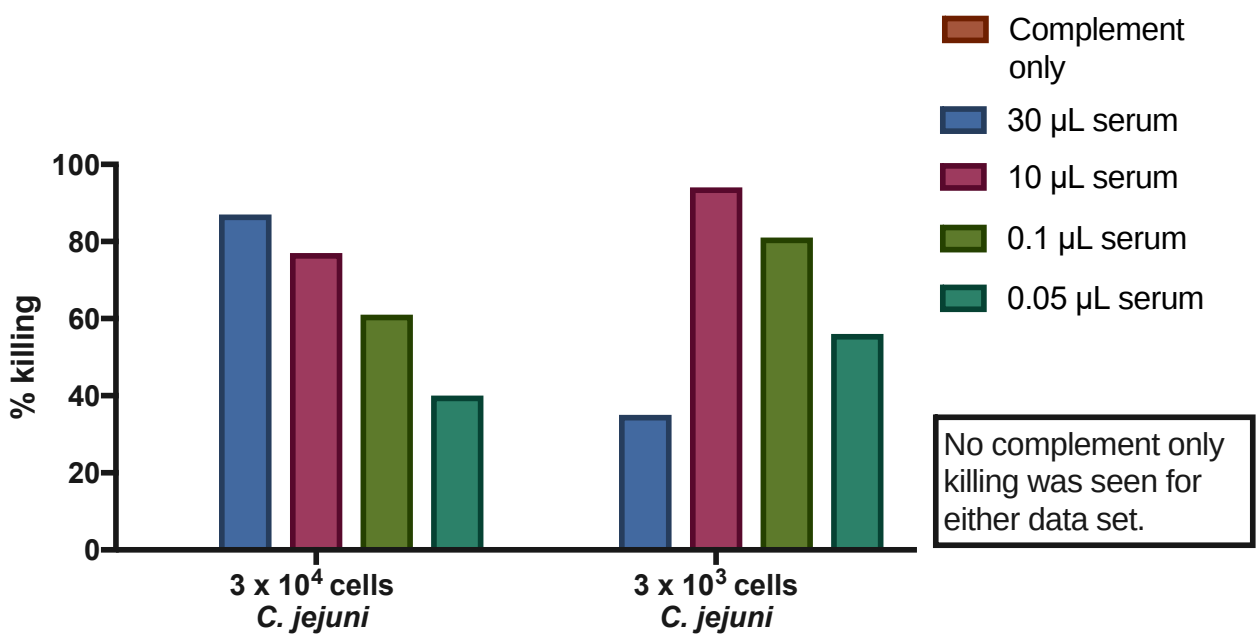


Figure 3.13 At lower serum volumes the percentage killing decreases as serum volume decreases.

Method version 4 was tested, for this method *C. jejuni* was grown using the revised method, section 2.5.3. In a 96-well plate *C. jejuni*, 3 x 10^4 or 3 x 10^3 was added to DGV and complement, the final volume giving a 10% complement concentration, and the non-control wells contained different volumes of serum (30, 10 0.1 or 0.05 µL). The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

In contrast with the previously observed trend of an inverse relationship between serum volume and % killing, these results generally suggest a decreased % killing trend as would be expected with a decreasing volume of serum. However, with the combination of the highest volume of serum (30 µL) and fewest bacteria, 3 x 10^3, there was still a lower % killing at 30 µL of serum than was demonstrated at lower serum volumes. This SBA was the first to extend the serum volumes to as low as 0.05 µL, and may demonstrate the loss of the pro-zone phenomenon, and the threshold at which it re-occurs. The results also show that the evolution of method conditions to method version 4; the use of diluent DGV and the revised shorter method of *C. jejuni* preparation,

still allow bactericidal activity to be demonstrated. Therefore method 4 continued to be used for further SBA method evolution.

3.2.8 Examining alternative sources and concentrations of complement

Given the high complement-only non-specific killing seen throughout the development of the SBA, the concentration of complement used was re-examined. Additionally alternative types of complement, human, guinea pig and rabbit were purchased and tested for complement-only killing effects, Figure 3.14. No serum was used in this assay. The Cedarlane BRC complement that had been used in the method development to this point was also tested in this comparison assay. The complement concentrations tested were 75, 50, 25 and 10%.

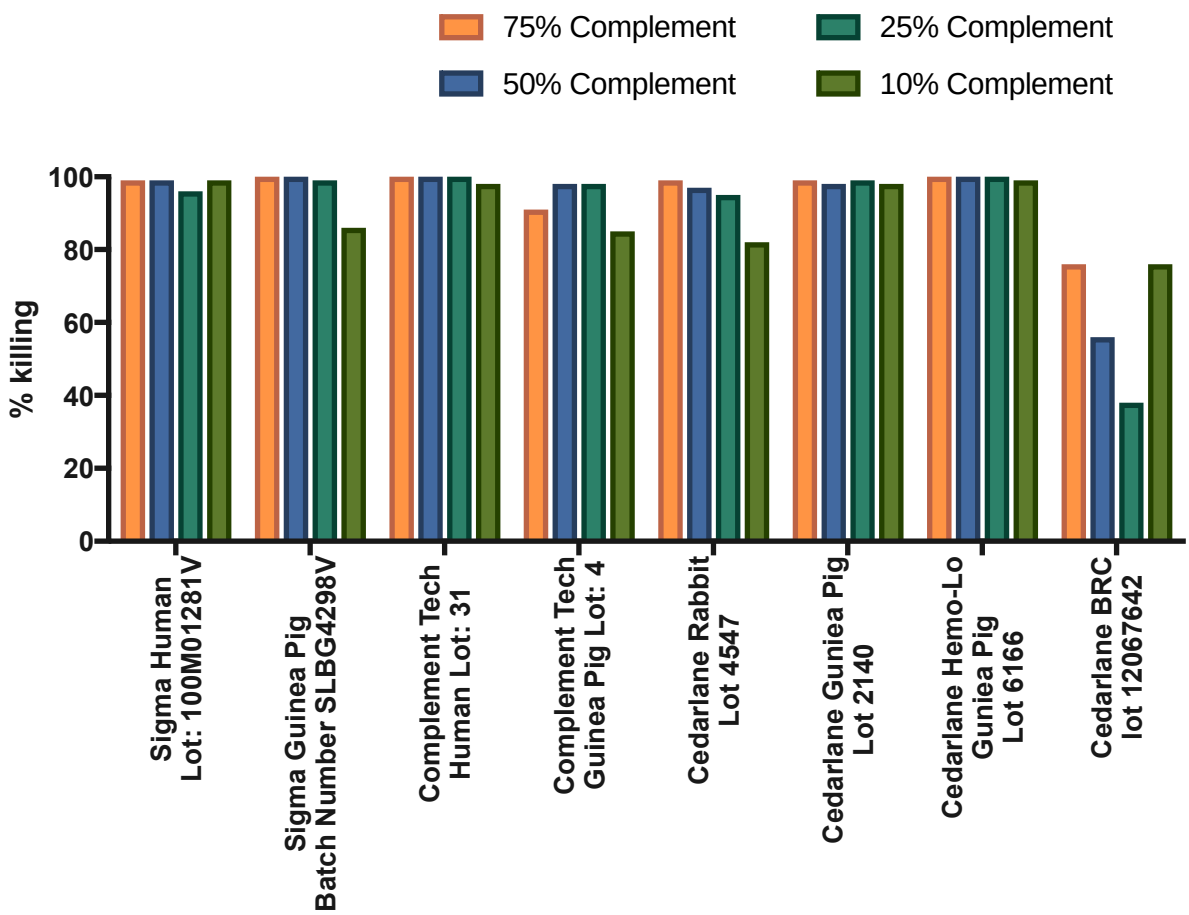


Figure 3.14 Alternative sources of complement have high complement-only killing effects

Method version 4 was used, *C. jejuni* was grown using the revised method, section 2.5.3. In a 96-well plate *C. jejuni*, 1 x 10^7 was added to DGV and complement, the final volume giving 75%, 50%, 25% or 10% complement concentrations. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

The alternative sources of complement demonstrated high complement only killing effects, even at 10% complement concentration. The CFU counts for all complement sources other than CEDARLANE BRC Lot Number 12067642, (the lot used throughout the assay development) were extremely low, suggesting a high complement-only killing effect. Therefore the CEDARLANE BRC Lot Number 12067642 continued to be used.

Having established that Cedarlane BRC would continue as the source of complement, the complement only and serum % killing effects were examined further using lower complement concentrations. The killing effect of CEDARLANE BRC lot 12067642 was compared at volumes equating to 10, 15, 18, 20 and 25% of the final well volume, in order to establish the complement-only killing effect seen at these different percentages by volume. The % killing effect of each complement concentration was also tested in the presence of 10 µL CJCV1 serum, the results of which are shown in Figure 3.15.

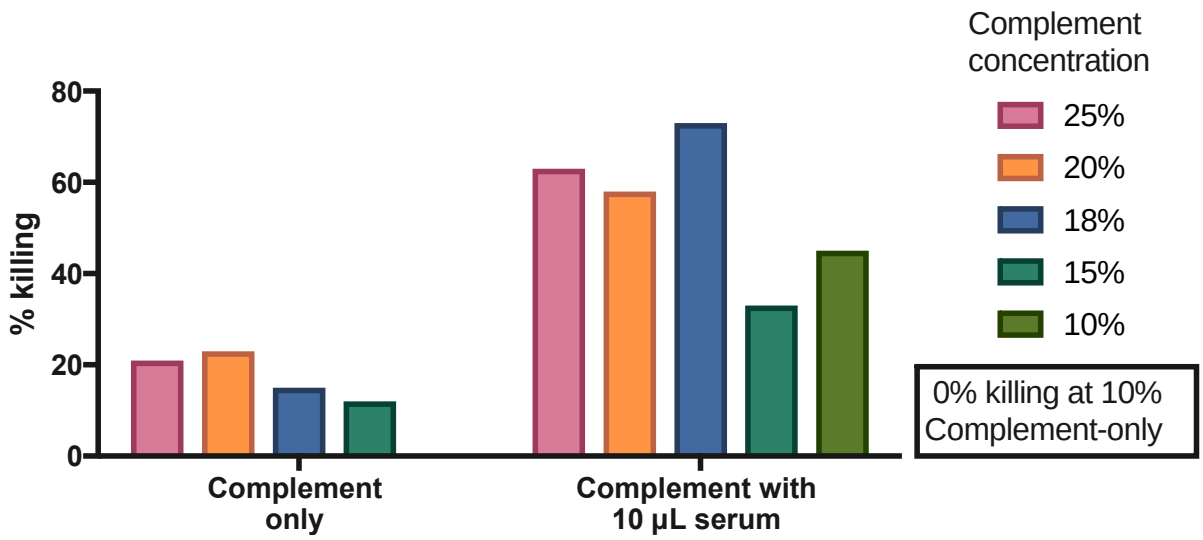


Figure 3.15 An 18% complement concentration demonstrates a low complement-only killing effect, and facilitates a high serum mediated killing effect.

Method version 4 was used; *C. jejuni* was grown using the revised method, section 2.5.3. In a 96-well plate *C. jejuni*, 1 x 10^7 was added to DGV and complement, the final volume giving 25%, 20%, 18%, 15% or 10% complement concentrations, and the non-control wells contained 10 µL of serum. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

A complement concentration of <20%, i.e. 10%, 15% and 18%, had a % killing effect of under 20%. At a complement concentration of 18%, as well as low complement-only killing, the serum mediated % killing effect was high. Replicates were not performed therefore there are no error

bars. This SBA was performed subsequent to the SBAs providing data for Figures 3.18 and 3.19, which used a 25% complement concentration, however these data informed the choice of complement concentration for that assay illustrated at Figure 3.20.

3.2.9 Attempts to eliminate possible bacterial aggregation

Throughout the SBA evolution there were occasions when % killing seemed erratic, or unexpectedly high. Occasionally a MHP agar plate had an unusually low CFU, the concern was that bacteria might be aggregating, and therefore the results giving a falsely high representation of the % killing occurring. No real time bacterial visualization tests, such as microscopy, were carried out to either prove or disprove aggregation. As *C. jejuni* is a flagellated organism, and the flagella could be linking, either within the assay wells or on the MHP agar plate, the following points were considered:

- Reducing the numbers of *C. jejuni;* scaling down the numbers of organisms had already been examined and described, however erratic counts still occurred.
- Vortexing between steps; this was carried out throughout the assay development, it was hoped that by thorough vortexing, the flagella might be sheared off, separating any aggregated bacteria. However, again, erratic counts still occurred.

Two further methods were therefore examined:

- Addition of the detergent Triton X 100 as a 0.05% solution to diluting tubes
- The use of an unflagellated strain, *C. jejuni* strain 2875, held in stock by the *Campylobacter* Working Group.

Viability tests, involving a 60-minute incubation period, suggested that at a concentration of 0.05%, Triton X 100 was not toxic to *C. jejuni.* Therefore, Triton X 100 was added as a 0.05%

solution to the dilution tubes, in an attempt to reduce aggregation prior to plating. Previous results have suggested that a complement concentration below 20% was optimal to avoid high levels of complement-only killing. However, in case the high level of apparent killing was actually a falsely high percentage, and the low numbers of CFU were a consequence of aggregation rather than *actual* complement-only killing, the higher percentage complement concentrations were retested. This assay was carried out, with a single data set for 10% complement concentration and with two replicates for 25%, 50% and 75% complement concentrations, in the presence of 1, 10 and 30 µL of serum, with triton X 100 detergent in the diluent, Figure 3.16.

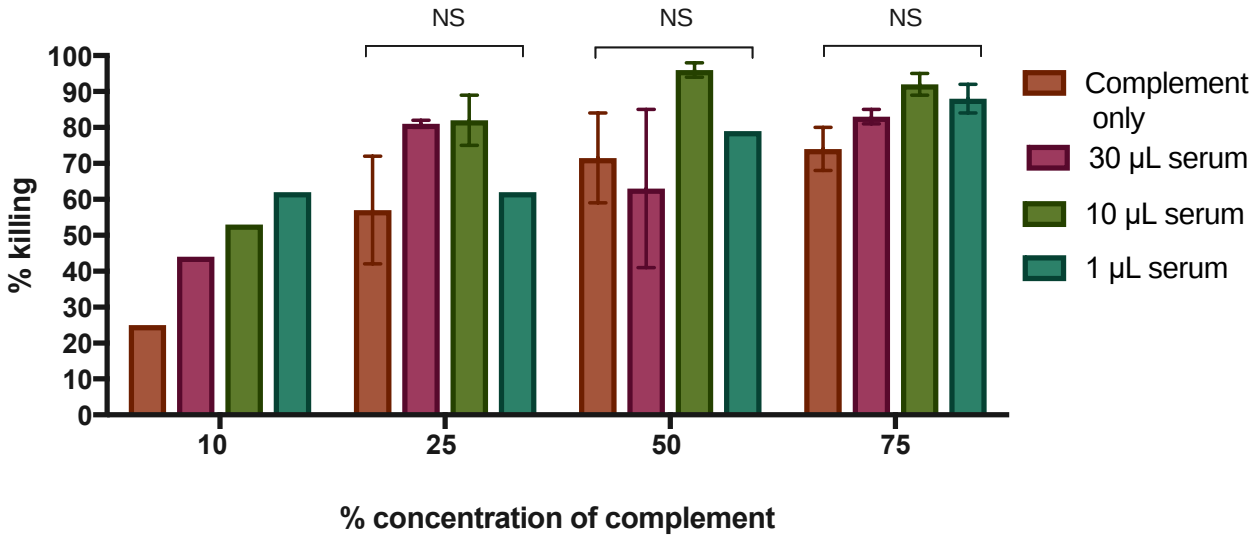


Figure 3.16 Adding Triton X 100 to dilution tubes does not reduce high apparent killing rates.

Method version 4 was used, *C. jejuni* was grown using the revised method, section 2.5.3. In a 96-well plate *C. jejuni*, which had been diluted down to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving 75%, 50%, 25% or 10% complement concentrations, the non-control wells contained 30, 10 or 1 µL of serum. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

Despite using Triton X 100, high killing rates were observed. This may suggests that the high killing rates were indeed true killing rates, and aggregation was not the cause of *apparent* high killing effects, or that Triton X 100 did not reduce aggregation. In addition the inverse trend of

increasing % killing with decreasing serum volumes was seen again, most clearly in the 10% complement concentration assay, and partially in the 75% complement concentration assay. There was no significant difference in % killing between serum volumes, including no serum (complement-only control) at the 25%, 50% and 75% complement concentrations; 2 replicates, one-way ANOVA, $p = 0.21$, $p = 0.41$ and $p = 0.12$ respectively. Error bars represent SEM, replicates were not performed for the assay using a 10% complement concentration. As Triton X 100 is not toxic to *C. jejuni*, it continued to be used in dilution tubes.

To further examine whether aggregation was caused by the presence of flagella, an assay using a non-flagellated strain of *C. jejuni*, strain 2875, was used in place of *C. jejuni* 81-176, Figure 3.17. Method version 4 was used with 3×10^4 cells added to each well, a 10% complement concentration with serum volumes of 30, 10, 0.1, and 0.05 μL.

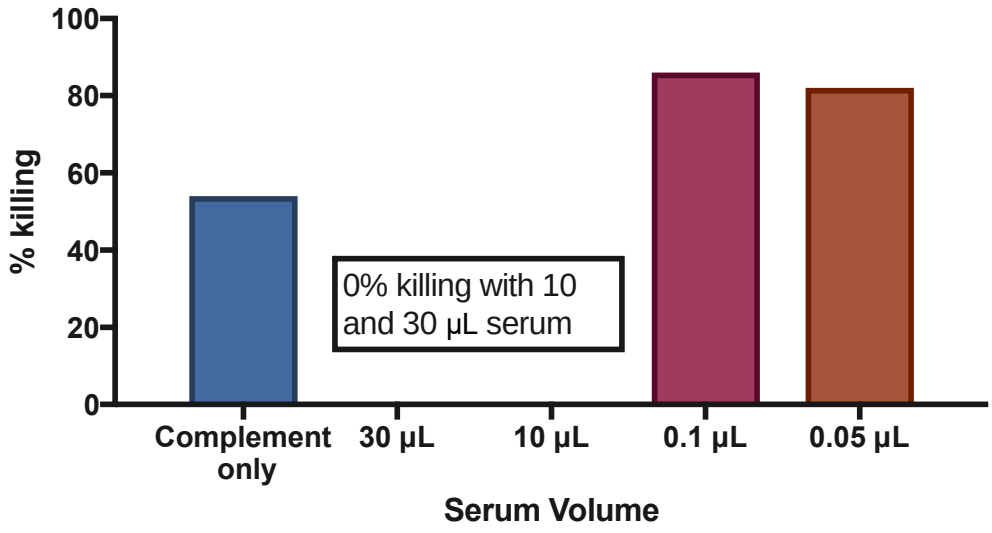


Figure 3.17 % killing using the unflagellated *C. jejuni* strain 2875 remains high
Method version 4 was used. In a 96-well plate *C. jejuni*, which had been diluted down to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 10% complement concentration, the non-control wells contained 30, 10, 0.1 or 0.05 µL of serum. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

If aggregation due to flagella caused falsely high % killing rates, then, without flagella, the % killing for all serum volumes, and complement-only, should appear lower. These results suggest that even in the absence of flagella, the complement-only killing effect remains high, suggesting that aggregation due to flagella is unlikely to cause a falsely high % killing result. As has been observed in previous assays, % killing was lower at the higher serum volumes. Replicates were not performed.

Again, Fig 3.17 shows a low percentage killing effect at higher serum volumes, as seen in previous results that raised the possibility of a pro-zone phenomenon. It was hoped that by performing serial dilutions of the sera, and titrating it such that the volumes of sera being used were indeed very low, this effect would be lost. This was the next evolution of SBA development.

3.2.10 Evolution of the SBA to examine five-fold serum dilutions

The role of a functional SBA is to facilitate the testing of the bactericidal activity of an antibody, and therefore be used to establish the highest serum titre that still achieves 50% killing when further dilutions fall below that threshold. As the intended final design of the functional SBA was to test different serum titres, the method evolved to use serially diluted serum titres rather than set volumes of serum. The titre recorded is the reciprocal of the highest dilution that achieves a greater than or equal to 50% killing effect. To test serial serum dilutions, method version 4 was used (Table 2.4), the CJCV1 serum volumes after serial five-fold dilutions were 25 µL (titre 12), 5 µL (titre 60), 1 µL (titre 300), 0.2 µL (titre 1500) and 0.04 µL (titre 7,500), the complement concentration used was 25%. The serum dilutions to be used in the assay were achieved by carrying out serial 5-fold dilutions in DGV. The results are shown in Figure 3.18.

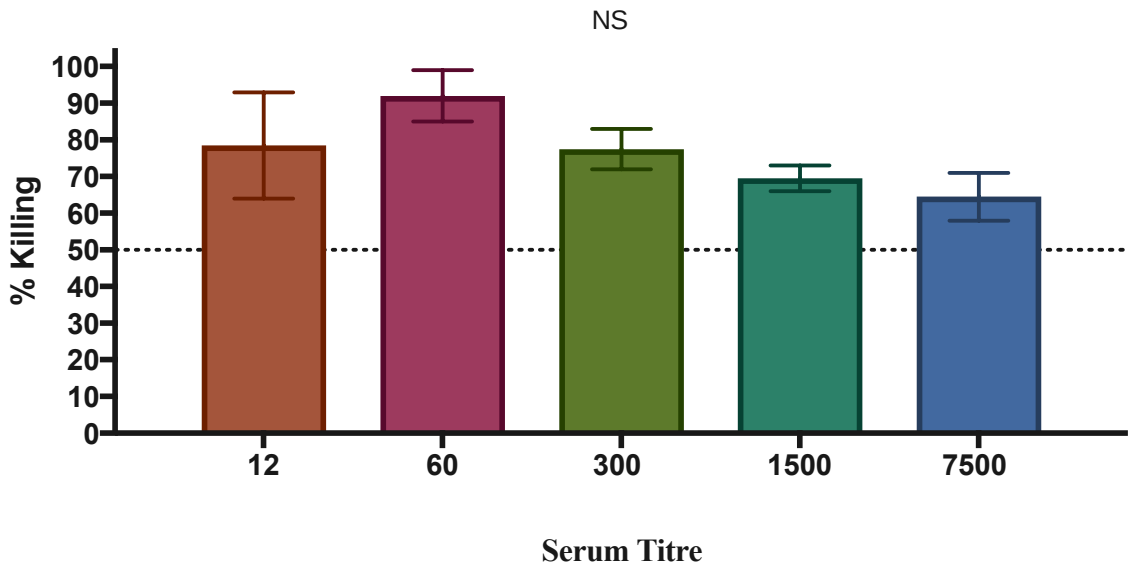


Figure 3.18 Serial dilutions to a titre of 7500 do not demonstrate the point at which 50% killing is lost

Method version 4 was used. In a 96-well plate *C. jejuni*, which had been diluted down to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 25% complement concentration, the non-control wells contained serum which had been serially diluted to give volumes of 25, 5, 1, 0.2 and 0.04 μL of serum. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

This assay demonstrates that as the titres increase, the killing effect falls, with the exception of the well containing a serum titre of 12, an anomaly similar to the inverse trend seen in previous assays at serum volumes >10 μL. However, as the titre increased to 60 and higher, at subsequent titres the SBA results follow a trend that would be expected, decreased % killing with higher titres. The dotted line at 50% killing effect is to illustrate the target threshold for this SBA. In this assay the titre at which the % killing effect fell below 50% killing was not reached. Using the data from 2 replicates there was no significant difference in % killing between titres 60 and 300, 300 and 1500 or 1500 and 7500 (unpaired t-test p = 0.24, p = 0.34, p = 0.57), or when titres 60, 300, 1500 and 7500 were analysed by ANOVA, p = 0.10). The error bars represent the SEM. The lack of

significance may be due to the low number of replicates; 2 replicates of the assay were performed. The assay was repeated with the 5-fold dilutions extended further thus examining the higher titres of 37,500 and 187,500 in an attempt to determine the highest titre to still achieve 50% killing, Figure 3.19.

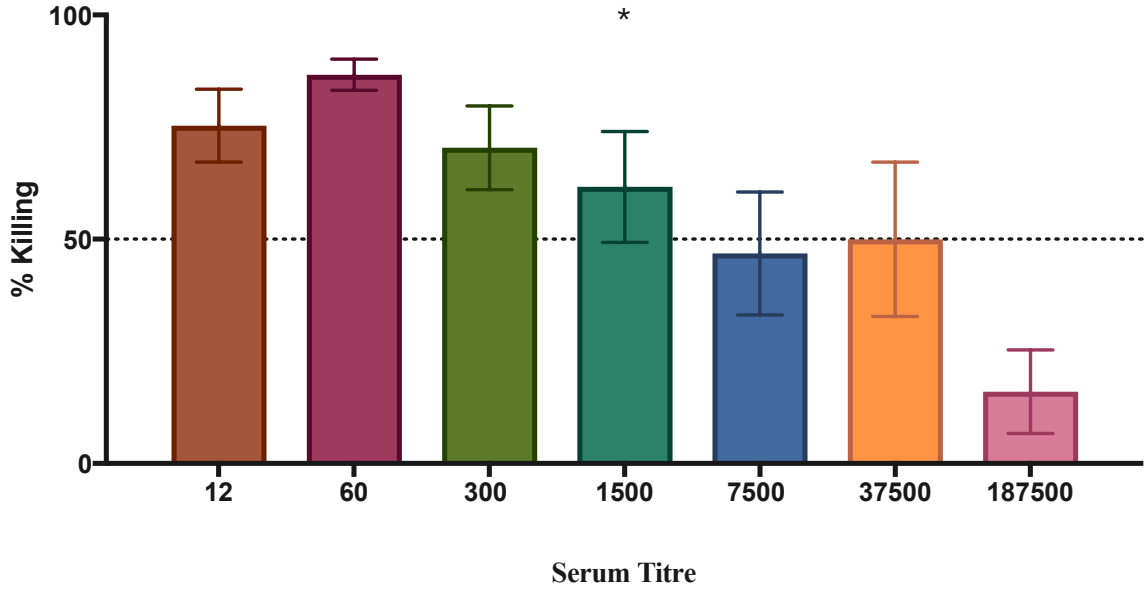


Figure 3.19 Serial dilutions to a titre of 187,500 identify highest titre achieving 50% killing as 1500.

Method version 4 was used. In a 96-well plate *C. jejuni*, which had been diluted down to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 25% complement concentration, the non-control wells contained serum which had been serially diluted to give volumes of 25, 5, 1, 0.2, 0.04, 0.008 and 0.0016 µL of serum. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

There is a clear reduction in % killing as the titre increases and it is possible to establish the point at which 50% killing is no longer achieved for this serum. The dotted line at 50% killing effect is to illustrate the target threshold for this SBA. The lowest serum titre to *still* achieve 50% killing is 1,500. These data were considered to be non-parametric so a Kruskal-Wallis test was applied, p = 0.0214. The error bars represent SEM, 6 replicates were performed. This assay demonstrates a

serum titre of 1500 that achieved a 50% killing effect. Were this SBA to be carried out using serum from serial time points during the vaccination schedule of a single subject, changes in titre could be observed. Therefore an increase, or fold-increase in titre, could be established between time-points. If the titre was sufficiently high, or a significantly high fold-increase was demonstrated when compared to baseline, it may have the potential to be used as a correlate for protection. The SBA was repeated using method version 4 (Table 2.4) with serial dilutions of CJCV1 serum, and an 18% complement concentration, Figure 3.20. An 18% complement concentration was chosen as it was shown to be an optimal concentration in Figure 3.15.

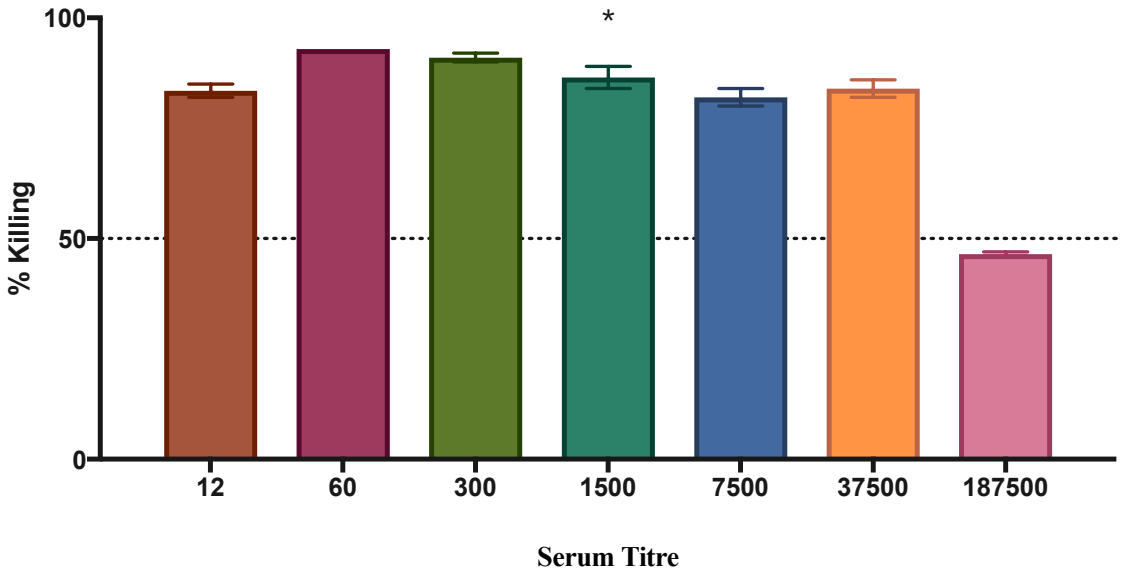


Figure 3.20 When using an 18% complement concentrations, the 50% killing titre of this serum is 37,500.

Method version 4 was used. In a 96-well plate *C. jejuni*, which had been diluted down to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 18% complement concentration, the non-control wells contained serum which had been serially diluted to give volumes of 25, 5, 1, 0.2, 0.04, 0.008 and 0.0016 µL of serum. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

The highest serum titre achieving a 50% killing effect is 37,500. These results differ from Figure 3.19, which demonstrated a titre of 1,500. However, if the results from Figure 3.18 were

extrapolated to higher titres then the results may have been similar to Figure 3.20. If more replicates had been performed for the data set in Figure 3.19 the dip in % killing at serum titre 7500 may have not been so pronounced, possibly leading to a titre reflective of the data in Figures 3.18 and 3.20. A Kruskal-Wallis test was applied, statistical significance was achieved when the killing effects of titres were compared, $p = 0.0104$. The error bars represent SEM, 2 replicates were performed.

The difference in 50% killing titre observed in Figures 3.19 and 3.20 may be due to the difference in complement concentration used, supported by the different percentage killing effects seen with different concentrations of complement over the course of the development of the SBA. Therefore, when a complement concentration has been determined as the concentration for the SBA, then it must remain constant. SBA method 4 had proven to be reliable in demonstrating bactericidal effects at different serum titres so was considered the optimal assay method. Having established the method and reagent concentrations to be used, the serum from the New Zealand White Rabbit Safety Study was tested, the details of the vaccination schedule are described in Chapter 1, Table 1.2.

3.3 CJCV1 NEW ZEALAND WHITE RABBIT SAFETY STUDY

Using the method described in Section 2.7, SBAs were performed to establish the % killing titres. Each data point represents a rabbit. Replicates were not performed. Half of the rabbits were sacrificed prior to Day-71, consequently there are fewer data points at Day-71. The titres plotted represent the highest titre to achieve a 50% killing effect. If the titre were to fall between baseline and the first time point after vaccination then that animal was excluded from the results. Also, if a baseline titre was not achieved, i.e. a baseline titre that gave <50% killing was not reached, then that animal was also excluded as it was deemed that it had had prior *C. jejuni* exposure, occasionally with this scenario the baseline CPS IgG ELISA titre established at the time of the safety study was also high, which was not expected as they were commercial animals.

The serum from Group 1, the group administered PBS rather than the CJCV1 vaccine, was not tested. Group 1 were not vaccinated with CJCV1, they were the control group for adverse events occurring following vaccination. For Groups 2 and 3 the individual pre-vaccination serum sample provided the control for each animal. The results for Group 2 (administered vaccine: 10 μg antigen:125 μg alhydrogel) are shown in Figure 3.21. The results for Group 3 (administered vaccine: 10 μg antigen:0 μg alhydrogel) are shown in Figure 3.22. The serum was tested at the time points: pre-vaccination, Day-56 (after 2 vaccinations given) and Day-71 (after 3 vaccinations given).

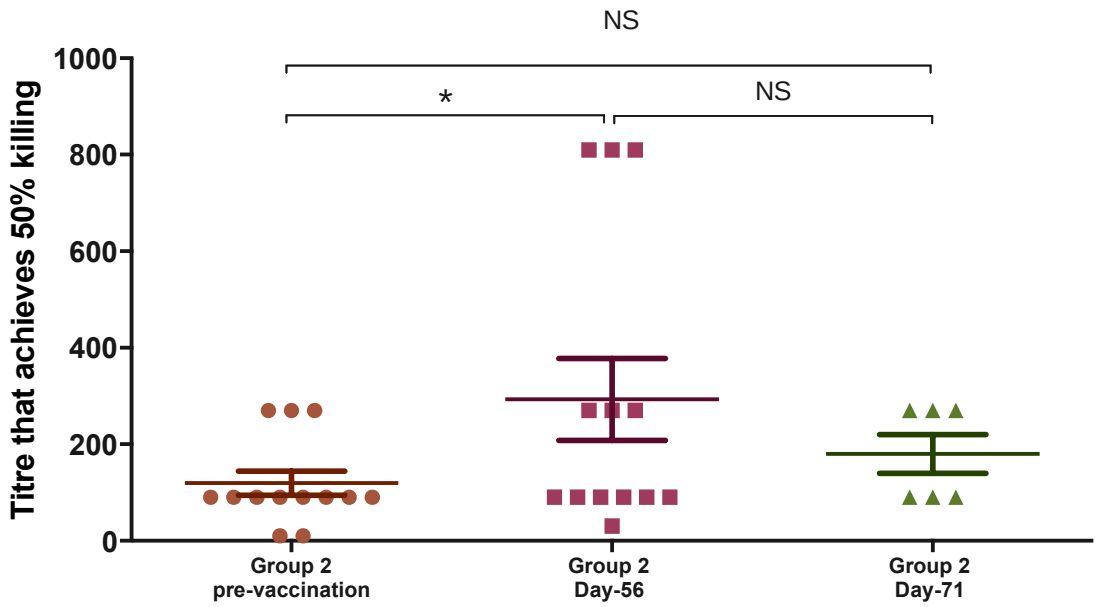


Figure 3.21 Group 2: The increase in titre between pre-vaccination and day-56 is significant whereas there is no significant increase in titre between pre-vaccination and day-71, or day-56 and day-71.

In a 96-well plate *C. jejuni*, which had been diluted down to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 18% complement concentration. The non-control wells contained serum from Group 2 rabbits from each time-point which had been serially diluted to give volumes of 10, 3.3, 1.1, 0.37 or 0.12 μL of serum. These were added to the wells according to Tables 2.5 and 2.6. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

Each data point represents a rabbit. These results show that there was a significant difference between the pre-vaccination and Day-56 titres (paired t-test, p = 0.048), however there was not a significant difference between pre-vaccination and Day-71 (paired t-test, p = 0.189), or between Day-56 and Day-71 (paired t-test, p = 0.420). Replicate testing of individual rabbit serum was not performed. If a sensitivity analysis is performed, and the 3 animals with the high titre at Day-56 are removed from the analysis, then there is no significant difference between pre-vaccination and Day-56 titres (paired t-test, p = 0.168), or between Day-56 and Day-71 (paired t-test, p = 0.102), however there is a significant difference between pre-vaccination and Day-71 titres (p = 0.020). Error bars represent the mean and SEM of the group. Group 3 animals were also tested, Figure 3.22.

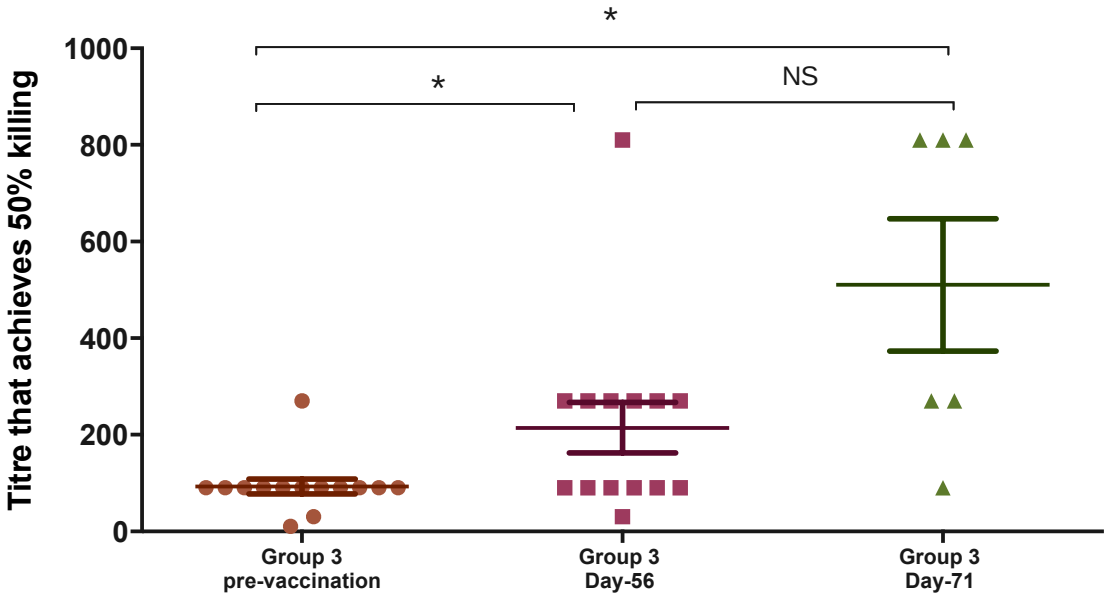


Figure 3.22 Group 3: The increase in titre between pre-vaccination and Day-56, as well as between pre-vaccination and Day-71 is significant whereas there is no significant increase between Day-56 and Day-71.

In a 96-well plate *C. jejuni*, which had been diluted down to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 18% complement concentration. The non-control wells contained serum from Group 3 rabbits, from each time-point, which had been serially diluted to give volumes of 10, 3.3, 1.1, 0.37 or 0.12 μL of serum. These were added to the wells according to Tables 2.5 and 2.6. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

Each data point represents a rabbit. These results show that there was a significant difference between baseline and Day-56 titres (paired t-test, p = 0.034), as well as a significant difference between baseline and Day-71 titres (paired t-test, p = 0.028). However there was no significant difference between Day-56 and Day-71 titres (paired t-test, p = 0.203). Each data point represents a rabbit in the group. Error bars represent the mean and SEM of the group.

Having established these results, fold increases in 50% killing titre from baseline to Day-56 and baseline to Day-71were determined, for Group 2, Figure 3.23 and for Group 3, Figure 3.24.

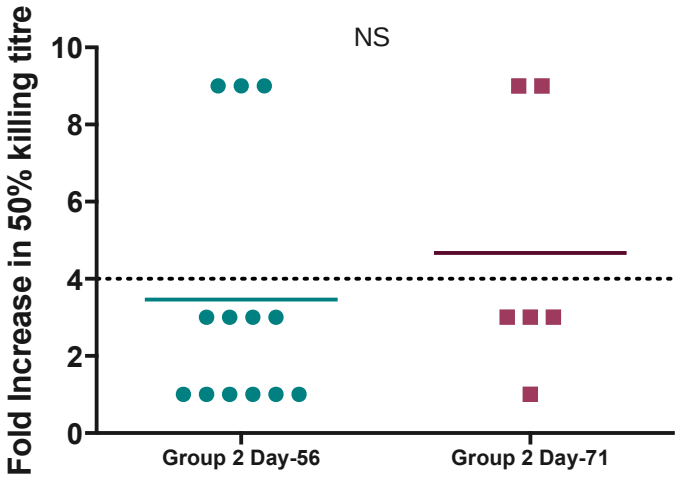


Figure 3.23 Group 2: Mean fold increase in 50% killing titre at Day-71 is 5.4

The data was achieved using the method of Figure 3.21 and interpreted to calculate a fold increase in killing compared with baseline.

The dotted line at 4 on the Y-axis is plotted as a fold-increase of 4 is considered a correlate to demonstrate protection following vaccination, Chapter 1. The horizontal lines represent the mean fold-increase in 50% killing titre. At Day-56 the mean fold increase is 3.7, this increases by Day-71 to 5.4. However, whilst the mean fold increase in 50% killing titre at Day-71 increases to 5.4, only 2 rabbits have a titre above the 4-fold increase threshold. The difference between fold-increases is not significant (unpaired t-test p = 0.474). Each data point represents an individual rabbit. Replicates for individual rabbits were not performed.

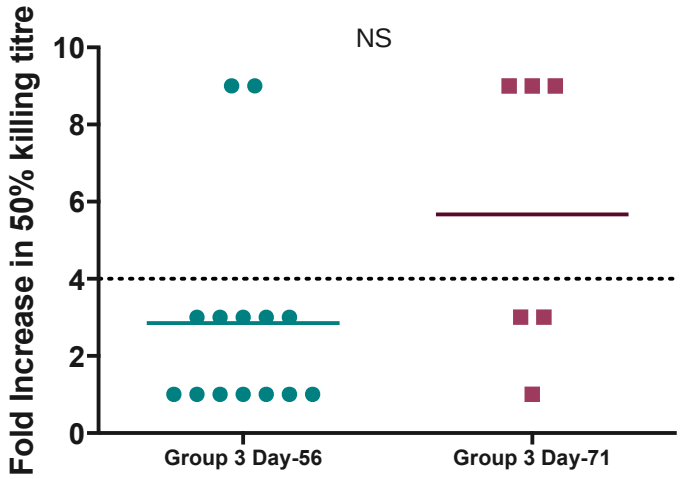


Figure 3.24 Group 3: Mean fold increase in 50% killing titre at Day-71 is 5.7
The data was achieved using the method of Figure 3.22 and interpreted to calculate a fold increase in killing compared with baseline.

At day-56 the mean fold increase is 2.9, this increases by Day-71 to 5.7. However, whilst the mean fold increase in 50% killing titre at Day-71 increases to 5.7, only 3 rabbits have a titre above the 4-fold increase threshold. The difference between fold increases is not significant (unpaired t-test $p = 0.077$). Each data point represents an individual rabbit. Replicates for individual rabbits were not performed.

Each group has a range of fold increases, however, after the third immunisation, the mean fold increase for Group 2 is 5.4, and for Group 3 is 5.7. Whilst at Day-71 the mean fold increase is > 4-fold for each group, this is not the case for every animal, indeed less than half the animals demonstrated a >4-fold titre increase. The significance of a fold increase ≥ 4-fold is expanded upon in the discussion.

3.4 NZWR SERUM RE-EXAMINED USING A NHP AND HUMAN SBA METHOD

The method used to evaluate the functional antibody response for the NZWR used in the safety study had proved to be very labour and material intensive. Whilst it did demonstrate 50% killing titres and fold-increases in 50% killing titres, it was not a practical way to proceed with subsequent SBAs. In addition, for the data presented above using the serum from the NZWR, there were occasions when the 50% killing titre would fall over the course of the vaccination and serum collection schedule. This is difficult to explain and is a shortcoming of either the SBA method, or the vaccine. Therefore the serum of NZWR that underwent all three vaccinations, and had serum collected at Day -1, was retested using the method described in Section 2.8, that had been developed to test the *A. nancymaae* and human CJCV1 serum.

As there were fewer animals to be tested (as half had been sacrificed prior to Day-71) and the method had been altered in an attempt to make it less labour and material intensive, the PBS control Group, Group 1, was tested as well as Groups 2 and 3. The results are shown in Appendix 5. The results demonstrated inconsistency between replicates. In some cases titres dropped over the course of the vaccination schedule, and when compared to the original SBA results, trends in titres and titre fold-increases in terms of increasing and decreasing over time, differed. It was expected that the 50% killing titre for Group 1 would be low and stable across the 3 different time-points as the animals received PBS only, or if there was variation then it might be within one titre higher or lower, however, there was a great deal of variability when some replicates were performed. Due to time and material constraints repeat replicates were only performed if there was a concern after the first assay. Such concerns included complement-only killing of greater than 35%, unexpectedly high (or immeasurable) titres where titres should be low as the rabbits were not vaccinated or exposed to *C. jejuni*, or low *C. jejuni* colony counts. It is recognised that

replicates are required and this is addressed in the discussion. For two of the Group 1 rabbits, 12114 and 12115, there is no data to show. Rabbit 12114 had 4 replicates performed. The first did not achieve a titre that fell below 50% killing, despite testing to a titre of 3645. The second had very poor growth of the bacteria, with the control CFU counts in single figures. The third and fourth replicates again did not achieve a titre that fell below 50% killing effect, despite testing to a titre of 32,805. With a finite volume of serum available the serum was not retested at this stage. This anomaly for a control group rabbit either suggests that the SBA method has a weakness, or that the rabbit had previously developed antibodies that were displaying functional killing properties against the *C. jejuni* bacteria, this is unlikely as they were commercial animals.

Whilst the sera from some vaccinated rabbits had a similar trend in fold-increase of 50% killing titre when tested with the different method, it was by no means true for all the serum samples, indeed for some the titre increased over time using one method, and decreased using another method. In addition the fold-increases, in some instances, are different for replicates of SBAs using a serum sample and the same method. This suggests a flaw within the SBA method, as retesting replicates of serum should show similar results. However, as suggested above and in the introduction, *C. jejuni* is an organism that employs a vast repertoire of evasive techniques, such as genomic phase variation and the ability to turn the expression of its capsule on and off, in order to elude the host immune response and those of competing pathogens, therefore it is not wholly surprising that the results are somewhat inconsistent, as it is possible that these evasive techniques may also be used, to varying degrees, during the course of the assay. Testing capsule expression at this stage would have been informative.

3.5 EXAMINING *A. NANCYMAAE* SERUM, FOLLOWING VACCINATION AND SUBSEQUENT CHALLENGE

A. nancymaae NHP, n = 20, were vaccinated with the research grade CCV vaccine, as described in Section 1.4.2; Group 1 (n-5) received 0.1 μg CPS, Group 2 (n-5) received 0.5 μg CPS, Group 3 (n-5) received 2.5 μg CPS and Group 4 (n-5), the Control group, received PBS. Following vaccination the NHP were challenged with $5x10^{11}$ CFU *C jejuni* strain 81-176, administered intra-gastrically via a feeding tube whilst under anaesthetic. During the vaccination study the diarrhoeal attack rates for the NHP that were immunized were as follows: Group 1, 40% (2/5), Group 2, 20% (1/5), Group 3, 0% (0/5), Group 4 (PBS control), 60% (3/5) [8]. When testing the *A. nancymaae* serum for bactericidal activity using the SBA method described in Section 2.8, very high titres of bactericidal activity were demonstrated, the titres are therefore shown below in a log-10 scale. The data for each time point, Day-0, Day-63 and Day-125 for individual groups is shown below, Figure 3.25.

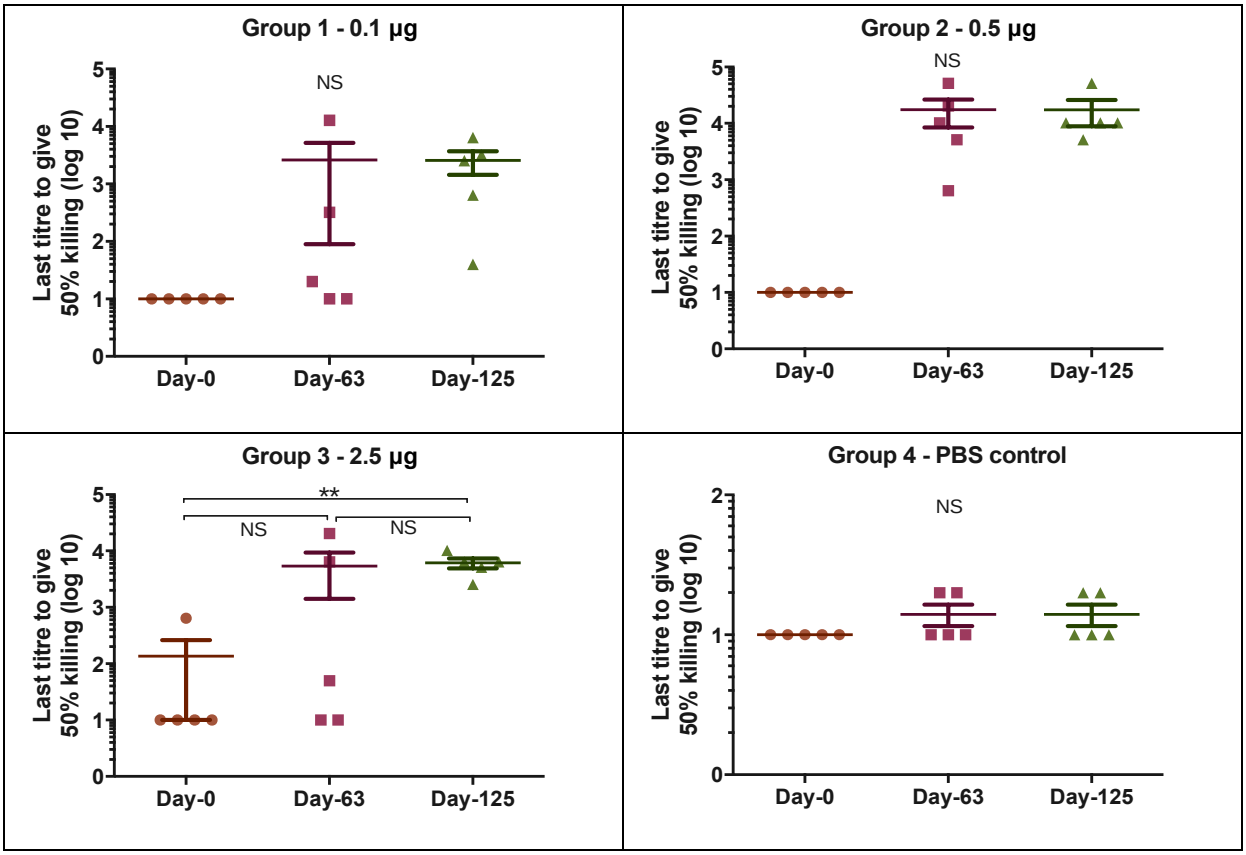


Figure 3.25 50% killing titres increase following vaccination

In a 96-well plate *C. jejuni*, diluted to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 16% complement concentration. The non-control wells contained serum from NHPs, from each time-point, which had been serially diluted to give final serum titres, when added to the well, from 20 to 20480, Table 2.7. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

For Group 1 there was no significance between Day-0 and Day-63, paired t-test p= 0.36, Day-0 and Day-125, paired t-test p= 0.085, or between Day-63 and Day-125, paired t-test p=0.98. For Group 2 there was no significance between Day-0 and Day-63, paired t-test p= 0.12, Day-0 and Day-125, paired t-test p= 0.11, or between Day-63 and Day-125, paired t-test p=0.97. For Group 3 there was no significance between Day-0 and Day-63, paired t-test p= 0.24, however there was a significant difference between the titres at Day-0 and Day-125, paired t-test p= 0.0092, but not between Day-63

and Day-125, paired t-test p=0.87. In Group 3 all NHPs were protected when challenged and did not develop diarrhoea. As is expected there are no significant differences between the titres in Group 4, the PBS control group.

These data, from Day-125 are plotted below, Figure 3.26, showing the final titres of each group.

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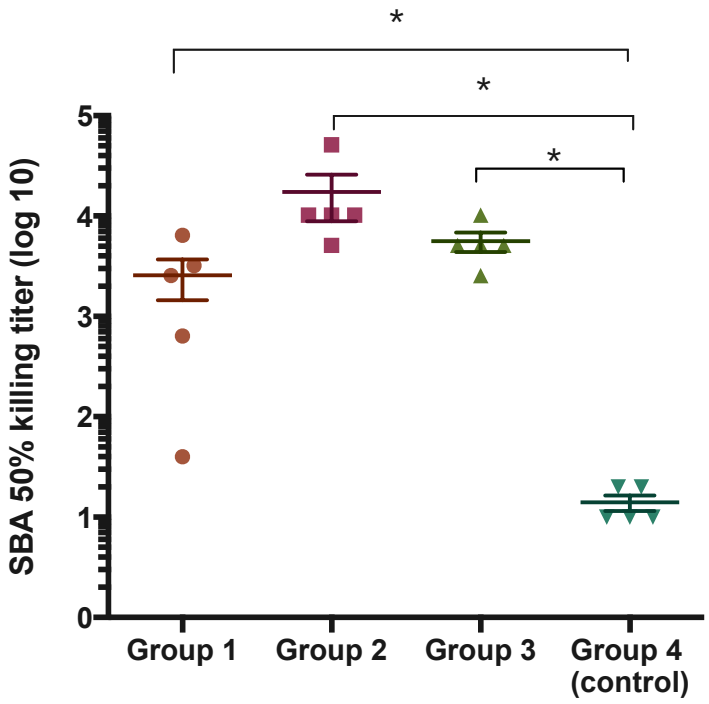


Figure 3.26 NHP: Day-125 50% killing titres, irrespective of vaccine dose, are significantly higher than the control group

The Day-125 data was plotted to illustrate a compariton of the 50% killing titres following the vaccination course, prior to challenge.

The mean 50% killing titres at Day-125 of Group 1, Group 2 and Group 3 are significantly increased when compared to Group 4. (Mann Whitney test, p = 0.011, p = 0.010, p = 0.010). Functional bactericidal activity does not increase as the dose of CPS increases; the mean 50% killing titre for Group 3 (2.5 µg CPS) is lower than for Group 2 (0.5 µg CPS). Each data point

represents a NHP. Replicates were not performed in individual serum samples. Error bars show the mean and SEM for the Group data.

This vaccination and challenge study allowed the 50% killing titres to be compared with the status post *C. jejuni* challenge, the challenge was given on Day-147. In terms of status allocation NHPs were deemed to be protected if they did not develop diarrhoea after *C. jejuni* challenge, or not protected if they did develop diarrhoea after *C. jejuni* challenge. Table 3.4 demonstrates the variability in Day-125 50% killing titres achieved, listed by status, protected or not protected, post-challenge. The number in brackets shows the vaccination group that the NHP was in. It is clear from the table that some NHP that were protected post-challenge had lower Day-125 titres than NHP that were not protected, and one NHP that was not protected had a much higher Day-125 titre than others that were protected. Therefore, with these data, a Day-125 titre did not predict whether protection had been achieved.

Table 3.4 Day-125 50% killing titres, protected vs. not protected

Day-125 50% killing titre	
Protected no diarrhoea post challenge	Not protected diarrhoea post challenge
640 (Group 1)	40 (Group 1)
3200 (Group 1)	2560 (Group 1)
6400 (Group 1)	10240 (Group 2)
5120 (Group 2)	10 (Group 4)
10240 (Group 2)	10 (Group 4)
51200 (Group 2)	10 (Group 4)
10240(Group 2)	
2560 (Group 3)	
10240 (Group 3)	
5120 (Group 3)	
6400 (Group 3)	
6400 (Group 3)	
10 (Group 4)	
20 (Group 4)	

As fold-increases from baseline have, for other vaccines, been used to determine successful protection, the individual and mean fold-increases were established and plotted against status following *C. jejuni* challenge; protected (did not develop diarrhoea after *C. jejuni* challenge) or not protected (did develop diarrhoea after *C. jejuni* challenge), Figure 3.27. Group 4 data has been removed, as this was the PBS control group.

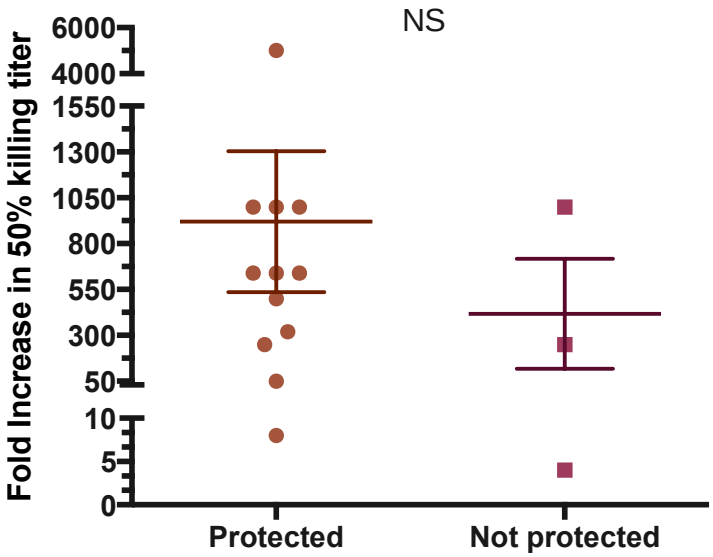


Figure 3.27 Fold-increases in 50% killing titre, grouped according to protection status following challenge, are not significantly different.
The data from Figure 3.26 was plotted to demonstrate 50% killing titres for those NHPs in the protected vs. not protected groups, Table 3.4.

Each data point represents a NHP, the data is grouped as those who did not develop diarrhoea, i.e. were protected, and those who did develop diarrhoea, i.e. were not protected when challenged. The mean fold-increase in bactericidal titre for NHP protected when challenged was 921-fold, and for those not protected, i.e. those who developed diarrhoea, it was 418-fold. This did not reach statistical significance (Mann Whitney test, $p = 0.421$). This may be due to the low number, and discrepancy in number, of animals in each group; 12 in the protected group and only 3 in the

unprotected group. Replicates were not performed on individual serum samples. Error bars show the mean and SEM.

If there had been an increase in the number of NHP in this study then it is possible that significant conclusions could be drawn from these data. With the data provided, there was an observed difference in mean fold-increase between those who were protected post-challenge and those not protected post-challenge. However some NHP who were protected had a lower fold-increase over the course of vaccinations than those who were not protected, therefore a correlate for protection could not be determined.

The data for the SBAs performed using NHP sera as well as sera from the NZWR safety study, was accepted as a poster presentation to the Vaccines for Enteric Diseases Conference 2015, at the Royal College of Physicians of Edinburgh. The poster is shown at Appendix 4.

3.6 EXAMINING HUMAN SERUM FROM THE CJCV1 PHASE 1 STUDY

The 48 human subjects in the Phase 1 Clinical Trial, described in Section 1.4.3, were vaccinated with the CJCV1 cGMP grade vaccine, with and without Alhydrogel. Those vaccinated with Alhydrogel had odd study numbers, and those vaccinated without Alhydrogel had even study numbers. The data is presented by Group, further divided into subsets with/without Alhydrogel. Using the same serum samples CPS IgG ELISA testing was carried out by Dr Renee Laird, Immunology Lead for the Campylobacter Research Group. The ELISA method and data is not shown, however, for certain subjects whose SBA results showed an increase in 50% killing titre following vaccination, the ELISA data from that serum samples is referred to. Where a subject showed a response to the vaccine their data points have been highlighted in colour, and discussed further.

3.6.1 SBA results, Group 1, vaccinated with 2 µg CJCV1

Tables 3.5 and 3.6, and Figures 3.28 and 3.29 below show data for subjects in Group 1, vaccinated with 2 µg CJCV1, with and without Alhydrogel. Despite 4 repeats, there is no data for Subject 107; for the first assay there was no titre achieved for days 0 or 56, for the second repeat the complement-only killing effect was 43 %, which increased to 75% at the third repeat, and on the forth repeat the *C. jejuni* growth was poor with low CFU counts, and the pre-vaccination titre was not reached, it was decided not to repeat the assay further. Subject 110 withdrew from the study prior to Day-56, therefore there is no data for this time-point, and the Day-56 titre for Subject 113 was not achieved despite testing up to a titre of 81,920, the fold-increase in 50% killing titre for that sample is therefore recorded as >8-fold.

Table 3.5 Data for Group 1, vaccinated with 2 µg CJCV1 with Alhydrogel

Subject Number	50% killing titre Day 0	50% killing titre Day 28	50% killing titre Day 56	Fold Increase Day 0-28	Fold increase Day 0-56	% Complement-only killing
101	10	10	20	1	2	0
103	10	10	10	1	1	0
105	10	10	10	1	1	25
107	no data	no data	no data	no data	no data	no data
109	10	10	40	1	4	0
111	10	10	640	1	64	18
113	10,240	20,480	Not achieved	2	>8	14
115	10	10	10	1	1	0

Table 3.6 Data for Group 1, vaccinated with 2 µg CJCV1 without Alhydrogel

Subject Number	50% killing titre Day 0	50% killing titre Day 28	50% killing titre Day 56	Fold Increase Day 0-28	Fold increase Day 0-56	% Complement-only Killing
102	10	10	10	1	1	0
104	10	10	40	1	4	30
106	10	10	10	1	1	12
108	10	80	160	8	16	0
110	10	10	no data	1	no data	0
112	10	10	20	1	2	0
114	20	10	40	1	2	0
116	10	10	10	1	1	0

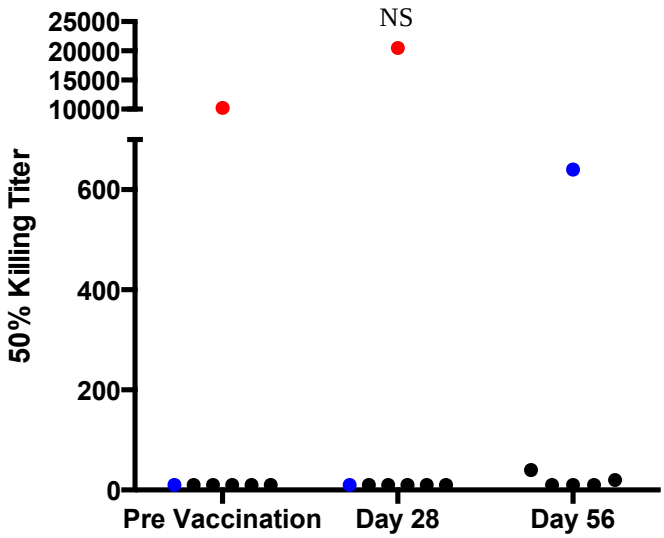


Figure 3.28 Group 1; 2 µg CJCV1 with Alhydrogel, 50% killing titres only increase in a small proportion of those vaccinated

In a 96-well plate *C. jejuni*, diluted to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 16% complement concentration. The non-control wells contained serum from human subjects in Group 1, vaccinated with 2 µg CJCV1 with Alhydrogel, from each time-point, which had been serially diluted to give final serum titres, when added to the well, from 20 to 20480, Table 2.7. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

Group 1 with Alhydrogel: Subject 113 demonstrated a high pre-vaccination titre, and the Day-56 titre was not achieved, therefore the results for this subject were removed from the statistical analysis. When that data set is removed there was no difference between increase in titre amongst this subset of the group from pre-vaccination to Day-28. There was no significant increase in titre between pre-vaccination and Day-56 (paired t-test p = 0.33) or Days 28-56 (paired t-test p = 0.33). Within this group there are two subjects singled out as having interesting results; Subject 111 (blue data point) had a 64-fold increase in 50% killing titre at Day-56, when this was compared with the CPS IgG ELISA titre data for that individual there was no increase in ELISA titre at Day-28 or 56. Subject 113 (red data point) had a very high 50% killing titre from the start, suggesting

that they may have had pre-existing functional antibodies, or a component of the immune system with a functional killing capability for *C. jejuni*, prior to vaccination. The Day-56 titre for Subject 113 was not achieved despite testing up to a titre of 81,920. Although the titres for this individual are high, the fold increase in 50% killing titre was only 2-fold at Day-28 then >8-fold at Day-56. Interestingly for this individual there was an increase in CPS IgG from pre-vaccination to Day-28 of 8-fold, and from pre-vaccination to Day-56 of 16-fold. Therefore this individual mounted an immune response to the vaccine, perhaps as a consequence of previous exposure to *C. jejuni*.

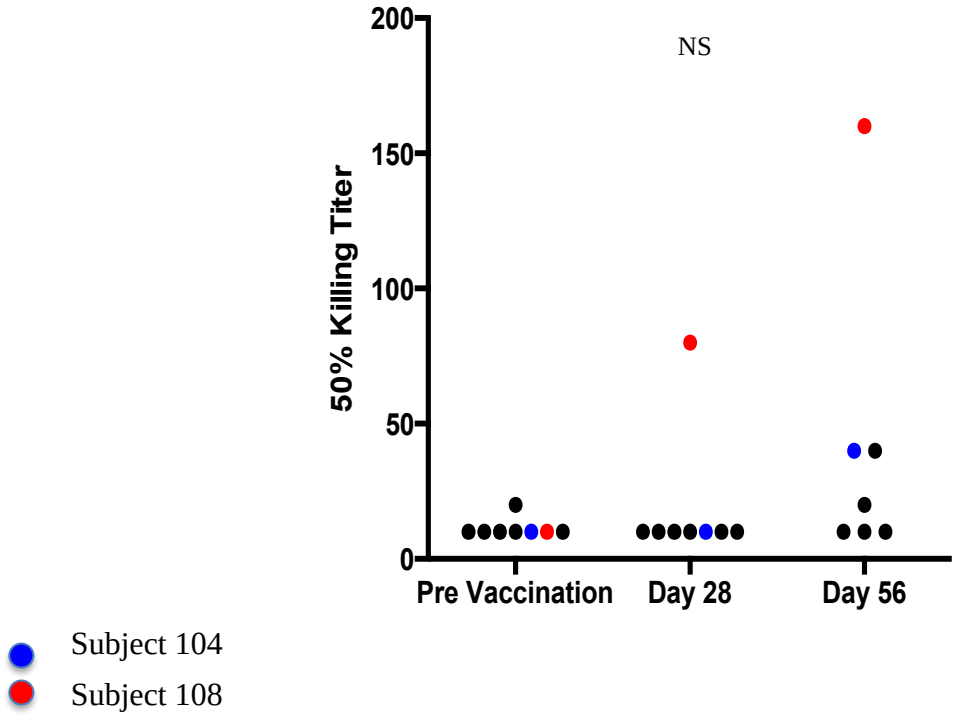


Figure 3.29 Group 1; 2 µg CJCV1 without Alhydrogel, killing titres only increase in a small proportion of those vaccinated

In a 96-well plate *C. jejuni*, diluted to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 16% complement concentration. The non-control wells contained serum from human subjects in Group 1, vaccinated with 2 µg CJCV1 without Alhydrogel, from each time-point, which had been serially diluted to give final serum titres, when added to the well, from 20 to 20480, Table 2.7. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

For Group 1, 2 μg CJCV1 without Alhydrogel, there is no significant difference between pre-vaccination and Day-28 (paired t-test p = 0.44), pre-vaccination and Day-56 (paired t-test p = 0.19) or Days 28-56 (paired t-test p = 0.10). The subjects that did show an increase in titre are Subject 104 (blue data point) and Subject 108 (red data point). Subject 104 had a 4-fold increase in 50% killing titre at Day-56, there was no increase in CPS IgG ELISA titre however. Subject 108 had an 8-fold then 16-fold increase in 50% killing titres at Days-28 and 56 respectively, however, no fold-increase in CPS IgG ELISA titres was observed at either time point.

3.6.2 SBA results, Group 2, vaccinated with 5 μg CJCV1

Tables 3.7 and 3.8, and Figures 3.30 and 3.31 below show data for subjects in Group 2, vaccinated with 5 μg CJCV1, with and without Alhydrogel.

Table 3.7 Data for Group 2, vaccinated with 5 μg CJCV1 with Alhydrogel

Subject Number	50% killing titre Day 0	50% killing titre Day 28	50% killing titre Day 56	Fold Increase Day 0-28	Fold increase Day 0-56	% Complement Killing
201	10	1280	5120	128	512	17
203	20	40	40	2	2	4
205	10	10	10	1	1	19
207	10	10	10	1	1	21
209	80	160	320	2	4	2
211	10	10	10	1	1	0
213	10	10	10	1	1	13
215	10	10	10	1	1	0

Table 3.8 Data for Group 2, vaccinated with 5 μg CJCV1 without Alhydrogel

Subject Number	50% killing titre Day 0	50% killing titre Day 28	50% killing titre Day 56	Fold Increase Day 0-28	Fold increase Day 0-56	% Complement Killing
202	10	10	10	1	1	2
204	10	10	10	1	1	2
206	10	10	10	1	1	30
208	160	640	640	4	4	0
210	80	80	160	1	2	22
212	10	10	10	1	1	31
214	10	10	10	1	1	22
216	10	10	10	1	1	26

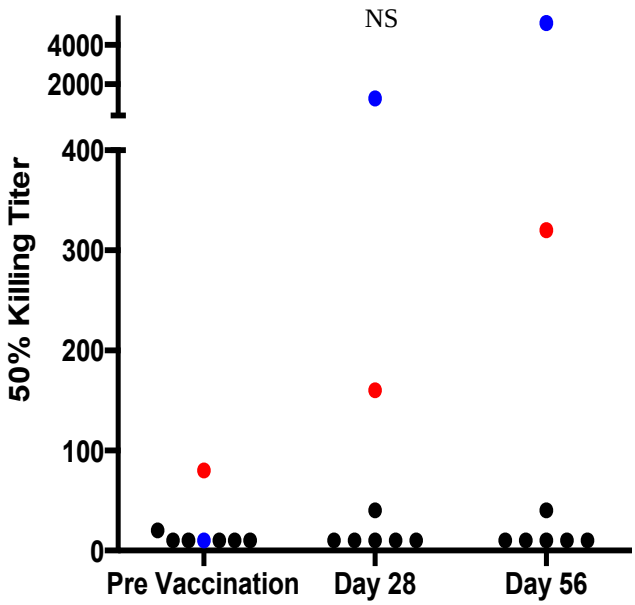
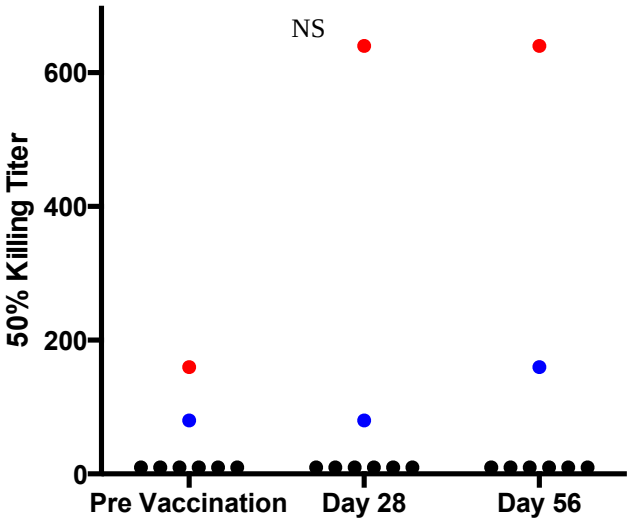


Figure 3.30 Group 2; 5 µg CJCV1 with Alhydrogel, 50% killing titres only increase in a small proportion of those vaccinated

In a 96-well plate *C. jejuni*, diluted to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 16% complement concentration. The non-control wells contained serum from human subjects in Group 2, vaccinated with 5 µg CJCV1 with Alhydrogel, from each time-point, which had been serially diluted to give final serum titres, when added to the well, from 20 to 20480, Table 2.7. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

Similar to Group 1, the results for Group 2 show no significant increase in the 50% killing titre following vaccination. For Group 2, 5 µg CJCV1 with Alhydrogel, there is no significant difference between pre-vaccination and Day-28 (paired t-test p = 0.31), pre-vaccination and Day-56 (paired t-test p = 0.33) or Days 28-56 (paired t-test p = 0.33). Again, within this group there are two subjects singled out as having interesting results; Subject 201 (blue data point) had a 128-fold increase in 50% killing titre at Day-28, and a 512-fold increase on Day-56. When this was compared with the CPS IgG ELISA titre data for that individual there was a 12-fold increase in ELISA titre at Day-28, and a 24-fold increase in ELISA titre at Day-56. Subject 209 started with

a pre-vaccination 50% killing titre of 80 which increased 2-fold on Day-28, and 4-fold on Day-56. For this individual the CPS IgG ELISA titre increased by 1.3-fold on Day-28 however there was no increase from the pre-vaccination ELISA titre on Day-56.



Subject 208

Subject 210

Figure 3.31 Group 2; 5 µg CJCV1 without Alhydrogel, 50% killing titres only increase in a small proportion of those vaccinated

In a 96-well plate *C. jejuni*, diluted to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 16% complement concentration. The non-control wells contained serum from human subjects in Group 2, vaccinated with 5 µg CJCV1 without Alhydrogel, from each time-point, which had been serially diluted to give final serum titres, when added to the well, from 20 to 20480, Table 2.7. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

For Group 2, 5 µg CJCV1 without Alhydrogel, there is no significant difference in titre between pre-vaccination and Day-28 (paired t-test p = 0.35), pre-vaccination and Day-56 (paired t-test p = 0.28) or Days 28-56 (paired t-test p = 0.35). The subjects that did show an increase in titre are subjects 208 (red data point) and 210 (blue data point). Subject 208 had a 4-fold increase in 50% killing titre at Days-28 and 56, there was also an increase in CPS IgG ELISA titres; 1.5-fold on

Day-28 and 6-fold on Day-56. Subject 210 had a 2-fold increase in 50% killing titre at Day-56, however, no fold-increase in CPS IgG ELISA titre was observed.

3.6.3 SBA results, Group 3 vaccinated with 10 μg CJCV1

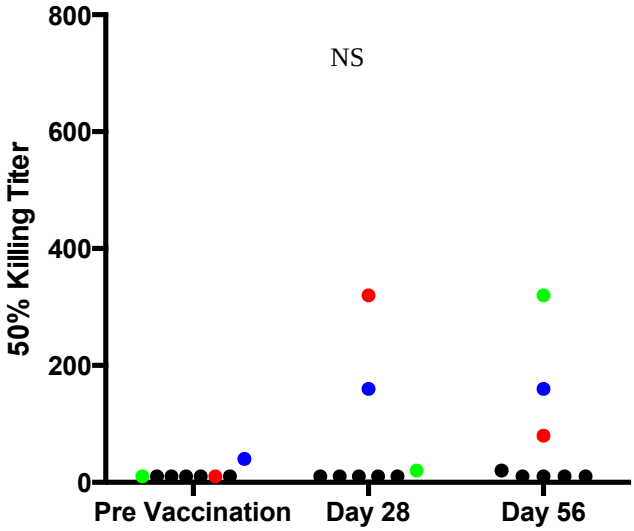
The Tables 3.9 and 3.10, and Figures 3.32 and 3.33 below show data for subjects in Group 3, vaccinated with 10 μg CJCV1, with and without Alhydrogel.

Table 3.9 Data for Group 3, vaccinated with 10 μg CJCV1 with Alhydrogel

Subject Number	50% killing titre Day 0	50% killing titre Day 28	50% killing titre Day 56	Fold Increase Day 0-28	Fold increase Day 0-56	% Complement Killing
301	10	10	10	1	1	19
303	10	10	10	1	1	12
305	40	160	160	4	4	0
307	10	320	80	32	8	23
309	10	10	10	1	1	0
311	10	10	10	1	1	0
313	10	20	320	2	32	0
315	10	10	20	1	2	0

Table 3.10 Data for Group 3, vaccinated with 10 μg CJCV1 without Alhydrogel

Subject Number	50% killing titre Day 0	50% killing titre Day 28	50% killing titre Day 56	Fold Increase Day 0-28	Fold increase Day 0-56	% Complement Killing
302	10	10	10	1	1	23
304	10	10	10	1	1	0
306	10	10	10	1	1	3
308	80	320	640	4	8	0
310	10	10	10	1	1	0
312	10	10	160	1	16	0
314	20	20	40	1	2	0
316	80	80	80	1	1	0



Subject 305

Subject 307

Subject 313

Figure 3.32 Group 3; 10 µg CJCV1 with Alhydrogel, 50% killing titres only increase in a small proportion of those vaccinated

In a 96-well plate *C. jejuni*, diluted to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 16% complement concentration. The non-control wells contained serum from human subjects in Group 3, vaccinated with 10 µg CJCV1 with Alhydrogel, from each time-point, which had been serially diluted to give final serum titres, when added to the well, from 20 to 20480, Table 2.7. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

Once more, in keeping with the results of Groups 1 and 2, and despite having the highest dose of CPS in the vaccine, the results for Group 3 show no significant increase in the 50% killing titre following vaccination. For Group 3, 10 µg CJCV1 with Alhydrogel, there is no significant difference between pre-vaccination and Day-28 (paired t-test p = 0.20), pre-vaccination and Day-56 (paired t-test p = 0.14) or Days 28-56 (paired t-test p = 0.87). Within this group there are three subjects singled out as having interesting results; Subject 305 (blue data points) had a 4-fold increase in 50% killing titre at Days-28 and 56. When this was compared with the CPS IgG ELISA titre data for that individual there was no increase in ELISA titre at Day-28, however there was a 6-fold increase in ELISA titre at Day-56. Subject 307 (red data points) demonstrated an

increase in 50% killing titre of 32-fold on Day-28, however this fell to 8-fold on Day-56. Interestingly, despite these SBA fold increases, for this individual the CPS IgG ELISA titre did not increase on Days-28 or 56. The third data set of interest is Subject 313 (green data points), this individual had a 2-fold increase in SBA titre at Day 28 but a 32-fold increase in SBA titre at Day-56. The IgG CPS ELISA data for this individual shows no increase at Day-28, and only a 1.5-fold increase at Day-56.

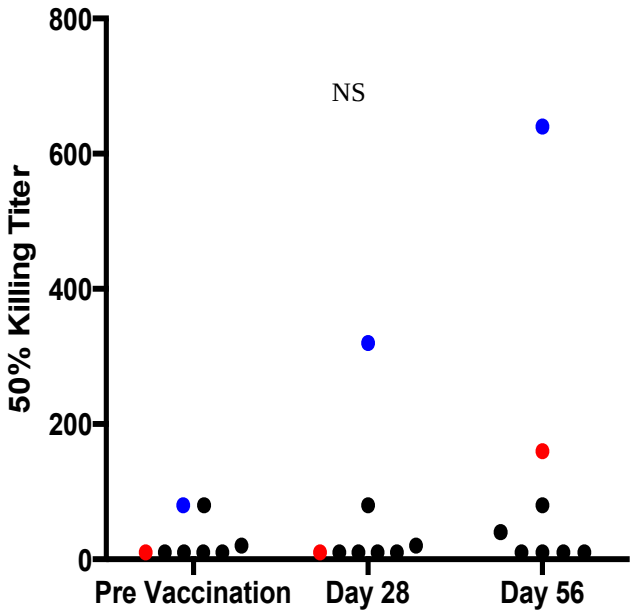


Figure 3.33 Group 3; 10 µg CJCV1 without Alhydrogel, 50% killing titres only increase in a small proportion of those vaccinated

In a 96-well plate *C. jejuni*, diluted to 1 x 10^7 in diluting tubes of DGV and 0.005% Triton X 100, was added to DGV and complement, the final volume giving a 16% complement concentration. The non-control wells contained serum from human subjects in Group 3, vaccinated with 10 µg CJCV1 without Alhydrogel, from each time-point, which had been serially diluted to give final serum titres, when added to the well, from 20 to 20480, Table 2.7. The 96-well plate was incubated at 37°C under microaerobic conditions for 60 minutes then well contents plated and incubated for a further 48 hours at 37°C under microaerobic conditions.

For Group 3, 10 µg CJCV1 without Alhydrogel, there is no significant difference between pre-vaccination and Day-28 (paired t-test p = 0.35), pre-vaccination and Day-56 (paired t-test p =

0.23) or Days 28-56 (paired t-test p = 0.18). The subjects that did show an increase in titre are subjects 308 (blue data points) and 312 (red data points). Subject 308 had a 4-fold increase in 50% killing titre at Day-28 and a 8-fold increase at Day-56, however, this was not reflected in the CPS IgG ELISA titres; which showed no titre increase on Day-28 and only a 1.5-fold increase on Day-56. Subject 312 had no increase in 50% killing titre at Day-28, however had a 16-fold increase in SBA titre on Day-56; again no fold-increase in CPS IgG ELISA titre was observed on Day-28, and only a 1.5 fold increase on Day-56.

3.6.4 Fold-increases in 50% killing titres, Groups 1, 2 and 3

In order to determine whether there is a dose dependent effect in achieving a higher level of functional antibody response, the 50% killing titre is not necessarily the best data point to use as some individuals have a higher measurable titre prior to vaccination than others. Instead the corresponding fold-increases in 50% killing titre achieved at different time points in comparison to baseline can be analysed as these compare the effect of vaccination to the pre-vaccination level, so the baseline is always 1. Figure 3.34 below shows the fold increases from pre-vaccination to Day-56 by Group.

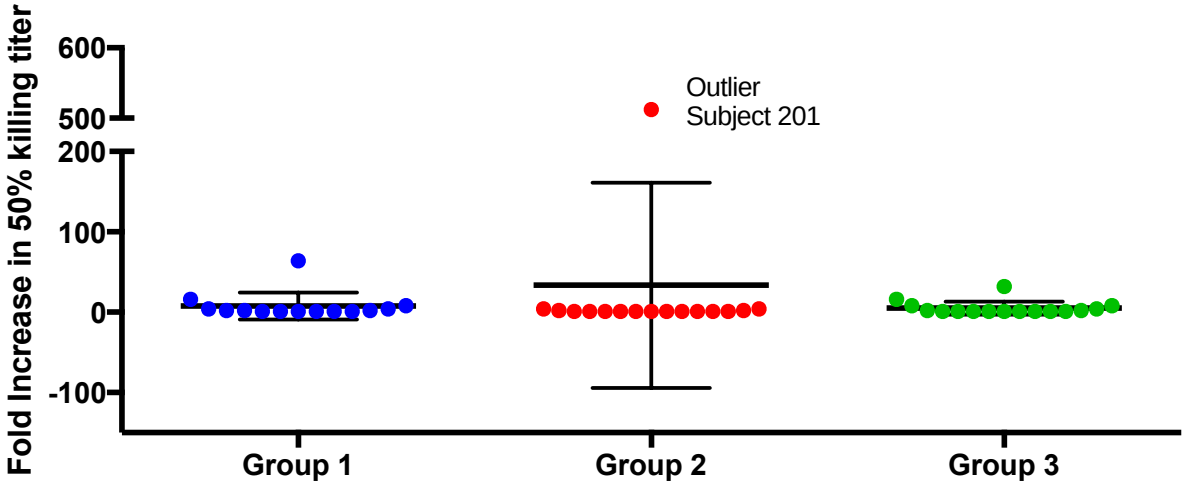


Figure 3.34 Very limited Day-56 fold-increase in 50% killing titre by Group

These data have been replotted from data determined in Figures 3.27 – 3.32 and shows fold increases in 50% killing titres. Each data point represents a Subject, the error bars show the Mean and SEM.

As there is clearly an outlier, Subject 201 with a 512-fold increase at Day 56, the data point has been removed in Figures 3.35 and 3.36.

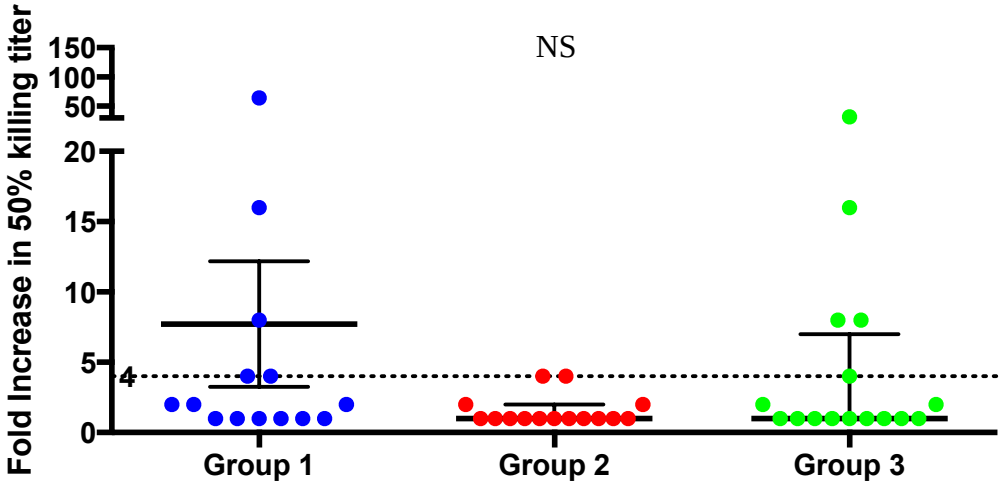


Figure 3.35 Very limited Day-56 fold-increase in 50% killing titre by Group, outlier removed, showing Mean and SEM

These data have been replotted from data determined in Figures 3.28 – 3.33 and shows fold increases in 50% killing titres. The outlier has been removed. Each data point represents a Subject, the error bars show the Mean and SEM.

Having removed the data point for Subject 201, there were no significant differences in the fold increases between Groups 1 and 2 (unpaired t-test p = 0.164), Groups 1 and 3 (unpaired t-test p = 0.570) or Groups 2 and 3 (unpaired t-test p = 0.113).

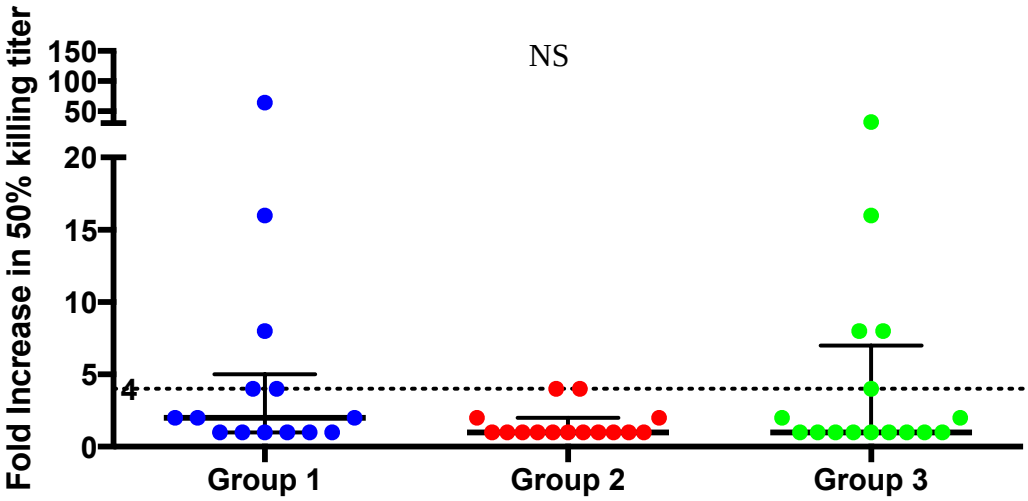


Figure 3.36 Day-56 fold-increase in 50% killing titre by Group, outlier removed, showing the Median and Interquartile Range

These data have been replotted from data determined in Figures 3.28 – 3.33 and shows fold increases in 50% killing titres. The outlier has been removed. Each data point represents a Subject, the error bars show the Median and Interquartile Range.

Figures 3.35 and 3.36 have a horizontal line at Y=4. The relevance of this line is that other capsule conjugate vaccines, specifically the meningococcal serogroup C conjugate (MCC), considers protective efficacy to have been achieved by demonstrating either a 50% killing titre >128, or, if a titre of between 8 - 128 is achieved, a $\geq$4-fold increase in 50% killing titre when tested by SBA using rabbit complement [90]. Therefore, for the MCC vaccine a $\geq$4-fold increase in 50% killing titre is considered to be a correlate for protection.

Whilst such a numerical value for a correlate or surrogate for protection cannot be applied directly to the CJCV1 vaccine until a vaccination and challenge study has been performed, it can act as a guide. Of the 45 subjects for whom a Day-56 data point has been entered in Figures 3.35

and 3.36, few demonstrated an increase in 50% killing titre, and only 12 (26%) met the ≥4-fold increase in titre threshold.

In view of the immunological data, including results from the SBA analysis, for this Phase 1 study, the CJCV1 vaccine did not proceed to a Phase 2 study.

3.7 DISCUSSION

3.7.1 The development of a functional SBA

Development of a functional SBA to determine the highest titre of serum, obtained from vaccinated Subjects, capable of achieving 50% killing of *C. jejuni* required the investigation of multiple conditions and method evolution. *C. jejuni* is an organism that requires very specific environmental conditions, not only for culture, but also in order to survive; the optimisation of this environment formed a large part of this thesis. There are significant limitations to these data. In view of the limitations and lack of robust reproducibility, the first objective of the thesis was not fully met. However whilst this SBA development process did not formally identify a correlate for protection, it advanced the process of towards finding a correlate for protection, an important step in the development of a commercially available vaccination for *C. jejuni.*

A major limitation to these data is the lack of replicates performed, which impacts upon statistical analysis. For many of the assays that contributed to the initial evolution and development of the SBA method replicates were not performed. This was due to the finite amount of serum from the single hyper-immune rabbit, the source of serum for the initial assay development. This is recognized as a limitation however towards the conclusion of the assay evolution, when serial dilutions and titres were examined, replicates were performed. Despite a lack of replicates, over the course of the SBA development many trends were observed, such as the inverse killing effect at higher volumes of serum, and the high complement-only killing effect.

The high level of complement killing throughout the SBA development was a cause for concern and the examination of different complement concentrations featured highly. The susceptibility of

C. jejuni to complement appears to be variable and may represent a feature of phase variation. Maintaining a constant complement concentration, once determined, in the assay method is important to mitigate variability due to complement-only killing, however other factors such as phase variation may well be implicated in the effect of complement and this cannot be controlled for.

Phase variation is a limitation as it alters the behaviour of *C. jejuni*, and it affects the sensitivity of the bacteria to specific environmental conditions as characteristics, such as the status of capsule expression [7]. Whilst this may alter the results, it is a phenomenon that will also occur in the natural environment and therefore, when a vaccine for *C. jejuni* is developed, the efficacy of the vaccine may be quite variable, depending upon the phase that the bacteria adopts upon contact with the host. As mentioned above, phase variation of the bacteria could not be controlled for.

Another limitation is that *C. jejuni* growth in the control well was often poor leading to the assay being discounted. This variability in both behaviour and growth suggests that SBA methods may not be the most reliable method to use to test bactericidal effects.

A limitation when testing replicates for the hyper-immune NZWR serum was that some results were highly non-reproducible. The possible reasons for this include bacterial phase variation, the amount of CPS expressed by the bacteria, which we did not demonstrate, and technical error.
Following the data collected in the Phase 1 trial the SBA method was further evolved by other members of the Campylobacter Working Group as the duration of my Military Exchange period with the US Navy had been completed. The new method is based upon flow cytometry.

3.7.1.1 The pro-zone or 'hook' effect

During the early stages of method development it became evident that, at the higher volumes of serum used, a lower % killing was seen, demonstrating a lesser bactericidal effect than at the lower volumes. Therefore the expected upward trend of an increase in serum volume leading to an increase in % killing effect was not achieved, indeed the inverse occurred. This appeared to be consistent, occurring with many assays using different methods, and suggested that the results were demonstrating the 'pro-zone', or 'hook' effect. This phenomenon is described in clinically used diagnostic tests when a false negative result is achieved in the presence of a high concentration of analyte, such as antibodies. The 'pro-zone' refers to the zone of higher analyte concentrations where no reaction occurs. It has been well established in tests for syphilis [123] and Cryptococcus [124] where a high concentration of antibody may lead to a false negative result, which becomes positive upon serial dilutions of the serum. This has also been seen in-vivo in patients with HIV, and therefore a dysregulated B cell immune response [125]. In addition, during outbreaks of *Salmonella* infection amongst populations with a high prevalence of HIV, individuals with HIV have a very severe form of salmonellosis despite high titres of antibody against the bacteria [125]. The possibility of a pro-zone effect in SBAs using serum following meningococcal polysaccharide vaccination has also been historically described [93].

3.7.2 Establishing a bactericidal killing titre in vaccinated NZWR, CJCV1 safety study

Campylobacter spp. Have a high epidemiological prevalence and prior exposure to the bacteria may have occurred in the safety study NZWR, which were not screened for prior exposure to *C. jejuni*, this may account for the higher baseline titres. A means of establishing seroconversion, and protection, following vaccination is by determining the 50% killing bactericidal titre of the

serum. As described in Chapter 1, a bactericidal titre of ≥4 has been established as the correlate for protection for other gram-negative encapsulated organisms [90], and a titre of ≥4 is applicable when human complement is used in the SBA; if rabbit complement is used the protective titre is 128. However, for those subjects with a titre, using rabbit complement, between 8 and 128, a fold-increase in 50% killing titre of ≥4-fold is also a correlate for protection [90]. If this primary endpoint of ≥4-fold increase in SBA titre following capsule conjugate vaccination were to be applied to the serum of the CJCV1 Safety Study NZWR, then it suggests that, were they to have been challenged with *C. jejuni*, those with a fold increase in titre ≥4-fold would be protected. The mean fold increase in 50% killing titre from baseline to day-71 was 5.4-fold for Group 2 and 5.6-fold for Group 3. Whilst both demonstrated a mean fold increase of ≥4, only 33% of animals in Group 2 and 50% of animals in Group 3 achieved this.

The New Zealand rabbits used in the safety study were 10-16 weeks old at the initiation of the study; there may be age related discrepancies in the robustness of the immune response produced. The effect of different IgG sub-classes causing differences in bactericidal properties due to different levels of complement activation is not an issue as rabbits produce only one class of IgG [126]. Rabbits produce different subclasses of IgA however IgA does not have such a strong effect on complement activation, which is required in a SBA.

3.7.3 Establishing a bactericidal killing titre in vaccinated and challenged *A. nancymaae*

The NHP used for the dose escalation study were pre-screened for evidence of prior exposure to *C. jejuni*. Despite this, SBA titres that achieved 50% killing were very high, with a mean fold increase of 921-fold in those animals who proved to be protected from diarrhoeal illness when

challenged. This, whilst an impressive fold increase, did not achieve statistical significance when compared to the mean fold increase of 418-fold in unprotected NHP. This may have been due to the low numbers of animals in the unprotected group. The high fold-increase in the unprotected group suggests that, for these animals, a ≥4-fold increase in 50% killing titre does not confer protection. However, the numbers are small, with only 3 animals in the unprotected group.

When considering the IgG subclass produced by *A. nancymaae* there is little information available. Not all NHP produce the same IgG subclasses as humans [127]. Human IgG subclasses, IgG1, IgG2, IgG3 and IgG4, have been compared to those of two Old World monkeys; the baboon and the macaque [127]. It was demonstrated that, similar to humans, baboons produce the IgG1, IgG2, IgG3, and IgG4 subclasses, while macaques produce only IgG1, IgG2, and IgG4. Phylogenetically, the great apes (chimpanzees, orangutans, gorillas and gibbons) are most closely related to humans, followed by the Old World monkeys (including baboons, African green monkeys, and macaques) [127]. The primate species the most distantly related to humans are the New World monkeys (including Aotus monkeys, capuchin monkeys, and squirrel monkeys) [127] therefore immune responses seen in *A. nancymaae* may not be seen in humans.

The sub-class of IgG produced by the *A. nancymaae* was not tested. Given the differences in IgG subclasses produced amongst members of the Old World monkey group, it would be interesting to investigate the differences in IgG subclass between humans and the New World monkey *A. nancymaae.* The major effector function for the IgG3 subclass in humans is activation of the complement system, more so than IgG1, IgG2 or IgG4 [49], so determining the *A. nancymaae* IgG subclasses and corresponding human subclass would help further understand the intricacies of the SBA.

3.7.4 Establishing a bactericidal killing titre using human sera, CJCV1 Phase 1 study

These data showed a large degree of variability in response to the vaccine, irrespective of whether the vaccine was administered with or without Alhydrogel, with only a few individuals responding in a way that could be detected by SBA, however in some cases these SBA titres were high. These discrepancies are hard to explain. Overall the immune response, as demonstrated by SBA, was very low, this may have been a fault of the SBA however when the study ELISA data was examined the CPS IgG response was also low. In view of this the vaccine CJCV1 did not proceed to Phase 2 Clinical Trials, instead the structure of the vaccine was examined and modified. Therefore, whilst the SBA was not able to demonstrate a correlate for protection, the results were informative and contributed to the development of a *C. jejuni* vaccine that may become commercially available.

It is clear from the results that there are large discrepancies in the titres achieved using the SBA method, firstly for the NZWR, then the NHP, and finally the human sera in the Phase 1 CJCV1 study. The titres achieved using the sera from the NHP were so high that a log scale had to be used in the presentation of results. This difference could possibly be explained by the different type of vaccine used for the studies. For the NHP vaccination and challenge study a non-GMP formulation of the vaccine was used, for the NZWR and human vaccination studies the cGMP grade vaccine CJCV1 was used. It is possible that in different formulations of the vaccine there are different amounts of either free capsular polysaccharide, or methyl phosphoramidate, present.

3.8 FUTURE SBA MODELS

The SBA methods described above were labour and resource intensive. Whilst a SBA method has been established for use with *C. jejuni*, and has been shown to demonstrate a functional bactericidal titre in immunized NZWR, *A. nancymaae* NHP and humans, this method is under constant revision by the Campylobacter Working Group at NMRC and at present a flow-cytometry based SBA is being developed.

3.9 PROPHYLAXIS FOR CAMPYLOBACTERIOSIS IN THE NEAR FUTURE

Vaccine development, including establishing a correlate or surrogate for protection, takes time. The requirement for prophylaxis against diarrhoeal disease remains high within US and UK Military research priorities. It is due to this requirement that an alternative to vaccination was investigated, and the clinical trial Double Blind, Placebo-Controlled Trial Assessing the Efficacy of Rifaximin in Preventing Campylobacteriosis in Subjects Challenged with *Campylobacter jejuni, ClinicalTrials.gov Identifier: NCT02280044, was carried out. The planning and* execution of this Clinical Trial fulfils the second objective of this thesis.

CHAPTER 4 RESULTS FOR OBJECTIVE 2 – TO DEMONSTRATE THE PROTECTIVE EFFICACY OF RIFAXIMIN VS. PLACEBO IN A *C. JEJUNI* CG8421 HUMAN CHALLENGE MODEL

4.1 INTRODUCTION

The development, testing and licensing of an efficacious vaccine for use in a select human population, military or civilian, for non-experimental purposes, takes many years. In the meantime, for health protection purposes, it is desirable to have an alternative method of prophylaxis to be used, in specific circumstances, for the prevention of diarrhoeal disease. As one of the bacterial pathogens recognized as being most prevalent, and responsible, for diarrhoeal disease, *C. jejuni* is an appropriate pathogen against which to evaluate the potential prophylactic pharmacological candidate, the minimally absorbed antibiotic rifaximin.

In order to facilitate this the following trial, funded by the United States Navy, Advanced Medical Development, was carried out:

A Double Blind, Placebo-Controlled Trial Assessing the Efficacy of Rifaximin in Preventing Campylobacteriosis in Subjects Challenged with *Campylobacter jejuni*

ClinicalTrials.gov Identifier: NCT02280044

4.1.1 Personnel in the Research Team

The Principal Investigator was initially Dr Clayton Harro, MD, of the Department of International Health, Johns Hopkins University, Baltimore. Sadly Dr Harro passed away during the study, therefore the role was taken over by Dr Kawsar R. Talaat, MD, also of the Department of International Health, Johns Hopkins University. My role was that of NMRC Lead Investigator, and I was very closely supported by members of the NMRC Enteric Diseases Department, both the Clinical Trials Group; Dr Mark Riddle, Dr Ramiro Gutierrez, Dr Chad Porter, Dr Robert Gormley, Dr Chris Duplessis, Vicky Chapman, Kayla Jaep, Ashley Alcala, and the Campylobacter Research Group, led by Dr Patricia Guerry.

When my exchange period with the US Navy finished, and I left NMRC to return to the UK, Dr Chad Porter took over my role as lead NMRC Investigator and he was responsible for co-ordination of data analysis for the clinical data collected during the trial.

Other NMRC Investigators for this trial were Dr Mark Riddle, Dr Ramiro Guttierez, Dr Chris Duplessis, Dr Chad Porter, Dr Patricia Guerry, Dr Alex Maue and Dr Renee Laird, as well as Dr David Tribble from the Infectious Disease Clinical Research Program, Uniform Services University, Bethesda, Maryland. The study was conducted at the Center for Immunization Research (CIR) Isolation Unit, Bayview Campus, Johns Hopkins Hospital, Baltimore, Maryland.

The clinical laboratories and other departments or institutions involved in the trial were as follows;

- Site investigational product accountability: Johns Hopkins Bayview Medical Research Pharmacy;
- Site investigational product accountability (challenge strain): CIR Enterics Research Laboratory (JHSPH);
- Clinical laboratories: Quest Diagnostics, Inc. and Johns Hopkins Bayview;
- Research laboratories: Patricia Guerry, PhD Molecular Biology Laboratory, Enteric Diseases Department, NMRC; Renee Laird, PhD Immunology Laboratory, Enteric Diseases Department, NMRC; Dr David Sack, MD, CIR Enteric Research Laboratory (JHSPH).

The statistician was Dr Chad Porter, PhD, MPH (EDD, NMRC), and Data Management was handled by the EMMES Corporation, 401 N Washington Street, Rockville, Maryland. The Institutional Review Boards (IRB) was the Western Institutional Review Board which collaborated with the NMRC Institutional Review Board.

4.1.2 Personnel involved in data collection and analysis

To determine whether or not each subject met the primary endpoint of campylobacteriosis, data relating to the number and volume of stools produced, as well as symptoms experienced, was used. These data were collected by the research team working within the Center for Immunization Research (CIR) Isolation Unit, Bayview Campus, Johns Hopkins Hospital, of which I was part. An independent adjudication board; Dr AL Bourgeois, Dr C Lyon and Dr D Blazer, used these data to make the final determination of whether or not subjects met the primary endpoint, Dr Chad Porter then analysed the data when it was unblinded.

4.1.3 My roles within the Clinical Trial

My role in this study as lead NMRC Investigator encompassed many aspects of study design and execution, including administrative, laboratory based and clinical roles.

4.1.3.1 Administrative Role

With regard to document preparation and the formalities of reporting events, as the lead NMRC Investigator I was responsible for the development of the Protocol, initially for submission to the Scientific Review Board and later to the IRB. I was also responsible for the development and editing of Study Specific Procedures (SSPs), liaising with all members of the research team for their input into the documents and then incorporating these into final versions of the for use in the trial. As the clinical trial progressed I was also responsible for the reporting of any significant adverse events (SAE), any protocol modifications, or memos to file that were required. For each of these steps my work was supervised by the NMRC Clinical Trials Group. When I left my post at NMRC and Dr Chad Porter assumed the role of Lead Navy Investigator the study data was still blinded. I do not have access to the Case Record Files as they are property of the US Navy, and data analysis was completed by the rest of the Investigative Team.

4.1.3.2 Laboratory Role

In terms of laboratory procedures I worked with JHSPH staff to prepare, transport and administer the challenge inoculum. Preparation of the challenge inoculum had to be practiced many times and it became evident from preparation re-runs that the final CFU in the inoculum was often variable. This led to the development of an inoculum preparation SSP that had some inbuilt flexibility so that as the preparation process was underway, modifications could be made to

increase the likelihood of reaching the target CFU in the final inoculum. This SSP is included at Appendix 6.

4.1.3.3 Clinical Role

I reviewed the subjects with the Primary Investigator daily during the inpatient phase and was present at outpatient visits to review subjects who required review, as determined by the study research nurses. When a subject required medical review by a secondary care specialist, as was the case when 2 subjects developed joint pains and required a Rheumatology review, I accompanied them. These cases will not be elaborated upon as it was determined that they did not have *C. jejuni* related reactive arthritis.

For the purposes of this thesis only the work directly that was carried out by myself, or towards which I contributed significantly, will be presented, this will include the clinical data collected during the inpatient and outpatient periods, which determined whether the primary endpoint of campylobacteriosis was met.

4.2 STUDY OBJECTIVES AND ENDPOINTS

The primary and secondary study objectives are described below. Only the results of those highlighted in bold will be described in this thesis, as they were the objectives specifically linked to my role in the study.

4.2.1 Primary Objective

- **Estimate the preliminary efficacy of a standard rifaximin dose (550 mg BID) in preventing campylobacteriosis subsequent to experimental infection.**

4.2.2 Secondary Objectives

- **Evaluate the efficacy of rifaximin chemoprophylaxis in reducing various clinical and microbiological outcomes (e.g. disease severity, fever, dysentery, stool frequency, shedding).**
- Evaluate the effect of rifaximin chemoprophylaxis on acquired resistance to *C. jejuni* experimental challenge strain.
- Evaluate the natural immune response to *C. jejuni* infections in humans.

The primary and secondary endpoints are described below:

4.2.3 Primary Endpoint

- The primary endpoint was Campylobacteriosis, defined as a clinical illness meeting at least one of the following patterns:
  • Moderate to severe diarrhoea.
  • Fever (present on at least 2 occasions, at least 20 minutes apart) without diarrhoea, plus an associated symptom (nausea, vomiting, abdominal cramps, tenesmus, or gross blood in ≥ 2 stools); with consideration of potential alternative diagnosis per clinical investigator based on illness time course and associated symptoms.

4.2.4 Secondary Endpoints

4.2.4.1 Efficacy Endpoints

The secondary efficacy endpoints were chosen to support the primary endpoint in determining the protective efficacy of rifaximin.

- Number of subjects with moderate-severe campylobacteriosis
- Mean total weight of grade 3-5 stools passed per subject
- Mean number of grade 3-5 stools per subject
- Number of subjects with nausea, vomiting, anorexia, or abdominal pain/cramps rated as moderate to severe
- Number of subjects with dysentery or fever
- Mean time to onset of diarrhoea
- Number of colony forming units of the challenge strain per gram of stool
- Number of subjects requiring early antibiotic treatment

4.2.4.2 Microbiology Endpoints

Intestinal colonization by the challenge strain was assessed by monitoring faecal shedding patterns (by stool culture) in subjects daily from Day-0, after the challenge inoculum was administered, up to discharge and at Days 14 (±1), 21 (±2), 28 (±2), 35 (±2), 56 (±4) and 84 (±4).

4.3 DEFINITIONS AND CLASSIFICATIONS

4.3.1 Diarrhoea

- One loose/liquid stool (grade 3-5, see Table 4.1, below) of ≥ 300 g within 144 hours of challenge with *Campylobacter;* OR
- At least two loose/liquid stools totalling ≥ 200 g during any 48-hour period within 144 hours of challenge with *Campylobacter*; OR
- ≥ 3 loose or liquid stools in a 24-hour period regardless of volume.

Table 4.1 Grading system for stools

Grade 1	Firm, formed (normal)
Grade 2	Soft, formed (normal)
Grade 3	Viscous opaque liquid or semi-liquid which assumes the shape of the container
Grade 4	Watery, non-viscous, opaque liquid which assumes the shape of the container
Grade 5	Clear or translucent, watery or mucoid liquid which assumes the shape of the container
Frank Blood	Demonstrated in Figure 4.1

Figure 4.1 Frank Blood in Stool

This is a photograph of a stool sample collected during the inpatient phase of the study, showing the degree to which *C. jejuni* infection results in frank blood in the stool.

4.3.2 Diarrhoea severity (within 144 hours of challenge)

Diarrhoea severity was determined by the frequency and weight of loose stools (Grade 3-5) during a 24-hour period. The severity grade selected was the higher severity by either frequency or weight. Table 4.2 illustrated the severity grading scheme.

Table 4.2 Diarrhoea Severity Grading Scheme

Severity Grade	Diarrhoea Frequency within 24 hrs	Diarrhoea Weight within 24 hours
Mild	3	1 stool ≥ 300 g 2–3 stools 200–400 g
Moderate	4–5	401–800 g Grade 3-5
Severe	≥ 6	> 800 g Grade 3-5

4.3.3 Fever

This was defined as an oral temperature ≥ 38.0°C (100.4° F) present on at least two occasions, 20 minutes or more apart.

4.4 STUDY PROCEDURES

4.4.1 Recruitment, randomisation and challenge

Subjects were recruited from the greater Baltimore, Washington DC and Philadelphia regions. The inclusion and exclusion criteria for this study were extensive, and are listed at Appendix 8. The power calculation for the study determined that a sample size of 14 subjects per group yielded >80% power to detect a significant difference in the rate of campylobacteriosis, therefore 28 subjects were required, with six alternates recruited to replace anyone who failed to attend, or were unable to participate at the time of planned admission or challenge administration. The following graphic, Figure 4.2, illustrates the numbers screened, randomized and challenged, it also illustrates the demographics of the 28 who were challenged and followed up during the study.

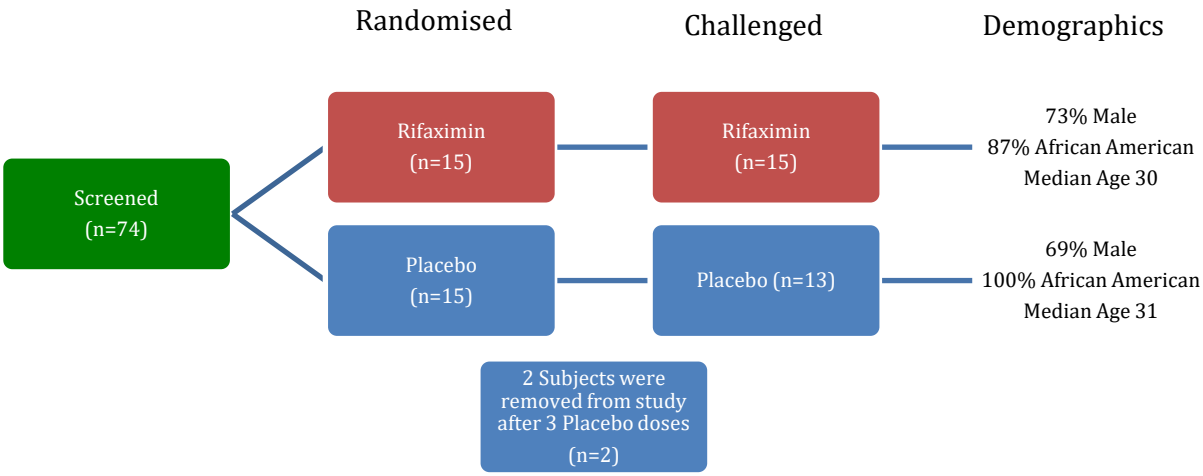


Figure 4.2 Subject Numbers and Demographics

15 Subjects received rifaximin and 13 subjects received the placebo. The Subjects were predominantly male and of African American Ethnicity, with a mean age of 31.

Thirty of the Subjects were randomised and received the first three doses of rifaximin 550 mg, or placebo, administered on days -1, and 0, however only 28 subjects received the oral *C. jejuni* CG8421 challenge inoculum and remained as inpatients. It had been intended that one Subject from each group, rifaximin or placebo, would be removed from the study prior to challenge, leaving 14 subjects in each group, however, the PI identified two individuals who were not suitable to remain in the inpatient unit, both were in the placebo group, and so, following discussion with the statistician, they were removed from the study.

4.4.2 Time-points for intervention and sample collection

This study had screening, inpatient and outpatient periods. The study timeline is detailed below in Figure 4.3, it is an abridged version of the formal study timeline; time points for stool and sera

collection for immunological analysis have been removed as the data for these will not be discussed.

Table 4.3 Study Timeline

Study Event	Screening visits (1-2)	-1	0	1	2	3	4	5	6	7	8	9	14	21	28	35	56	84	180
Compliance Range (study day)	-60 to -3	--	--	--	--	--	--	--	--	--	--	± 2	± 1	± 2	± 2	± 2	± 4	± 4	±30
Outpatient Visits																			
Inpatient[1]																			
Admission to JHU CIR		x																	
Informed Consent & Comprehension test	x																		
Medical interview/Physical assessment[2]	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Campylobacter prior exposure tests[3]	x																		
Screening clinical labs[4]	x																		
Stool sample pre-challenge[5]	x																		
Rifaximin Treatment		x	x	x	x														
C. jejuni challenge			x																
Stool weighing/grading			x	x	x	x	x	x	x	x	x	x							
Stool collection (bacteriology)[6]			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Antibiotic therapy initiated[7]									x										
Approximate Hospital discharge[8]												x							
Telephone follow up																			x
Study completion[9]																			x

Legend for Study timeline:

1. Inpatient period may be reduced if discharge criteria met prior to day 9. In this case the subject will present to the clinic on days 7 and/or 9 as applicable (not day 8).
2. Baseline medical interview establishes eligibility. During the inpatient phase of the study, interview (plus targeted exam based on symptoms) updates medical history and confirms on-going eligibility. During the follow up phase interview, assessments and as needed targeted physical exam will assess safety.
3. *Campylobacter jejuni* prior exposure tests; of these tests, only strain-specific anti-glycine extract IgA serology will determine eligibility for the study.
4. Screening clinical Labs: Haematology; complete blood count and WBC differential; Serum chemistry; Creatinine, glucose, ALT; Additional; HIV, HBsAg, HCV, IgA, HLA-B27 and a serum pregnancy test for females.
5. The pre-challenge stool specimen is for research purposes only, and includes assessing the subject's pre-trial microbiota. This is very important and whilst failure to produce one is not an exclusion criteria, they will be encouraged to provide one during the screening period or on admission, prior to any medication or challenge. If they fail to produce a stool then a rectal swab will be taken and if faecal matter is not seen on the swab they will be re-swabbed until faecal matter is obtained.
6. Faecal samples will be collected for detection of *C. jejuni* strain CG8421 and for exploratory host microbiota assays. During Inpatient, for subjects who meet criteria for early discharge stools for culture will not be collected on the day subject is eligible for discharge through the end of the inpatient period. Subjects will resume stool testing for culture at the Day 14 visit.
7. Azithromycin 500 mg daily x 5 days concurrently with ciprofloxacin 500 mg twice daily x 5 days initiated on day 6 unless criteria met for early antibiotic treatment.
8. If subject fails to meet discharge criteria on day 9, s/he will receive a medical interview, vital signs, and physical exam and stool will be collected on each subsequent inpatient day, until they meet discharge criteria.
9. Study completion at day 180 after telephone follow-up, 6-month post-inoculation (+/- 1 month) phone calls will inquire about new onset serious health events.

4.4.3 *C. jejuni* CG8421 challenge inoculum

A human challenge model for studies involving the use of *C. jejuni* has been established. Previously strain 81-176 had been used for these studies however due to ganglioside mimicry (described in Chapter 1) and the risk of precipitating GBS, this has been superseded. For human challenge studies, C. *jejuni* CG8421 is now the strain used as it is considered to be safe. The lack of ganglioside mimicry in its lipooligosaccharide mitigates the risk of GBS associated with *Campylobacter* infection [128], and the strain provides a consistently high attack rate and robust immune responses when using a low concentration of inoculum ($5x10^5$ CFUs [69]. The benefit of this is that human challenge studies can be performed without the risk of causing GBS, however it does have limitations. The main limitation is the data may not reflect the effect that prophylaxis with rifaximin has upon other strains with different levels of virulence, such as strain 81-176. In order to examine protection against strains encountered by travellers a field-study is required.

4.4.3.1 Inoculum preparation and administration

The challenge inoculum was administered on Day-0 of the inpatient period (Table 4.4) having been cultured at the JHSPH, Appendix 6. The challenge dose was confirmed by colony counts to have been approximately 1.8×10^5 CFU. The inoculum was administered in a bicarbonate buffer solution. Subsequent *C. jejuni* CG8421 faecal shedding was confirmed in all subjects.

4.4.4 Antibiotic treatment with Azithromycin and Ciprofloxacin

All subjects received treatment antibiotics, Ciprofloxacin (500 mg twice daily for 5 days) and Azithromycin (500 mg once daily for 5 days) on the morning of study day 6 (approximately

144 hours after challenge) unless they met early treatment criteria. The challenge strain was known to be susceptible to both antibiotics. Earlier treatment was provided to Subjects meeting any of the following criteria as they would have achieved the primary endpoint:

• Severe diarrhoea (> 6 grade 3–5 stools in 24 hours or > 800 g of Grade 3–5 stools in 24 hours)

• Moderate diarrhoea for 24 hours (4–5 loose stools in 24 hours or 401–800 g of grade 3–5 stools in 24 hours)

• Diarrhoea (any severity) AND two or more of the following symptoms: severe abdominal pain/cramps, severe nausea, severe headache, severe myalgia, severe arthralgia, gross blood in loose stools (2 specimens), any fever 38.0ºC (100.4ºF), or any vomiting

• Early treatment warranted by a study physician for other reasons

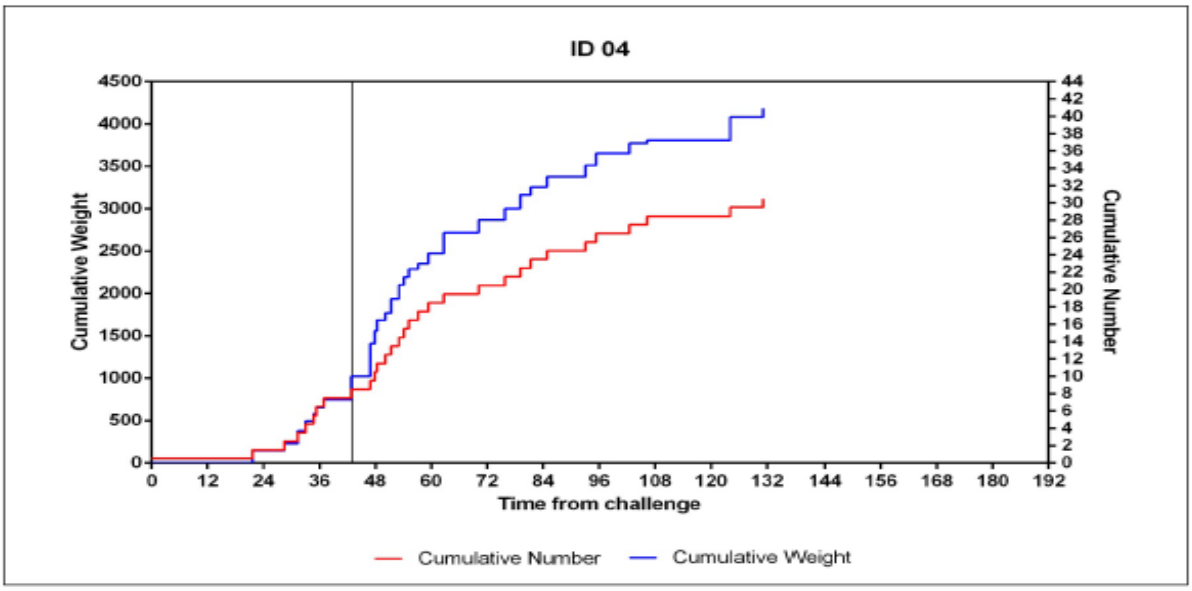
4.5 DATA COLLECTION; DATA ENTRY AND MONITORING

Data was entered onto paper case report files at the time of each Subject routine review, or at the time that they were symptomatic, as an ad-hoc entry. This paper file was maintained throughout both the inpatient and outpatient periods. Clinical observations including pulse, blood pressure and temperature were also recorded at set times, with additional readings taken if a subject was symptomatic, as determined by the protocol and by medical and nursing staff. Stool logs were maintained, including time of stool, grade, weight, and the presence of frank blood, which was confirmed by haemoccult testing. A log of which stools were sent for *C. jejuni* strain CG8421 testing, as well as immunology and exploratory outcomes was also maintained. The Investigative Team at the Center for Immunization Research transcribed

these data into electronic Case Report Files, for data analysis. Data monitoring was carried out by the EMMES Corporation.

4.6 RESULTS ANALYSIS BY ADJUDICATION BOARD

Prior to the data being unblinded an independent adjudication board sat to determine whether or not the primary endpoint of campylobacteriosis had been met. The relevant data was entered into a spread sheet for each Subject by the NMRC Investigative team and presented to the members of the Adjudication Board in a teleconference. An example of one of these spread sheets is shown at Figure 4.3, data compiled by Dr Chad Porter and the NMRC Clinical Trials Group.



ID	0-24 Hours	24-48 Hours	48-72 Hours	72-96 Hours	96-120 Hours	120-144 Hours	144-168 Hours	168-192 Hours
04	147 @ 21.6 Hours	81 @ 28.5 Hours	128 @ 48.3 Hours	137 @ 75.8 Hours	124 @ 102.5 Hours	270 @ 124.1 Hours		
04		152 @ 31.3 Hours	82 @ 50.2 Hours	163 @ 79.1 Hours	34 @ 106.3 Hours	105 @ 131.2 Hours		
04		*118 @ 33.1 Hours	168 @ 51.4 Hours	88 @ 81.3 Hours				
04		83 @ 34.7 Hours	163 @ 53.0 Hours	126 @ 84.8 Hours				
04		*73 @ 35.3 Hours	93 @ 54.2 Hours	135 @ 93.1 Hours				
04		95 @ 37.0 Hours	95 @ 55.2 Hours	137 @ 95.4 Hours				
04		273 @ 42.8 Hours	65 @ 57.1 Hours					
04		387 @ 46.9 Hours	118 @ 59.4 Hours					
04		151 @ 47.8 Hours	249 @ 62.7 Hours					
04			145 @ 70.2 Hours					

Figure 4.3 Example of data for an individual Study Subject, presented to the Adjudication Board.

These data were used by the Adjudication board to determine whether the Study Subject 04 met the primary endpoint of campylobacteriosis. The maximum weight of stool in a 24-hour period was 2061 g, and the maximum number of stools in a 24 hour period was 14. This subject developed campylobacteriosis at 44 hours post challenge.

In Figure 4.3 the graph illustrates the cumulative stool weight (blue line, left Y axis), and cumulative number (red line, right Y axis) of loose stools, classified as diarrhoeal stool (Grade 3-5), passed since challenge (hours, X-axis). In addition, the table allowed the total weight of stool in any 24-hour period to be calculated, which allowed the clinical endpoint of campylobacteriosis to be determined. The vertical line on the X-axis indicates the time at which the subject met the criteria for early treatment with antibiotics, section 4.4.4. The data collection finishes at 144 hours post-challenge as this was the point when the clinical end-point

of campylobacteriosis was determined, and antibiotic therapy was given if not already started. For interpretation of the table in Figure 4.3, the guide for provided, Table 4.4.

Table 4.4 Guide to interpreting the Adjudication Board spread sheets

Symbol	Definition
xxxxx	Text highlighted yellow indicates observations that were included in maximum number of stools in a 24 hour period
xxxxx	Text boxed in red indicates observations that were included in maximum weight of stools in a 24 hour period.
xxxxx	Text written in red indicates a loose stool with gross blood
*	Indicates when subject met definition for moderate diarrhoea (4-5 grade 3-5 stools in 24 hours or 401-800 g of grade 3-5 stools in 24 hours), in Figure 4.4 this is at 33.1 hours.
Λ	Indicates when subject met definition for severe diarrhoea (≥ 6 grade 3-5 stools in 24 hours, or > 800 g of grade 3-5 stools in 24 hours), in Figure 4.4 this is at 35.3 hours.

The outcome of the Adjudication Board regarding whether or not individual Subjects met the primary endpoint, campylobacteriosis, is shown in Table 4.5. Subjects are listed by their study Subject Identification (ID) number. Table 4.5 details the maximum number and weight of stool produced in any 24 hour period within the first 144 hours, and whether or not the subject met the criteria for moderate or severe diarrhoea based on these observations, as well as the time of onset of moderate or severe diarrhoea. It then goes on to identify whether the primary endpoint was met based upon stool output, or fever with another associated symptom, then, if the primary endpoint was met, the time that elapsed from challenge to meeting the primary endpoint. The final column details whether or not the Subject met the criteria for early treatment with antibiotics.

Table 4.5 Outcome of the Independent Adjudication Board

This table was produced by the NMRC Clinical Trials Group

CIR296 (Rifaximin Chemoprophylaxis) Campylobacter Challenge Data Adjudication Form
Tuesday, 04 August 2015

Occurrence of Primary Endpoint, Campylobacteriosis

	Did the Subject Meet the Definition of Moderate or Severe Diarrhea based on peak 24 hr outputs (either by volume or number of grade 3-5 stools)					*Primary Endpoint			Early Abx
Subject ID	Yes/No If 'Yes', Severity based on max volume in 24hrs?	Max volume in 24hrs	Yes/No If 'Yes' Severity based on max number in 24hrs?	Max no. stools per 24 hrs.	Time of onset of episode post challenge (HH:MM)	Yes/No ≥401g grade 3-5 stool in 24hr OR ≥4 grade 3-5 stool in 24hr	Yes/No Fever plus an associated symptom	If 'Yes', Time to primary endpoint post challenge (HH:MM)	Yes/No Received early antibiotic
01	Y	489	N	3	44.7	Y	N	93.0	N
03	Y	476	N	3	56.8	Y	N	59.9	N
04	Y	2061	Y	14	21.6	Y	N	33.1	Y
05	Y	1027	Y	4	89.4	Y	Y	89.4[a]	Y
06	N	0	N	0	-	N	N	-	N
08	Y	946	Y	10	68.5	Y	N	74.8	Y
09	Y	1592	Y	15	44.7	Y	N	50.6	Y
11	Y	553	N	3	44.2	Y	Y	66.6[a]	N
12	Y	1708	Y	9	43.9	Y	N	53.2	Y
13	Y	1023	Y	14	7.4	Y	N	56.0	Y
14	Y	1089	Y	8	29.7	Y	N	45.2	Y
15	N	252	Y	6	52.5	Y	Y	69.7	N
16[b]	N	0	N	0	-	N	N	-	N
17	Y	865	Y	15	39.9	Y	N	50.2	Y
19	Y	1056	Y	13	73.7	Y	Y	75.7[c]	Y
20	Y	1323	Y	8	27.5	Y	N	29.0	Y

	Did the Subject Meet the Definition of Moderate or Severe Diarrhea based on peak 24 hr outputs (either by volume or number of grade 3-5 stools)					*Primary Endpoint			Early Abx
Subject ID	Yes/No If 'Yes', Severity based on max volume in 24hrs?	Max volume in 24hrs	Yes/No If 'Yes' Severity based on max number in 24hrs?	Max no. stools per 24 hrs.	Time of onset of episode post challenge (HH:MM)	Yes/No ≥401g grade 3-5 stool in 24hr OR ≥4 grade 3-5 stool in 24hr	Yes/No Fever plus an associated symptom	If 'Yes', Time to primary endpoint post challenge (HH:MM)	Yes/No Received early antibiotic
21	Y	1063	Y	10	27.4	Y	Y	45.4[c]	Y
22	Y	998	Y	8	30.6	Y	N	41.3	Y
24	Y	1441	Y	11	37.3	Y	N	43.9	Y
25	Y	1516	Y	9	44.5	Y	N	47.8	Y
26	Y	696	Y	7	83.8	Y	N	93.0	N
27	Y	851	Y	9	20.0	Y	N	58.0	Y
28	N	0	N	0	-	N	N	-	N
31	Y	887	Y	6	33.1	Y	Y	40.3[a]	Y
32	Y	1039	Y	12	56.4	Y	Y	60.1[c]	Y
33	Y	1157	Y	7	22.4	Y	Y	29.8[a]	Y
34	N	312	N	3	-	N	N	-	N
36	Y	1490	Y	11	53.9	Y	Y	45.3[c]	Y

*Primary Endpoint: The primary efficacy endpoint of this prophylaxis-challenge study is campylobacteriosis, defined as a clinical illness within the 144 hours after challenge, meeting at least one of the following patterns:

- Moderate to severe diarrhea (a diarrhea episode is based on a 48 hr window)
  - Severe diarrhea (> 6 grade 3–5 stools in 24 hours or > 800 g of Grade 3–5 stools in 24 hours)
  - Moderate diarrhea for 24 hours (4–5 loose stools in 24 hours or 401–800 g of grade 3–5 stools in 24 hours)
- Fever (present on at least 2 occasions, at least 20 minutes apart) without diarrhea, plus an associated symptom (nausea, vomiting, abdominal cramps, tenesmus, or gross blood in ≥ 2 stools); with consideration of potential alternative diagnosis per clinical investigator based on illness time course and associated symptoms.

[a]The time to primary endpoint was met based on the time to meeting the definition of moderate-severe diarrhea.
[b]Subject 16 experienced delayed onset of diarrheal episode at 152.4 hours post challenge, which did not meet the primary endpoint timeline of 144 hours post-challenge.
[c]The time to primary endpoint was met based on the time of documented fever concurrent with an associated symptom.

The data was un-blinded and the allocation of Subject to group was revealed, shown in Table 4.6.

Table 4.6 Allocation of Subject to group, by Study ID

Group	Subject Study ID
Rifaximin	1, 3, 6, 11, 13, 15, 17, 19, 20, 25, 26, 28, 31, 32, 33
Placebo	4, 5, 8, 9, 12, 14, 16, 21, 22, 24, 27, 34, 36

The clinical data, captured in the Subject Case Report forms, was analysed and, following the decisions made by the independent adjudication board regarding whether the primary endpoint had been achieved, the protective efficacy of rifaximin was determined.

The primary endpoint of campylobacteriosis was reached in 86.7% of subjects in the rifaximin group and 84.6% of subjects in the placebo group, p=1.00. Rifaximin did not provide protective efficacy against campylobacteriosis, when using 1.8×10^5 CFU of *C. jejuni* strain CG842 in this human challenge model, Figure 4.4.

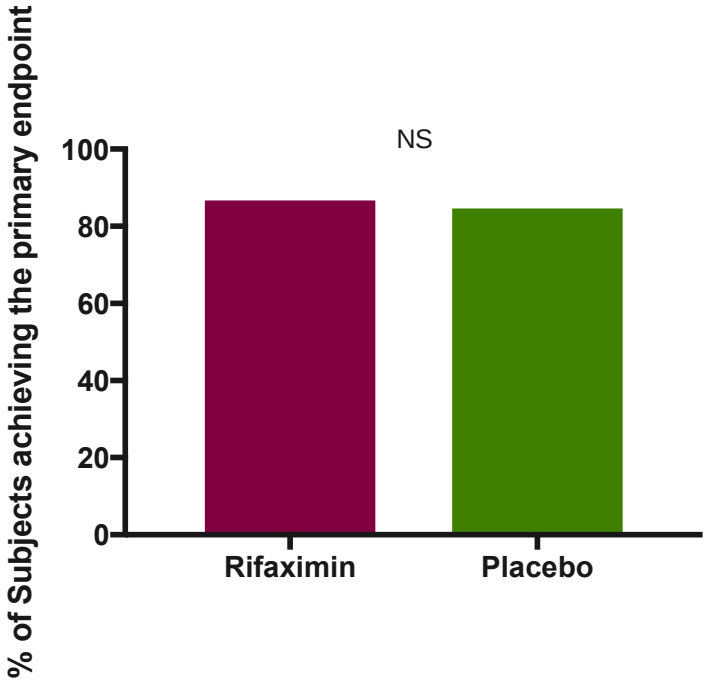


Figure 4.4 Rifaximin does not provide significant protection from Campylobacteriosis

Data logs were interrogated by the adjudication board in order to determine whether the primary endpoint was achieved.

The primary endpoint of campylobacteriosis was achieved in 86.7% of subjects in the rifaximin group and 84.6% of subjects in the placebo group, p=1.00. There is therefore no statistical significance in reaching the primary endpoint, between the groups, and rifaximin does not have protective efficacy against campylobacteriosis, when using 1.8×10^5 CFU of *C. jejuni* strain CG8421.

From the clinical data it is evident that the only statistically significant difference between the groups is the incidence of severe diarrhoea, when measured by total stool weight. Total stool weight is significantly reduced in the group that received rifaximin, p = 0.05, Table 4.7 and Figure 4.5

Table 4.7 Incidence of Symptoms, by Group

	Placebo (%) (n = 13)	Rifaximin (%) (n = 15)	P-value
Campylobacteriosis	84.6	86.7	1.00
Severe Diarrhoea (Weight/g)	**84.6**	**46.7**	**0.05**
Severe Diarrhoea (Number)	76.9	66.7	0.69
Dysentery	30.8	26.7	1.00
Abdominal Pain or Cramps	69.2	73.3	1.00
Nausea	38.5	40.0	1.00
Vomiting	0.0	20.0	0.23
Fever	46.2	46.7	1.00

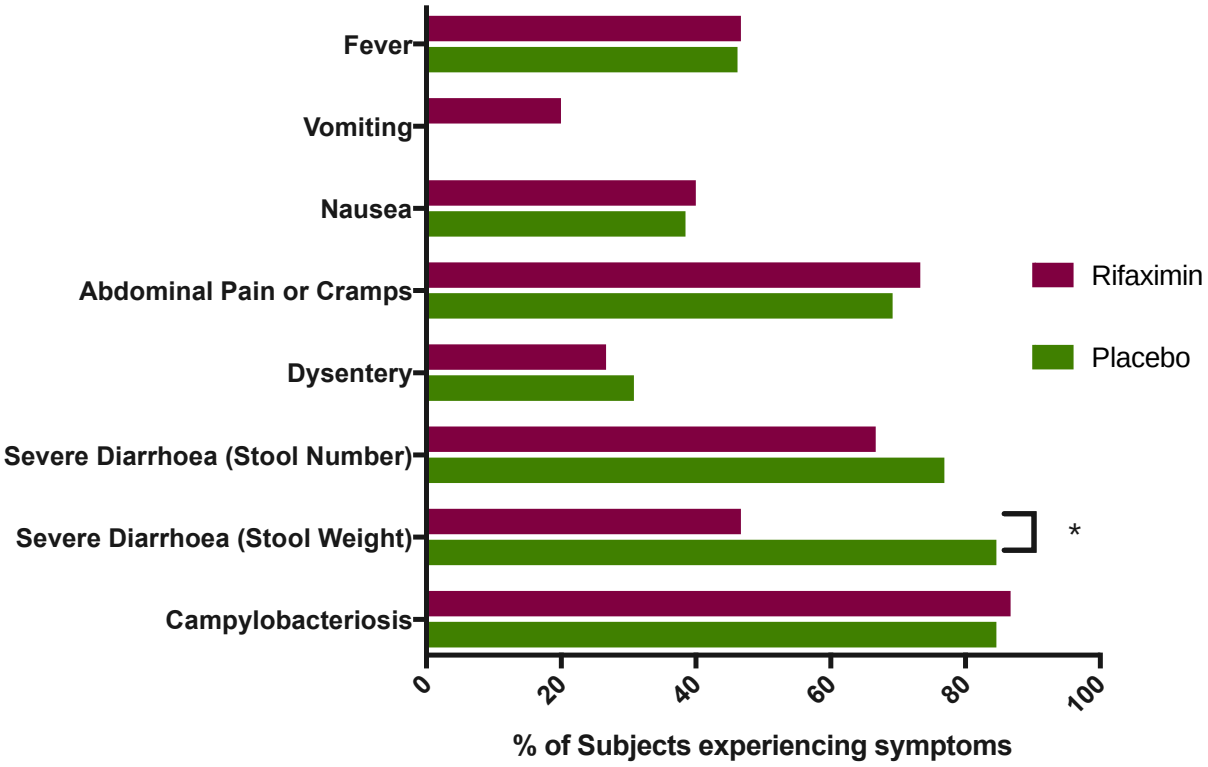


Figure 4.5 Rifaximin reduced the incidence of severe diarrhoea, by total weight

Interrogation of data logs allowed analysis of symptom reporting by Subjects

The only statistically significant difference in clinical data between the groups is the incidence of severe diarrhoea, when measured by total stool weight. The stool weight is significantly reduced in the rifaximin group, p = 0.05. When the different characteristics of stool data are individually examined, in addition to the statistically significant reduction in total stool weight, a reduction in the maximum weight of loose stools in any 24-hour period is also observed, although this does not reach statistical significance, p = 0.07, Table 4.8 and Figures 4.6 and 4.7.

Table 4.8 Stool Data, by Group, Rifaximin or placebo, with p-values.

The data is presented as the median and interquartile ranges.

	Placebo (n = 13)	Rifaximin (n = 15)	P-value
Total number loose stools	18 (11, 23)	12 (7, 18)	0.13
Maximum number loose stools/24 hrs	9 (8, 11)	7 (3, 12)	0.27
Total weight (g) of loose stool	**2565 (1777, 3183)**	**1437 (531, 2213)**	**0.05**
Maximum weight (g) of loose stool/24 hrs	**1063 (946, 1490)**	**704 (476, 1056)**	**0.07**
Time to first loose stool (hrs)	34.0 (24.5, 49.3)	44.5 (33.1, 56.4)	0.34
Duration of Diarrhoea (hrs)	100.4 (83.3, 115.2)	75.2 (71.2, 113.6)	0.36

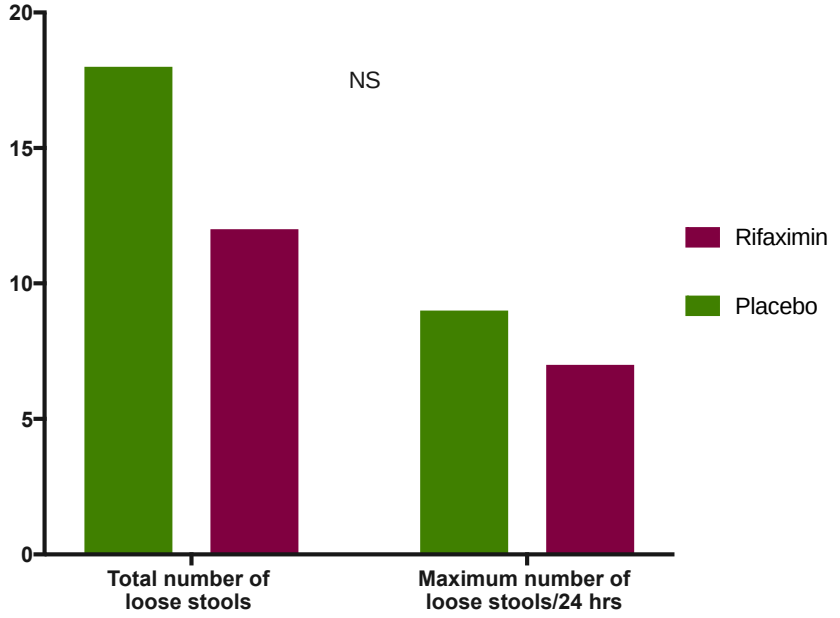


Figure 4.6 Rifaximin did not reduce the number of loose stools

Interrogation of data logs allowed calculation of total number of loose stools, and maximum number of loose stools/24 hrs.

There is no significant difference between the two groups in the total number of loose stools, (p=0.13) or maximum number of loose stools (p=0.27) in any 24-hour period.

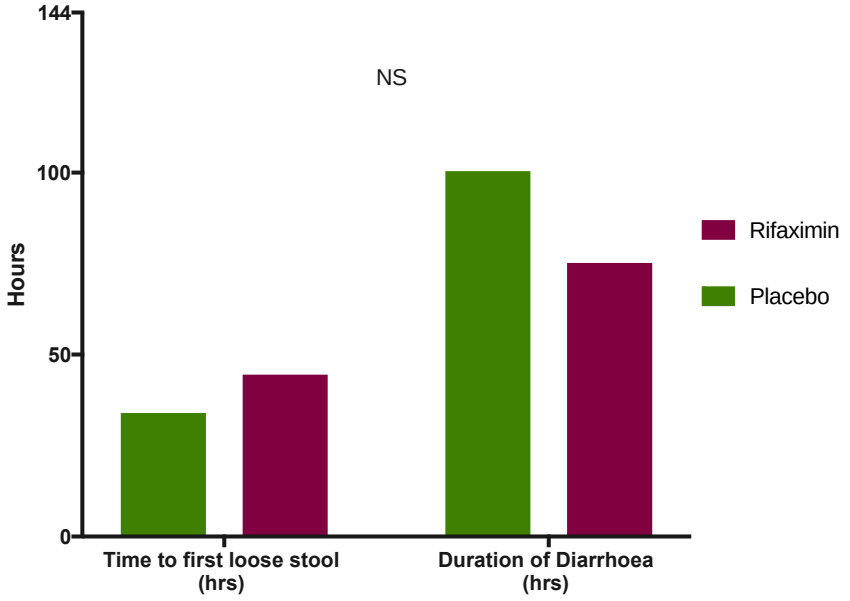


Figure 4.7 Rifaximin did not reduce the time to first loose stool or duration of diarrhoea

Interrogation of data logs allowed calculation of the time to the first loose stool, and duration of diarrhoea.

There is no significant difference between the two groups in the time to first loose stool (p=0.34) or duration of diarrhoea (p=0.36).

The following figures illustrate the stool data further. The mean total loose stool output, by weight, over a period of 144 hours, for both groups is illustrated in Figure 4.8, which shows the discrepancy between the two groups in terms of cumulative loose stool by weight, over this time period.

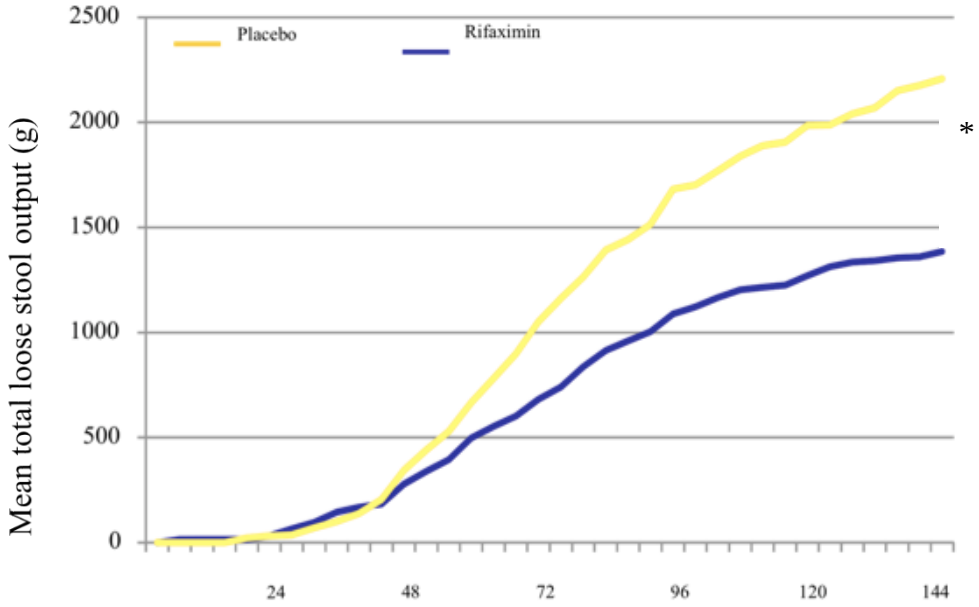


Figure 4.8 Total stool weight is significantly higher in the placebo group at 144 hrs

Interrogation of data logs allowed calculation of the mean loose stool output by weight for each group over time.

The mean stool weight of those in the placebo group becomes higher than that of subjects in the rifaximin group after the 44 hour point, this difference continues to increase as the gap between the two lines widens, at the 144 hours point the difference between the two is significant, p = 0.05. The cut off time of 144 hours was chosen as this was the period during which the decision of whether the subject met the primary endpoint of campylobacteriosis

was made, and at 144 hours antibiotic therapy was started if the criteria for initiation had not already been met.

Whilst the figure above shows the significant differences in total stool output by weight, the data captured also allowed analysis of the maximum weight of stool output in a 24-hour period. The difference between the two groups is shown in Figure 4.9, the difference is not statistically significant.

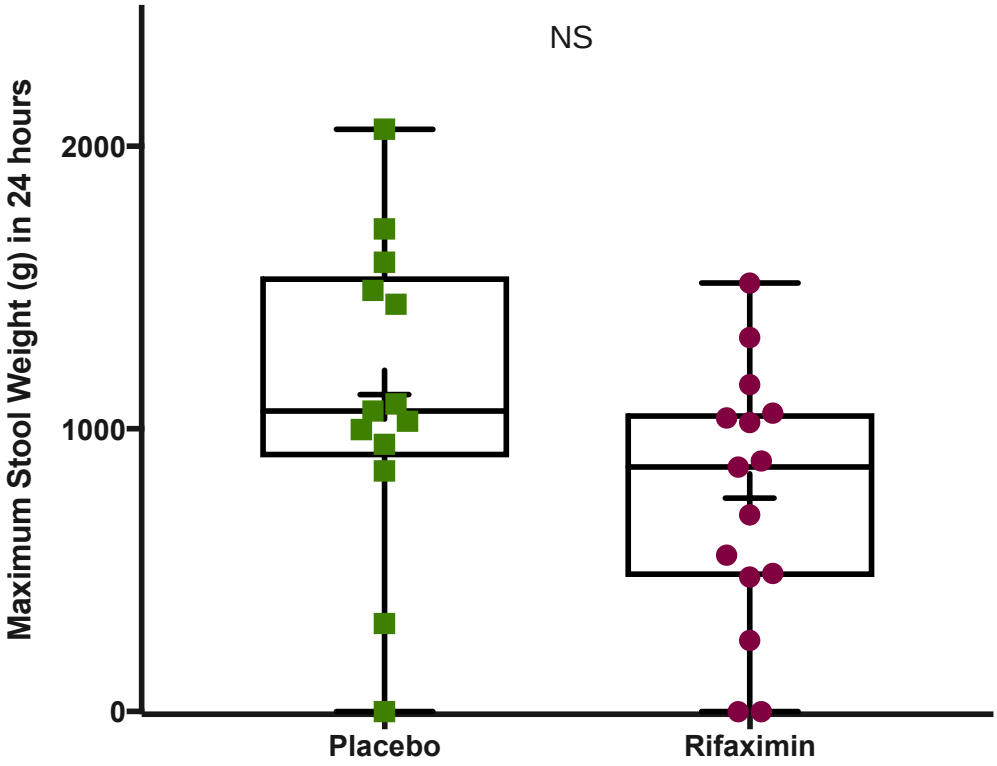


Figure 4.9 The maximum stool output by weight/24 hours is not significantly different

Interrogation of data logs allowed calculation of the maximum stool output by weight, in a given 24hr period, for each group.

Each data point represents the maximum stool produced in a 24-hour period by a Subject. Whilst the difference observed is not significant, p=0.07, it does illustrate the observation that the maximum weight of stool output in a 24-hour period is lower in the rifaximin group. The

box extends from the 25th to the 75th percentile, the line inside the box represents the median weight and the cross illustrates the mean weight, the whiskers represent the minimum and maximum weights (g).

4.6.1 Asymptomatic recrudescence

Symptomatic recrudescence of campylobacteriosis was monitored for, however, was not reported by any subjects. Asymptomatic recrudescence of *C. jejuni* CG8421, demonstrated by shedding *C. jejuni* CG8421 in stool, was monitored for using stool samples provided at the outpatient visits on days 14, 21, 28, 35, 56 and 84 post challenge, Table 4.3. Asymptomatic recrudescence was observed in 5 subjects. Two (13.1%) of these subjects had received rifaximin and both positive stool samples were provided on Day-56. The remaining three (23.1%) of these subjects had received the placebo, Figure 4.10, one subject provided a positive stool sample on Day-21 and 2 subjects provided positive stool samples on Day-56. Of the recrudescent cases, 80.0% were repeatedly culture negative following additional combined antibiotic treatment for 10 days. The subject who experienced a recrudescence at Day-21 also experienced a second recrudescence 56 days after starting the second dual antibiotic course (77 days after challenge). The subject was started on amoxicillin/clavulanic acid for 7 days with the probiotic Lactinex however, treatment compliance was questionable and stool cultures remained positive 20 days after antibiotic initiation. The subject was subsequently lost to follow-up.

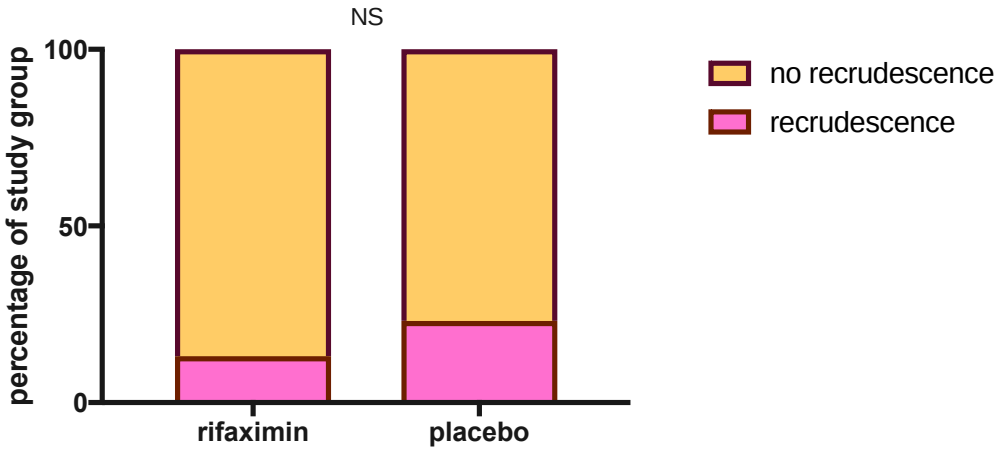


Figure 4.10 Rifaximin did not affect the rate of recrudescence

Subjects had to provide stool samples at designated follow-up study visits (Table 4.3) microbiological analysis of these determined recrudescence of disease.

The rate of recrudescence was not affected by Group. 13.1% of those who received rifaximin and 23.1% of those who received placebo demonstrated recrudescence, paired t-test p=0.099.

4.6.2 Presentation and publication of results

The data was published in the manuscript: Rimmer, J.E., et Al., Rifaximin Fails to Prevent Campylobacteriosis in the Human Challenge Model: A Randomized, Double-Blind, Placebo-Controlled Trial. Clin Infect Dis, 2018. **66**(9): p. 1435-1441, Appendix 1.

The data was also presented as a poster presentation at the Infectious Diseases Society of America (IDSA) Infectious Diseases week, San Diego, 2015, Appendix 3.

C. jejuni CG8421 isolates from recrudescent episodes were examined by Croft et. Al for transcriptional and genetic adaptation alterations, Crofts A.A., et. Al., *Campylobacter jejuni* transcriptional and genetic adaptation during human infection. Nat Microbiol. 2018 Apr;3(4):494-502, Appendix 2.

4.7 CONCLUSION

This study demonstrated a successful and robust human challenge model as the primary endpoint was met in >80% of subjects. The data from the study shows that there is no significant difference in the development of campylobacteriosis, the primary endpoint, between the two groups rifaximin prophylaxis or placebo (p=1.00). However, the mean difference in total weight of loose stool output (g) is significantly reduced in the rifaximin group (p=0.05), and a reduction in the maximum weight of loose stools in any 24-hour period was also observed (p=0.07). Therefore, whilst rifaximin has not been proven to protect against campylobacteriosis, the severity of disease with regard to stool output appears moderated. This suggests that it may be useful in preventing sequelae associated with fluid loss. Therefore these data should be considered when evaluating the efficacy of rifaximin in reducing the severity of disease burden in multi-pathogenic bacterial travellers' diarrhoea. Based on the high diarrhoea attack rates noted in this human challenge model, the efficacy of rifaximin prophylaxis against campylobacteriosis in a field setting requires further evaluation, and is underway in the clinical trial PREVENT TD, ClinicalTrials.gov Identifier: NCT02498301.

4.8 DISCUSSION

In this human challenge model Rifaximin did not protect against Campylobacteriosis. This was an unexpected outcome given the proven efficacy of approximately 60% protection against TD in field trials. However, in such studies, where the protective efficacy was 60%, the study had been carried out in areas where the predominant pathogen was *E. coli* [70]. When tested in Asia, where the prevalence of *campylobacter* is higher, this efficacy reduced to 48% [120]. It is also surprising given that rifaximin provided full protective efficacy

against the gastrointestinal pathogen, *Shigella* f*lexneri*, in a human challenge study [103]. It is possible that the inoculum dose given in this study exceeds that which may be encountered in the natural environment and therefore despite the high predicted concentration of rifaximin in the stool as described in Chapter 1, campylobacteriosis ensued. This is supported by the fact that in the initial *C. jejuni* human challenge studies using strains A3249 and 81-176, illness was induced by doses as low as 8×10^2 CFU [76].

The bioavailability of rifaximin at the site of invasion of *C. jejuni* may be a factor for consideration; rifaximin is water in-soluble and requires bile salts in order to be active against pathogens, and has little effect against pathogens, such as *E. coli,* in the aqueous environment of the colon [129], indeed solubility studies demonstrated that rifaximin became 70- to 120-fold more soluble in bile salts (approximately 30% in 4 mM bile acids) than in aqueous solution, therefore is more likely to be active throughout the small intestine [129]. Bile salts do not only affect rifaximin, the specific bile salt, sodium taurocholate, has been found to increase the number of outer membrane vesicles of *C. jejuni*, as well as increase the synthesis of virulence factors secreted via these vesicles [130]. This may suggest a higher pathogenicity within an environment of higher bile salt concentration, however there is little evidence to support this as human small intestine tissue samples are hard to obtain. Prominent features of human *C. jejuni* enteritis are terminal ileitis and inflammation of the caecum with mesenteric adenitis, as well as colitis seen at colonoscopy [131], therefore areas where the concentration of bile acid is lower than more proximally in the small intestine.

An interesting finding of this study was the high recrudescence rate; asymptomatic recrudescence was observed in 5 subjects; 2 (13.1%) receiving rifaximin (both on Day-56)

and 3 (23.1%) receiving placebo (1 on day 21 and 2 on day 56) [70]. *Campylobacter* recrudescence in an immunocompetent individual who had received antibiotic treatment for the bacterial infection was first described in 2010, the recrudescence was identified during the follow-up surveillance period of a previous *C. jejuni* CG8421 challenge study [132]. Our challenge study demonstrated an 18% recrudescence rate, this rate is significantly higher than has been seen previously in CG8421 challenge studies, where it has been recorded as 4% [70]. This increase may be due to a difference in the storage and subsequent culture of stool, yielding a higher rate of culture positive stool. At the time of each recrudescence episode, subjects were asymptomatic, raising the possibility that asymptomatic shedding may occur more frequently than is currently thought as asymptomatic individuals are unlikely present to primary or secondary healthcare facilities. Extended follow up with stool culture of patients experiencing naturally acquired *Campylobacter* infection is required to determine the natural recrudescence rate.

Given the unexpectedly high rate of recrudescence in this study, the *C. jejuni* isolates cultured from the recrudescence stool samples were further investigated by Croft et Al. for transcriptional and genetic adaptations, which could have occurred in vivo. The manuscript by Croft et Al is attached at Appendix 2. This research demonstrated variation in 11 genes that were associated with either acute or persistent human infections, including products involved in host cell invasion, bile sensing and flagella modification [20], all of which are characteristics contributing to virulence. Specifically, a functional version of the cell invasion protein A (cipA) gene product was strongly associated with isolated bacteria. Its biochemical role in flagella modification was identified [20]. This genetic adaptation represents another example of the of phase variation that *C. jejuni* is capable of, and was observed during this

challenge study. *C. jejuni* has been shown to possess phase variable genes that can provide a reversible and adaptive genetic mechanism for switching key characteristics on and off depending on the environment's selective pressures, which include exposure to the host immune system [133] as well as oxidative and aerobic stress responses [134].

In order to further understand the course of *C. jejuni* infection occurring in the natural environment, from both a host and pathogen perspective, a prospective, extended follow-up study recruiting individuals presenting with campylobacteriosis is required.

CHAPTER 5 FINAL DISCUSSION

Prevention of campylobacteriosis is important given the disease severity and chronic sequelae experienced as a result of *C. jejuni* infection. Disease prevention by either vaccination or antimicrobial chemoprophylaxis is a field in which research is on-going. Prophylaxis by vaccination is challenging as there are many variables; the host response to a vaccination can differ between individuals and for some the vaccine may not work. This thesis has evaluated the functional bactericidal effect of antibodies produced in response to vaccination with experimental vaccines for *C. jejuni*, chapter 3. The data in chapter 3 demonstrated that whilst NZWR and NHP developed a measurable response to vaccination, the NHP in particular achieving high bactericidal titres, this effect was not seen in humans. Where replicates of the SBA for individual samples of sera were performed the results were not always reproducible; this may be an error within the SBA method, or a result of changes in the behaviour of *C. jejuni* due to phase variation. The lack of increase in bactericidal titre demonstrated in humans contributed to the immunological data of the Phase I clinical trial that ultimately resulted in the structure and delivery of the vaccine being reviewed, rather than direct progression to a Phase II study.

In order to demonstrate the bactericidal effect of antibodies, the development of a SBA method was required before serum from vaccinated subjects could be tested. Chapter 3 also describes the development and evolution of the SBA method. The first objective of this thesis; to develop a serum bactericidal assay capable of demonstrating, by titre, the functional bactericidal effect of antibodies following *C. jejuni* vaccination, was not wholly achieved; there were significant limitations to the data, as described in chapter 3. However, the process

provided advances in the understanding of the optimal conditions for a successful SBA method. Importantly this work initiated the process of developing a SBA and therefore significantly contributed to the on-going work, evolving the method of the SBA in order that a bactericidal effect by titre can be measured following vaccination. The aspiration is that a robust SBA will be developed and applied in a human vaccination and challenge study, and so establish a serum titre that is subsequently recognised as a correlate for protection. It may be the case that, although SBA methods are used for other encapsulated pathogens in order to determine a serum titre that represents a correlate for protection, it is not appropriate for *C. jejuni* due to factors such as phase variation and complement sensitivity.

This thesis also evaluated antimicrobial chemoprophylaxis for *C. jejuni.* The second objective of this thesis was to demonstrate the protective efficacy of rifaximin vs. placebo in a *C. jejuni* CG8421 human challenge model. This objective was completed, rifaximin does not protect against campylobacteriosis in the *C. jejuni* CG8421 human challenge model. It does however reduce the burden of disease by reducing the total stool output, by weight. This is important when considering that may travellers and deployed Military personnel may have limited access to medical care and safe water for rehydration, so moderation of high stool output is beneficial.

Whilst rifaximin has been a successful prophylactic agent for other GI pathogens it was not successful against the *C. jejuni* inoculum in this study. There are limitations to antibiotic chemoprophylaxis. Antimicrobial resistance is an increasing problem globally and so the type of antibiotic used must be given careful consideration. In addition if an antibiotic is successful in achieving prophylaxis then the host immune system may not be exposed to the

bacteria and therefore development of immunity may be impeded, antimicrobial chemoprophylaxis is therefore a short-term solution. However, as human challenge studies have shown, [69] challenge with *C. jejuni* CG8421 does not prevent campylobacteriosis upon subsequent re-challenge 3 months later. Therefore naturally acquired immunity after infection is not always achieved. For some cohorts of individuals, antibiotic chemoprophylaxis, if successful, may be the ideal form of prophylaxis. According to the results in chapter 4, rifaximin is not the agent to be used for campylobacteriosis prophylaxis. However, if lower numbers of bacteria are encountered in the natural environment then it may have some protective efficacy, especially as the burden of diarrhoea was reduced in this study.

Importantly, this study demonstrated a successful *C. jejuni* CG8421 human challenge model with >80% of the subjects meeting the primary endpoint, campylobacteriosis. The ability to create a robust and reliable human challenge model is pivotal in assessing the efficacy of a prophylactic intervention, whether that intervention is an antibiotic or a vaccine. Therefore the success of the human challenge model will allow efficacy of the *C. jejuni* vaccine to be evaluated in due course. A vaccination and challenge study allows investigators to measure the degree of protection achieved by vaccination, in terms of clinical symptoms experienced following challenge. This type of study also measures the immune response using serum and/or faecal immune markers, and can therefore compare the serological and/or faecal immune response with incidence and severity of symptoms post challenge, to determine if the level of clinical protection correlates with immunological data. Without a reliable challenge model the protective efficacy of a vaccine is not known, and so a correlate for protection cannot be determined. The success of the *C. jejuni* CG8421 human challenge model, described in Chapter 4, has therefore contributed significantly to the advancement of future *C.*

jejuni vaccination trials. When the *C. jejuni* vaccine proceeds to a Phase II vaccination and challenge study, the human challenge model has already been proven to be successful. The limitation of this particular human challenge model was that the challenge only uses one strain of *C. jejuni*. Challenging human subjects with *C. jejuni* is difficult due to the risk of precipitating GBS. In order to test for protective efficacy against multiple strains, a vaccination field study will be required.

Campylobacteriosis continues to cause significant morbidity and mortality. Therefore work in the development of a prophylaxis will continue to be focussed upon the development of both vaccine and antimicrobial agents. When a vaccine for *C. jejuni* is able to demonstrate a robust immune response in Phase I immunogenicity studies, and progresses to Phase II testing, then a correlate for protection may be established. As discussed the SBA method developed in chapter 3 may not be employed for this, currently the *Campylobacter* Research Group is working on a flow cytometry-based method. In terms of antimicrobial chemoprophylaxis, the protective efficacy of rifaximin for Travellers Diarrhoea is currently under investigation in the clinical trial 'PREVENT TD' ClinicalTrials.gov Identifier: NCT02498301.

C. jejuni remains a fascinating bacterium. Whilst its ability to undergo genomic phase variation in response to the environment does provide challenges for investigators, it also provides opportunities to further examine the properties and mechanisms contributing to virulence and pathogenicity. If virulence and pathogenicity are more widely understood then the goal of achieving disease prevention is closer.

LIST OF REFERENCES

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APPENDIX 1

Rimmer, J.E., et Al., Rifaximin Fails to Prevent Campylobacteriosis in the Human Challenge Model: A Randomized, Double-Blind, Placebo-Controlled Trial. Clin Infect Dis, 2018. **66**(9): p. 1435-1441

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MAJOR ARTICLE

Rifaximin Fails to Prevent Campylobacteriosis in the Human Challenge Model: A Randomized, Double-Blind, Placebo-Controlled Trial

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Background. *Campylobacter* species are a leading cause of diarrheal disease globally with significant morbidity. Primary prevention efforts have yielded limited results. Rifaximin chemoprophylaxis decreases rates of travelers' diarrhea and may be suitable for high-risk persons. We assessed the efficacy of rifaximin in the controlled human infection model for *Campylobacter jejuni*.

Methods. Twenty-eight subjects were admitted to an inpatient facility and randomized to a twice-daily dose of 550 mg rifaximin or placebo. The following day, subjects ingested 1.7×10^5 colony-forming units of *C. jejuni* strain CG8421. Subjects continued prophylaxis for 3 additional days, were followed for campylobacteriosis for 144 hours, and were subsequently treated with azithromycin and ciprofloxacin. Samples were collected to assess immunologic responses to CG8421.

Results. There was no difference ($P = 1.0$) in the frequency of campylobacteriosis in those receiving rifaximin (86.7%) or placebo (84.6%). Additionally, there were no differences in the clinical signs and symptoms of *C. jejuni* infection to include abdominal pain/cramps ($P = 1.0$), nausea ($P = 1.0$), vomiting ($P = .2$), or fever ($P = 1.0$) across study groups. Immune responses to the CG8421 strain were comparable across treatment groups.

Conclusions. Rifaximin did not prevent campylobacteriosis in this controlled human infection model. Given the morbidity associated with *Campylobacter* infection, primary prevention efforts remain a significant need.

Clinical Trials Registration. NCT02280044.

Keywords. *Campylobacter*; rifaximin; controlled human infection model; prophylaxis.

Campylobacter is a leading cause of food and waterborne disease globally, with >1 million episodes annually in the United States alone [1]; additionally, in much of the developed world, rates are increasing [2, 3]. *Campylobacter* is also a leading cause of travelers' diarrhea (TD), accounting for >32% of all TD cases in certain regions [4–6]. In these populations, *Campylobacter* is known to be associated with several sequelae including chronic gastrointestinal conditions [7], reactive arthritis [8], and Guillain-Barré syndrome [9]. In low- to middle-income countries, *Campylobacter* is one of the most commonly isolated organisms in pediatric populations and has been associated with growth shortfalls [10–12].

While vaccine development efforts are under way [13], chemoprophylaxis may be a more immediate option, particularly for high-risk travel populations [14]. Rifaximin has shown preventive and therapeutic activity against bacterial enteropathogens, particularly diarrheagenic *Escherichia coli* [14–17]. Additionally, rifaximin prevented shigellosis in the controlled human infection model (CHIM) [17]. Given these data, we assessed the efficacy of twice-daily 550 mg rifaximin against campylobacteriosis in the CHIM [18, 19].

METHODS

The study was a randomized, double-blind, placebo-controlled trial to assess the efficacy of rifaximin against campylobacteriosis in a CHIM. After screening and informed consent, subjects were admitted to the inpatient facility at the Johns Hopkins University Center for Immunization Research (JHU CIR) on day –1 and started on rifaximin or placebo. On day 0, subjects received 1.7×10^5 colony-forming units (CFUs) of *Campylobacter jejuni* strain CG8421 [18–20], and were followed for the primary outcome.

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Clinical Endpoints and Definitions

The primary outcome was campylobacteriosis within 144 hours of challenge, defined as moderate to severe diarrhea (≥4 loose stools or ≥401 g of loose stools in 24 hours) or fever (oral temperature ≥38.0°C) with an associated symptom of nausea, vomiting, abdominal cramps, tenesmus, or gross blood in ≥2 loose stools. An illness severity index was also estimated [20].

Subject Recruitment and Inclusion and Exclusion Criteria

Healthy adults (18–50 years of age) were recruited from the mid-Atlantic area. All participants were briefed on study procedures, passed a comprehension test, signed informed consent, and met eligibility criteria. Subjects had no personal history of clinically significant diseases, immunosuppressive disorders, or medication. Subjects using antidiarrhea, anticonstipation, or antacid therapy, regularly ingesting raw milk, or with abnormal stool patterns were excluded. Subjects with a personal or family history of Guillain-Barré syndrome, inflammatory arthritis, or who were positive for HLA-B27 were excluded. Subjects with prior *Campylobacter* exposure history or serum immunoglobulin A (IgA) titer to *C. jejuni* CG8421 glycine extract (GE) >1:4000 were excluded [18].

Investigational Products

Eligible subjects were randomized (1:1) to rifaximin or placebo. Each 550-mg tablet of rifaximin was cut into 4 pieces, each placed into a gelatin capsule with microcrystalline cellulose to fill the capsule. The placebo consisted of 4 identical gelatin capsules filled with microcrystalline cellulose. Investigators and subjects remained blinded for the study duration. Subjects received the investigational product twice daily for 4 days starting on day –1 unless they met the primary endpoint.

Challenge Strain and Administration

After 3 doses of rifaximin or placebo, subjects received the *C. jejuni* challenge inoculum on study day 0. *Campylobacter jejuni* strain CG8421 [18, 19] was grown on Mueller-Hinton agar and incubated under microaerophilic conditions at 40°C for 20.5 hours. Colonies were harvested and diluted in phosphate-buffered saline (PBS) to a targeted concentration of 5×10^5 CFU/mL based on the prior work with this strain [18, 20]. One milliliter was added to 30 mL sodium bicarbonate buffer ($NaHCO_3$) immediately prior to inoculation. One minute prior to inoculation, subjects ingested 120 mL of the same $NaHCO_3$ solution (2 grams per 150 mL). The dose was verified by enumeration of CFUs on *Campylobacter* CVA (cefoperazone, vancomycin, and amphotericin B) agar.

Clinical Monitoring and Management

During the inpatient period, a physician conducted daily medical interviews and examinations as described previously [18, 20]. Fluids were replaced orally or intravenously as clinically appropriate. Azithromycin (500 mg by mouth once daily) concurrently with ciprofloxacin (500 mg by mouth twice daily) was used for 5 days to treat all subjects starting 144 hours postchallenge unless meeting early treatment criteria. Early treatment criteria included severe diarrhea, moderate diarrhea for 2 days, mild or moderate diarrhea and ≥2 associated symptoms, or any illness for which the study physician thought necessitated early treatment. At discharge, all symptoms were resolved or resolving and subjects had ≥2 stools negative for *C. jejuni*. Subjects were provided remaining antibiotic treatment to be taken at home and were seen as outpatients 14, 21, 28, 35, 56, and 84 days after challenge and contacted by telephone 6 months after challenge. For microbial recrudescence [20–22], extra visits were necessary and ciprofloxacin and azithromycin were given for 10 additional days.

Microbiology

For culture, tips of swabs with fresh stool were placed in thioglycollate (Hardy Diagnostics, Santa Maria, California). The vials were incubated at 40°C (±2°C) until shipment to the Enteric Laboratory at JHU CIR. The swab was used to inoculate the first quadrant of 2 CVA plates followed by streaking with a sterile loop across the remaining 3 quadrants. Post–antibiotic administration, 200 µL of stool/thioglycollate solution was spread onto 4 CVA plates. The CVA plates were incubated for approximately 48 hours at 40°C (allowable range: 37°C–42°C) in a microaerobic environment generated by filling a polyethylene bag with *Campylobacter* gas (85% nitrogen, 10% carbon dioxide, and 5% oxygen) and sealing with a rubber band. *Campylobacter* was confirmed by oxidase and catalase tests and Gram stain.

Frozen stools were extracted with the POWERsoil DNAisolation kit (MoBIO) following manufacturer protocol. The DNA were used to perform quantitative real-time polymerase chain reaction (qRT-PCR) using a *C. jejuni*–specific primer set lpxA F (5′-ACAACTTGGTGACGATGTTGTA-3′) and lpxA R (5′-CAATCATGDGCDATATGASAATAHGCCAT-3′) [23]. RT-PCR was performed on a QuantStudioT 6 (Applied Biosystems) using iTaqT Universal SYBR Green Supermix (Bio-Rad). Extrapolation of CFUs was made by establishing a standard curve using *C. jejuni* DNA of known concentration. Linear regression between RT-PCR cycle thresholds vs DNA concentration was used to estimate the numbers of CFUs per gram. The theoretical number of chromosome copies per nanogram of DNA was estimated using 6×10^5 copies/ng of DNA.

Immune Response

Serum was analyzed for CG8421-specific responses using GE as previously described [24]. In brief, microtiter plates were coated overnight with GE at 3 µg protein/mL. After washing and blocking, serum was serially diluted, added to wells, and incubated for 2 hours at 37°C. Plates were washed and specific antibodies detected by addition of horseradish

peroxidase–conjugated antihuman immunoglobulin G (IgG) or IgA (KPL, Gaithersburg, Maryland). TMB (eBiosciences, San Diego, California) was used as a detection reagent and the optical density at 450 nanometers was measured. The mean optical density of negative control wells (coating buffer alone) + 3 standard deviations was used to determine the endpoint titer.

Frozen stool was thawed on ice and resuspended in extraction buffer (ethylenediaminetetraacetic acid [EDTA], soy trypsin inhibitor, bovine serum albumin, phenylmethysulfonyl fluoride in PBS) at 500 mg/mL. Samples were vortexed for 30 minutes, then centrifuged at 20 000*g* for 30 minutes at 4°C. Supernatants were collected and assayed for total IgA content. Samples were normalized to a starting concentration of 20 µg/mL total IgA and serially diluted. GE-specific IgA was performed as described above. A response was defined as a ≥4-fold increase over the baseline values.

Whole blood was collected in EDTA to assess for *Campylobacter*-specific antibody-secreting cells (ASCs) and interferon gamma (IFN-γ) production. GE-specific ASCs were measured by coating nitrocellulose 96-well plates (MAHAS4510, Millipore, Billerica, Massachusetts) with 3 µg/mL CG8421 GE and incubating 5×10^5 peripheral blood mononuclear cells (PBMCs) per well at 37°C for 16–18 hours. ASCs were detected with peroxidase-conjugated goat antihuman IgG or IgA and 3-amino-9-ethylcarbazole (AEC, Millipore). ASCs were enumerated using a CTL analyzer (*Cellular Technology Ltd,* Cleveland, Ohio) and expressed per 10^6 PBMCs. A positive response was ≥5 antigen-specific ASCs per 10^6 PBMCs. *Campylobacter*-specific IFN-γ production was measured by stimulating 10^5 PBMCs with media alone or 10^5 CFUs of fixed CG8421 cells at 37°C for 72 hours. Culture supernatants were collected and analyzed with a Bio-Plex Pro Human Cytokine IFN-γ Magnetic Bead kit (Bio-Rad, Hercules, California). IFN-γ produced by media-alone wells was subtracted from CG8421-stimulated wells to estimate *Campylobacter*-specific IFN-γ. IFN-γ responders were defined as a ≥2-fold increase over baseline per 10^5 PBMCs.

Statistical Analysis

Dichotomous results were analyzed using Fisher exact test and quantitative parameters using Wilcoxon rank-sum test for variables not normally distributed. Serologic titers were $\log_{10}$-transformed for statistical analysis. Statistical analyses were performed with SAS version 9.4 software (SAS Institute, Cary, North Carolina). Statistical significance was set at $P < .05$.

Power

Using a 2-group continuity-adjusted χ^2 test with a 2-sided $\alpha = .05$, a targeted rifaximin efficacy of 60% [14], and a campylobacteriosis rate of 93.3% in placebo recipients [18, 20], a sample size of 14 subjects per group yielded >80% power to detect a significant difference in the rate of campylobacteriosis.

Regulatory Approvals

The study protocol was approved by the Naval Medical Research Center and the Western institutional review boards in compliance with all applicable local, federal, and Department of Defense regulations governing the protection of human subjects. The study was registered at ClinicalTrials.gov on 29 October 2014 as NCT02280044.

RESULTS

Of 74 screened subjects, 44 were excluded, 68.2% due to high serum anti-GE IgA titers (Figure 1). A total of 28 subjects (13 placebo, 15 rifaximin) received the challenge inoculum on day 0. Subjects were predominately male (71.4%) and African American (92.9%) with a median age of 30 years (Table 1). Rifaximin was well tolerated with no related adverse events.

Following challenge with 1.7×10^5 CFUs of CG8421, the rate of campylobacteriosis was 84.6% in placebo recipients and 86.7% in rifaximin recipients (protective efficacy: –2.5%; $P = 1.0$) (Table 2). Dysentery was observed in 30.8% of placebo recipients and 26.7% of rifaximin recipients ($P = 1.0$). No differences were observed in the frequency of clinical signs and symptoms of *Campylobacter* infection to include abdominal pain/cramps, nausea, vomiting, or fever. Additionally, there was no difference in median disease severity index (rifaximin; 7.3 vs placebo, 8.2; $P = .6$). There was a slight decrease in the total number of loose stools (rifaximin, 12 vs placebo, 18) and a significant decrease in total weight (rifaximin, 1437 g vs placebo, 2565 g) of loose stools in rifaximin recipients compared with placebo ($P = .13$ and $P = .05$, respectively).

All subjects shed the challenge strain with no difference ($P = .9$) in maximum shedding between placebo ($10^{7.2}$ CFU/g of stool) and rifaximin ($10^{7.5}$ CFU/g of stool) recipients. There was no evidence of acquired antirifampicin resistance in subjects receiving rifaximin (data not shown). Post–azithromycin/ciprofloxacin treatment, all subjects were repeatedly stool culture negative for *Campylobacter.* Asymptomatic recrudescence was observed in 5 subjects, 2 (13.1%) receiving rifaximin (both on day 56) and 3 (23.1%) receiving placebo (1 on day 21 and 2 on day 56). Of the recrudescent cases, 80.0% were repeatedly culture negative following additional combined antibiotic treatment for 10 days. One placebo recipient experienced a second recrudescence 56 days after starting the second dual antibiotic course (77 days after challenge). The subject was started on amoxicillin-clavulanate for 7 days with a probiotic (Lactinex, BD Diagnostic Systems, Sparks, Maryland); however, treatment compliance was questionable and stool cultures remained positive 20 days after initiation. The subject was subsequently lost to follow-up. All isolates remained sensitive to azithromycin and ciprofloxacin.

Serologic responses were observed in 92.9% (IgA) and 75.0% (IgG) of challenged subjects. Responses peaked on days 9 and

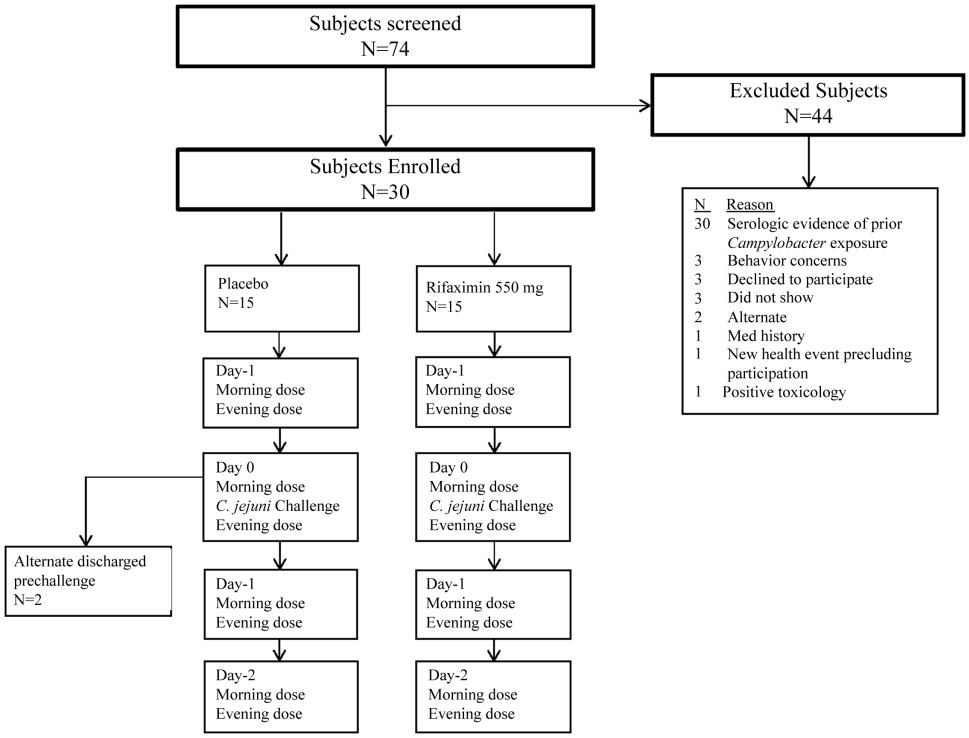

Figure 1. Study participation diagram for subjects included in the randomized, double-blind, placebo-controlled clinical trial assessing rifaximin chemoprophylaxis in the *Campylobacter jejuni* human challenge model.

14, respectively (Figure 2A). Fecal IgA response was observed in 85.7% of subjects, with no difference in the frequency ($P = .7$) or magnitude ($P = 1.0$) of the responses by treatment. Responses peaked on day 9, decreased over time, but remained elevated through day 84 (Figure 2B).

ASC responses to GE were observed in 75.0% (IgG) and 96.4% (IgA) of subjects with no difference across study groups. The maximum number of ASCs per 10^6 PBMCs varied greatly (Figure 2C). *Campylobacter*-specific IFN-γ responses were observed in 60.7% of subjects, with a median fold increase of 5.0 (Figure 3). There was no difference in response frequency ($P = 1.0$) or magnitude ($P = .8$) across treatments.

Table 1. Demographic Data of Study Subjects Challenged With *Campylobacter jejuni*

Characteristic	Placebo	Rifaximin	Total
No.	13	15	28
Median age, y (IQR)	31 (22–39)	30 (29–37)	30 (29–37)
Sex, No. (%)			
Male	9 (69.2)	11 (73.3)	20 (71.4)
Female	4 (30.8)	4 (26.7)	8 (28.6)
Race, No. (%)			
White	0 (0.0)	1 (6.7)	1 (3.6)
Black	13 (100.0)	13 (86.7)	26 (92.9)
Other	0 (0.0)	1 (6.7)	1 (3.6)

Two alternate subjects (1 white female and 1 African American male) receiving placebo were discharged prior to challenge.

Abbreviation: IQR, interquartile range.

DISCUSSION

The use of the *C. jejuni* CHIM provided a clear and specific result and demonstrated that rifaximin does not protect against campylobacteriosis in this model. In contrast, 2 separate systematic reviews of the prophylactic efficacy of rifaximin have estimated efficacy of approximately 60% against TD [14, 25]; however, many of these studies were in diarrheagenic *E. coli* predominate regions [4, 26]. A 48% efficacy was observed in a trial of rifaximin prophylaxis (200 mg twice daily) in travelers to Asia, a region with higher rates of *Campylobacter*-attributed TD [27]. The dose for this study, 550 mg twice daily, was selected to optimize efficacy given the findings from travelers to Asia. Perhaps more akin to this study, albeit at a different antibiotic dose, rifaximin provided 100% protection from shigellosis in the CHIM of *Shigella flexneri* [17].

These findings may be partially explainable by rifaximin pharmacology and *Campylobacter* infection. Hopkins et al tested the susceptibility to rifaximin of several enteropathogens recovered from travelers returning to the United Kingdom and showed that although rifaximin appeared to have broad coverage against most organisms, *Campylobacter* appeared to be broadly resistant (≥128 μg/mL) in vitro [28]. However, Gomi et al reported a minimum inhibitory concentration 90 (MIC_{90}) for rifaximin of 32 μg/mL using *C. jejuni* recovered from individuals with

Table 2. Clinical Endpoints and Stool Output by Treatment Arm

Endpoint	Placebo (n = 13)	Rifaximin (n = 15)	*P* Value
Campylobacteriosis	11 (84.6)	13 (86.7)	1.00
Dysentery[a]	4 (30.8)	4 (26.7)	1.00
Abdominal pain or cramps	9 (69.2)	11 (73.3)	1.00
Nausea	5 (38.5)	6 (40.0)	1.00
Vomiting	0 (0.0)	3 (20.0)	.23
Fever	6 (46.2)	7 (46.7)	1.00
Total No. of loose stools, median (Q1, Q3)	18 (11, 23)	12 (7, 18)	.13
Maximum No. of loose stools in 24 h, median (Q1, Q3)	9 (8, 11)	7 (3, 12)	.27
Total weight of loose stools, g, median (Q1, Q3)	2565 (1777, 3183)	1437 (531, 2213)	.05
Maximum weight of loose stool in 24 h, g, median (Q1, Q3)	1063 (946, 1490)	704 (476, 1056)	.07
Time to first loose stool, h, median (Q1, Q3)	34.0 (24.5, 49.3)	44.5 (33.1, 56.4)	.34
Duration of diarrhea, h, median (Q1, Q3)	100.4 (83.3, 115.2)	75.2 (71.2, 113.6)	.36
Campylobacteriosis score, median (IQR)	8 (6, 10)	7 (6, 10)	.63

Data are presented as No. (%) unless otherwise indicated.

Abbreviation: IQR, interquartile range.

[a]Gross blood, confirmed by hemoccult, in at least 2 loose stools.

TD from Kenya, and Novoa-Farias et al reported that 100% of campylobacteriosis isolates in Mexico were susceptible to rifaximin at <200 μg/mL [29, 30]. The pharmacological properties of rifaximin precluded determination of the CG8421 minimum inhibitory concentration; however, the minimum inhibitory concentration 50 (MIC_{50}) against rifampicin, an absorbable form of rifaximin, was 32 μg/mL (data not shown). Nonetheless, the applicability of these in vitro assays is unclear given the high levels of fecal concentrations of rifaximin following repeated oral dosing. For example, in stool following a 3-day 400-mg twice-daily dosing, there is a mean concentration of 8000 μg rifaximin/mL of stool at the therapeutic site of action [31].

We utilized a 550-mg rifaximin dose twice daily approximately 12 hours apart to maximize the potential efficacy against campylobacteriosis. Rifaximin was given with meals as it was anticipated that free molecular rifaximin would be enhanced in the small intestine following a meal [32]. It was presumed that rifaximin would intercept the pathogen in transit to the large intestine; however, rifaximin is water insoluble and requires physiological concentrations of bile acid to be active against diarrheal pathogens [33]. The primary site of human *Campylobacter* infection is the distal ileum and colon [34–36]. As such, decreased drug

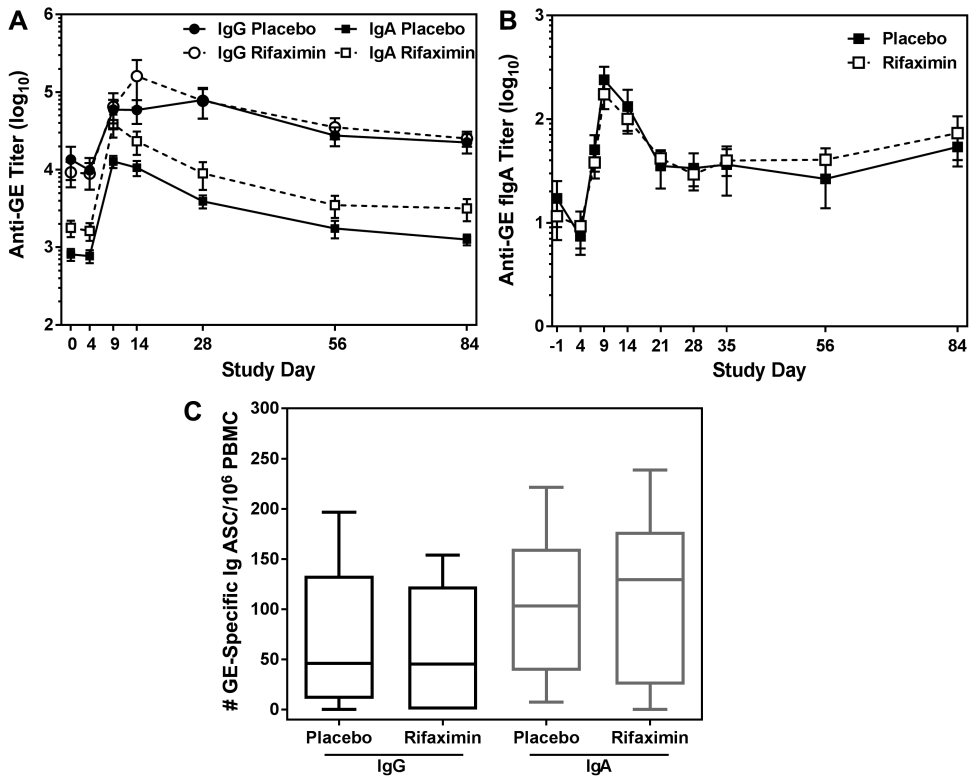

Figure 2. Humoral response to challenge including serum immunoglobulin responses (*A*), glycine extract (GE)–specific fecal immunoglobulin A (*B*), and GE-specific antibody-secreting cells (*C*). Abbreviations: ASC, antibody-secreting cell; GE, glycine extract; IgA, immunoglobulin A; IgG, immunoglobulin G; PBMC, peripheral blood mononuclear cell.

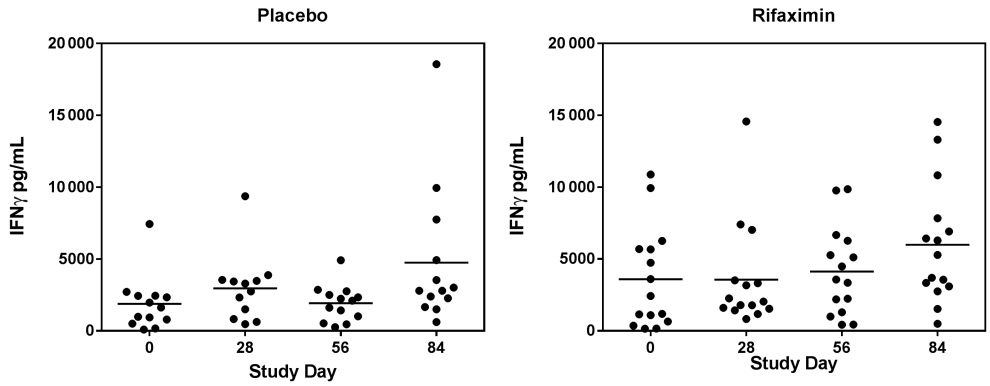

Figure 3. Interferon gamma (IFN-γ) response by group.

bioavailability at the infection site may have negatively affected efficacy. Rifamycin formulations targeting colonic bioavailability are under development and may be more effective [37]. However, rifaximin prevented shigellosis in the CHIM where the colon is an active site of infection, pointing to potential differences in pathogen susceptibility or antibiotic action during pathogen transit of the small intestine. Interestingly, in the shigellosis model, rifaximin-treated subjects did not develop IgA ASCs, a standard mucosal-mediated immune response, whereas placebo subjects had a geometric mean of 73 IgA ASCs per 10^6 PBMCs to anti–*S. flexneri* lipopolysaccharide ($P = .008$). In contrast, we observed no difference in ASC responses between placebo- and rifaximin-treated subjects.

The CG8421 CHIM-induced disease is comparable to prior studies despite a modification in immunologic screening from prior studies with this strain, which excluded subjects with an IFN-γ level >400 pg/mL after in vitro stimulation of PBMCs with formalin-fixed CG8421 whole cells [18, 20]. Such screening would have excluded 22 of 28 (78.6%) of our subjects; however, there was no difference in disease endpoints across levels of baseline IFN-γ (data not shown). Of note, *C. jejuni* 81–176 CHIM confers 100% short-term protection on homologous rechallenge compared to the lack of protection in the CG8421 CHIM, highlighting questions on protective immunity and immunologic screening [20, 24].

We observed an 18% microbial recrudescence rate, higher than seen previously (4%; $P = .03$) and not associated with treatment group. It is unclear whether this represents a true increase in recrudescence or is due to enhanced microbiological methods. Prior studies maintained whole stool specimens at refrigerator temperature until plating [18, 20]. Our use of short-term incubation in thioglycollate broth may have enhanced our ability to detect recrudescent events. Importantly, all subjects appeared to be *Campylobacter* negative prior to discharge. Additionally, all subjects remained asymptomatic during their recrudescence. Microbial recrudescence of *Campylobacter* following treatment with appropriate antibiotics was first described by Baqar et al in a CHIM with *C. jejuni* CG8421 [21]. It remains unclear whether this phenomenon occurs as part of the natural course of *C. jejuni* infection. The experience with the CG8421 CHIM has shown the majority, but not all, to be asymptomatic, so conceivably unrecognized recrudescence occurs in a minority of treated infections. The recrudescent bacteria likely are capable of securing a protective niche during the period of high-dose antibiotic exposure, particularly given that subjects are treated with multiple doses of both azithromycin and ciprofloxacin, which provide high fecal and intracellular drug levels with long half-lives. Among 9 subjects with recrudescence, there has been a single emergent antibiotic resistance to azithromycin and none against ciprofloxacin, a well-described phenomenon [22, 38]. Recrudescence may play a role in recurrent infections and sustained colonization among developing-world children; however, there are no reports or studies of *Campylobacter* recrudescence following community-acquired exposures [12]. Follow-up studies of naturally acquired *Campylobacter* infections are needed to determine the extent to which recrudescent infections occur.

CONCLUSIONS

Despite the high rifaximin dose, no efficacy against campylobacteriosis was observed in this CHIM. While disappointing, the CHIM model is a robust infection with inocula exceeding natural infection, and field studies with higher rifaximin doses in *Campylobacter* predominant regions are under way. While chemoprophylaxis for TD is a subject of debate, there are circumstances in which disease mitigation may tip the risk–benefit equipoise. Recent guidelines critically appraised the evidence of rifaximin chemoprophylaxis effectiveness in the context of potential benefits and risks [39, 40]. Recommendations continue to support short-term antibiotics to prevent TD in high-risk groups potentially susceptible to serious health consequences, or in whom illness may have negative travel implications. Rifaximin may represent a safer alternative to fluoroquinolones; however, potential lack of efficacy against *Campylobacter*-attributed TD may limit its utility. Perhaps most importantly, given the acute illness severity and the growing

number of attributable chronic long-term health consequences of *Campylobacter* infection [41], primary prevention strategies, such as vaccines, are clearly needed.

Notes

Disclaimer. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the US government.

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APPENDIX 2

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Campylobacter jejuni transcriptional and genetic adaptation during human infection

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Campylobacter jejuni **infections are a leading cause of bacterial food-borne diarrhoeal illness worldwide, and** ***Campylobacter*** **infections in children are associated with stunted growth and therefore long-term deficits into adulthood. Despite this global impact on health and human capital, how zoonotic** ***C. jejuni*** **responds to the human host remains unclear. Unlike other intestinal pathogens,** ***C. jejuni*** **does not harbour pathogen-defining toxins that explicitly contribute to disease in humans. This makes understanding** ***Campylobacter*** **pathogenesis challenging and supports a broad examination of bacterial factors that contribute to** ***C. jejuni*** **infection. Here, we use a controlled human infection model to characterize** ***C. jejuni*** **transcriptional and genetic adaptations in vivo, along with a non-human primate infection model to validate our approach. We found that variation in 11 genes is associated with either acute or persistent human infections and includes products involved in host cell invasion, bile sensing and flagella modification, plus additional potential therapeutic targets. In particular, a functional version of the cell invasion protein A (*cipA*) gene product is strongly associated with persistently infecting bacteria and we identified its biochemical role in flagella modification. These data characterize the adaptive** ***C. jejuni*** **response to primate infections and suggest therapy design should consider the intrinsic differences between acute and persistently infecting bacteria. In addition, RNA sequencing revealed conserved responses during natural host commensalism and human infections. Thirty-nine genes were differentially regulated in vivo across hosts, lifestyles and** ***C. jejuni*** **strains. This conserved in vivo response highlights important** ***C. jejuni*** **survival mechanisms such as iron acquisition and evasion of the host mucosal immune response. These advances highlight pathogen adaptability across host species and demonstrate the utility of multidisciplinary collaborations in future clinical trials to study pathogens in vivo.**

Studying bacterial evolution during infection exposes how pathogens adapt and survive in the host. High-resolution whole-genome sequencing enables accurate tracing of pathogen transmission between patients, identification of antibiotic resistance loci and understanding of selective pressures in vivo[1–6]. These developments have led to the identification of pathogen genetic variants that predict the success of treatments and guide therapy design. Such studies reveal the extent of bacterial adaptability in vivo while regarding the genetic fitness compromises that would only arise in the complex host environment.

Some bacteria adapt to new hosts and environments using a common population-level adaptation method known as phase variability[7]. Phase variation is driven by relatively high-frequency genome mutations in homopolynucleotide tracts which result in a functionally 'on' or 'off' phase of affected gene(s)[7]. Subpopulations of on/off gene variants are intrinsically more fit for a new environment and therefore increase in frequency in a population as they outcompete less fit variants. This adaptive process genetically regulates virulence factors and is important for pathogen colonization and immune evasion[8–10]. To fully understand phase variable populations, whole-genome sequencing can be used to identify the frequency of every 'on' or 'off' gene variant at the population level and then identify influential variants that are selected for during adaptation to new host environments[11,12].

C. jejuni is a phase variable Gram-negative intestinal pathogen that causes bloody diarrhoea, fever,and abdominal pain in humans. Notable secondary sequelae include Guillain–Barré syndrome, a potentially fatal paralytic autoimmune disorder. *C. jejuni* is a prevalent commensal bacterium in the intestinal tracts of chickens and other livestock, and consumption of contaminated animal products makes *C. jejuni* a leading cause of food-borne bacterial diarrhoea worldwide. In low-resource areas, asymptomatic and sometimes persistent *Campylobacter* infections are common in children younger than one year and correlate with stunted growth and

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therefore life-long physical and cognitive deficits[13]. In line with our understanding of in vivo pathogen evolution, small animal models of *C. jejuni* infection show host-passaged isolates are more fit in the host than the initial inoculum, and this genetic advantage remains even after multiple passages in vitro[14]. Here we use samples from a controlled human infection model to characterize the *C. jejuni* response to the human host. High-resolution genomic sequencing was used to track pathogen adaptation and phase variation in vivo, from inoculum through acute and persistent disease. In the challenge, a single liquid inoculum of *C. jejuni* strain CG8421 was used to simultaneously infect a group of human volunteers, making this study a highly controlled pathogen adaptation experiment in humans. The challenge was designed to evaluate the prophylactic efficacy of rifaximin on *C. jejuni* infection in humans and is described elsewhere (ClinicalTrials.gov Identifier NCT02280044)[15]. Briefly, rifaximin treatment had no effect on the primary clinical outcome of campylobacteriosis. To be released from the trial, volunteers were treated with azithromycin and ciprofloxacin and produced faeces negative for *C. jejuni*. However, five volunteers experienced recrudescent[16] infections. The clinical trial outcomes enabled us to compare pathogen gene variants that were selected for during both acute and recrudescent[16] infections, as well as in volunteers who experienced severe disease or received prophylactic antibiotic treatment. We found variants of genes involved in host cell invasion, bile sensing and flagella modification are selected for in recurrent human infections. To validate our approach, we performed a similar analysis in a symptomatic non-human primate infection model. Beyond defining genetic adaptation in primate hosts, we also used RNA sequencing to determine the *C. jejuni* transcriptome directly in human infection samples.

Campylobacter gene expression in human infection samples

Responding to the host environment via transcriptional changes is essential for *C. jejuni* colonization and infection[17,18]. To identify the transcriptional adaptations that occur in a human host we determined the *C. jejuni* transcriptome directly in infected human faeces of three volunteers. Compared with in vitro microaerobic growth on blood agar, 264 genes were differentially regulated in vivo by at least threefold (false-discovery rate adjusted $P<0.05$) (Fig. 1a, Supplementary Table 1). A similar number of genes were differentially regulated compared with growth on Mueller–Hinton agar and in Mueller–Hinton broth (Fig. 1a). No matter which comparison is made, a conserved group of 110 genes was differentially regulated in vivo. These gene products include diverse characterized colonization factors, including Peb1A, PldA, CsrA and the capsule polysaccharide transporter KpsM (Supplementary Table 1)[19–22]. These data also reveal only between 10 and 15% of annotated open reading frames show significant differential regulation in infected human faeces compared with growth in standard laboratory conditions.

Although primates can experience disease symptoms during infection, *C. jejuni* has a commensal lifestyle in the natural bird host. To relate transcriptional adaptations that occur during infection and commensalism, we compared our data with a previously published RNA sequencing data set of *C. jejuni* strain 81–176 in the chick caecum (Fig. 1b, Supplementary Table 2)[23]. We identified a core set of 39 conserved genes that are differentially regulated in vivo across hosts, colonization lifestyles and strains. These gene products share strikingly similar roles, including iron acquisition, inorganic phosphate uptake and protection from peroxide stress (Fig. 1c). Epithelial cell production of hydrogen peroxide plays an important role in mucosal immunity against *C. jejuni*. Hydrogen peroxide-mediated inactivation of the *C. jejuni* outer membrane tyrosine kinase Cjtk leads to a decrease in capsule production and therefore decreased virulence[24]. Here we see the importance and conserved nature of this response as *cjtK* along with two major hydrogen peroxide scavengers, *katA* and *ahpC*, are upregulated during both chicken commensalism and human infection. Finally, 7 of these 39 genes showed discrepant expression patterns. Notably, the *fdhTU operon*, which is important for formate dehydrogenase activity[25], is oppositely expressed between the human and chick data.

The most upregulated genes in human infection samples represent two distinct iron acquisition pathways. The *chuA* and *chuD* haemin uptake genes, and *cfrA*, a ferric-enterobactin uptake gene, are all upregulated >100-fold in vivo (Fig. 1d). *chuA* is not required for chicken colonization[26] and is modestly upregulated in the chick caecum (<tenfold increase in vivo), although it is highly upregulated in human infection samples (>1000-fold increase in vivo). There are 161 genes that are differentially regulated specifically in our human samples and not in the chick caecum (Fig. 1b), including the highly upregulated *cfrA*. *C. jejuni* does not produce siderophores, but CfrA enables import of a wide array of siderophores made by other organisms and, unlike *chuA*, is required for chicken colonization[27]. This suggests microbiome-derived siderophores and *cfrA* play an important role in *C. jejuni* infection of humans. Interestingly, components of the Sap antimicrobial peptide resistance efflux pump, which is required for chicken colonization[28], are also uniquely upregulated in human infection (Fig. 1e).

Genome variant selection during human infection

To identify genetic variants that are selected for during human infection we examined the genome of *C. jejuni* before and during acute and persistent human infections. We sequenced the genomes of 49 *C. jejuni* CG8421 infection isolate populations from 14 volunteers during acute and recrudescent infections and compared them with the inoculum genome (Fig. 2a, Supplementary Fig. 1). These infection isolate populations represent bacterial colonies harvested directly from primary isolation plates and are pooled together per sample with no further outgrowth, and is consistent with previous work that validated *C. jejuni* isolate sequencing to study genetic variation in vivo[7]. We achieved exceptional coverage of the infection population genomes across all samples, with an average genome sequencing coverage >1000× and 97% of genes had at least 25× coverage at every nucleotide position (Supplementary Fig. 2). Owing to the complexity of phase variable populations, different subjective methods have been used to determine noteworthy changes in phase variant frequencies over time[7,11,29]. We first called any variant that occurred in at least 1% of any sample population, which resulted in over 600 variants identified across all samples (Supplementary Table 3). To focus on variants that had both a large change in frequency during infection and represented a large portion of the population, we called variants in a sample if they occurred in at least 25% of the population. This enriched for variants that had, on average, a large statistically significant fold change in frequency between the inoculum and an infection isolate populations (~12-fold) while also representing a majority of the infection population sample (~60%) (Supplementary Fig. 3).

This enrichment analysis resulted in 48 unique genomic variants with major changes in frequency in at least one sample, including small nucleotide polymorphisms (SNPs), phase variations (PVs) and multinucleotide variants (MNVs) (Supplementary Table 4). Forty-seven of the variants clearly affected 30 annotated coding sequences and/or promoters and are noted per isolate population in Fig. 2b. Slip-strand mutations in poly G/C nucleotide tracts are the dominant phase variable mechanisms in *C. jejuni*[7], and 19 of 28 tracts in the CG8421 genome varied in at least one infection isolate population (Supplementary Fig. 4). The inoculum population had 12 genetic variants that differed from the reference genome, 4 of which remained intact in all infection isolate populations (Supplementary Table 5).

Three genetic variants were selected for in all 49 infection isolate populations. One of these completely conserved variants is a PV that occurs in the in last 10% of the *fedA* gene open reading frame

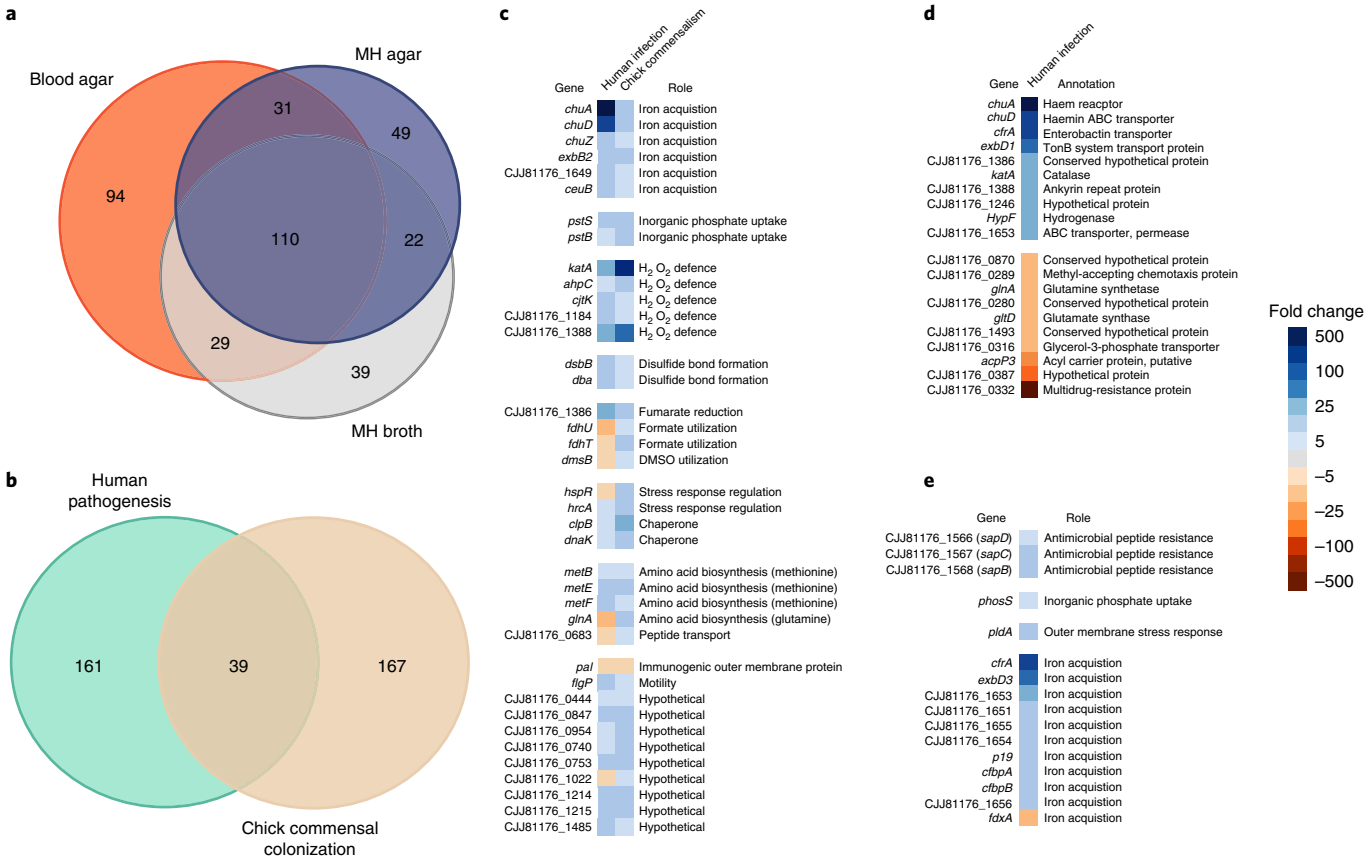

Fig. 1 | The *C. jejuni* transcriptome in human infection and chick commensalism. a, Differentially regulated genes in vivo. A Venn diagram showing the number of differentially regulated *C. jejuni* CG8421 genes (fold change >|3|, false-discovery-rate adjusted (FDR) $P < 0.05$) in the infected faeces of three volunteers compared with laboratory control growths which each represent three biological replicates. **b**, Human infection versus chicken commensalism transcriptomes. A Venn diagram showing the number of differentially regulated (fold change >|3|, FDR $P < 0.05$) gene homologues in vivo between *C. jejuni* CG8421-infected human faeces (three samples) and a previously published RNA-sequencing transcriptome of *C. jejuni* 81-176 in the chicken caecum, which represents three pools of five infected chick caeca each. Both data sets use mid-log phase growth in Mueller–Hinton (MH) broth biological triplicates as the in vitro laboratory comparison. **c**, Conserved differential gene regulation across hosts, lifestyles and strains. The 39 differentially regulated homologues from Fig. 1b conserved between human infection and chicken commensalism with transcriptional fold changes noted. **d**, The top most upregulated and downregulated CG8421 genes in human infection samples. **e**, Notable genes uniquely differentially regulated in human infection samples.

and in the −10 promoter region of an overlapping downstream gene of unknown function. *fedA* is putative hemerythrin that contributes to *C. jejuni* colonization in chicks and is coregulated with flagellar gene expression[30]. A second completely conserved variant is a SNP in the housekeeping gene adenine phosphoribosyltransferase (*apt*), which salvages adenine to make adenosine monophosphate. The inoculum population contained an *apt* SNP that is selected for during osmotic stress[29]; however, the wild-type *apt* sequence was selected for in vivo. The final completely conserved variant is a nine-nucleotide in-frame deletion in the CG8421 homologue of the DNA response regulator CJJ81176_1483 that was selected against in vivo. Selection for the wild-type version CJJ81176_1483 in vivo was confirmed by structural genomic variant analysis (Supplementary Table 6). CJJ81176_1483 is part of a newly identified two-component system that regulates a gluconate dehydrogenase complex important for chick colonization[31]. These data represent three distinct genetic variations (PV, SNP and MNV) in genes with diverse roles (chicken colonization, osmotic stress/housekeeping, two-component system) and demonstrate the genetic adaptability of *C. jejuni* in vivo.

Gene variants associated with human disease or treatment

Rifaximin prophylactic treatment did not impact the rate of campylobacteriosis during the clinical trial. Nevertheless, we hypothesized the antibiotic treatment environment in the host may have selected for unique genomic variants in these volunteers. However, we found no variants were more likely to be called in these volunteers than in control volunteers (Supplementary Table 7) using Fisher's exact test. We concluded rifaximin did not exert a noticeable pressure on *C. jejuni* in vivo. We also hypothesized some variants may be more virulent, and, therefore would be more likely to be found in volunteers who experienced severe disease symptoms. However, a Fisher exact test showed no variants were associated with isolates from volunteers experiencing severe disease compared with typical symptomatic disease isolate populations (Supplementary Table 8). This indicates host factors, more so than genetic drift of more virulent genotypes, may influence the severity of disease symptoms.

Despite being treated with azithromycin and ciprofloxacin and repeatedly producing faeces negative for *C. jejuni* isolates at the end of the trial, five volunteers returned to the clinic with at least one bout of recrudescent infection (Fig. 2a). This rate of recrudescence is higher than previous trials, probably because of enhanced culture techniques, such as not refrigerating the stool before culturing, that were employed specifically for increased vigilance of recrudescence[15]. Recrudescent infection isolates were confirmed to be the inoculum strain and, in all cases, were sensitive to azithromycin and ciprofloxacin. Remarkably, relapse infection isolate populations

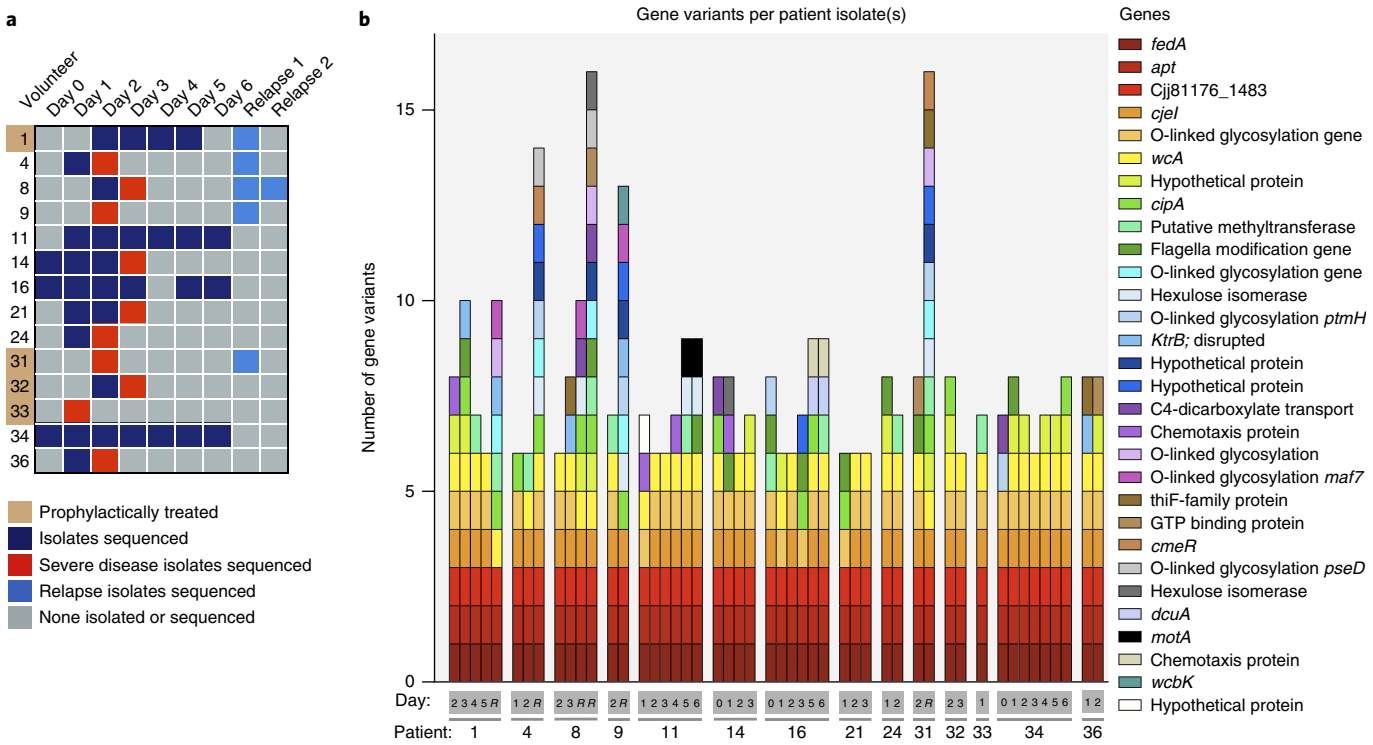

Fig. 2 | *C. jejuni* genomic variants are consistently selected for during human infections. a, Infection populations sequenced. Whole-genome sequencing was performed on 49 infection isolate populations, taken from 14 volunteers across 6 days of primary infection. Five volunteers relapsed after the clinical trial, and those infection isolate populations were also sequenced. Some volunteers experienced severe disease on the noted days (red) and therefore received early antibiotic intervention. Some volunteers received exploratory prophylactic treatment with the antibiotic rifaximin as noted. Sequencing details are noted in Supplementary Fig. 2. **b**, Genomic variants detected per volunteer isolate population, noted by the genes the variants affect. The number of genomic variants detected per infection population is noted on the *y*-axis, and the *x*-axis denotes the day and volunteer the sample was taken from, with R denoting a relapse sample. The corresponding genes are listed by their gene name or annotation when considering homologues across CG8421, 11168 and 81176 *C. jejuni* strains.

had twice as many genomic variants as primary infection isolates (Fig. 3a). We hypothesized the increased variant count would correlate with increased time in vivo. To test this, we compared the number of variants called per sample on every day of infection. Interestingly, there was not an increase in the number of variants called over time during the primary infection (Fig. 3b) and a Fisher exact test showed no particular variants are associated with early or late primary infection periods (Supplementary Table 9). These data suggest there is an immediate and consistent selection pressure during primary infection, and that either a secondary selection event or additional time within the host results in increased genetic variation of relapse isolates.

There are a considerable number of genetic variants that are associated with relapse infection isolates as determined by the Fisher exact test (FDR corrected $P < 0.05$) (Fig. 3c, Supplementary Table 10). Of the ten gene variants associated with relapse infection only three are characterized, named genes. The most striking is a phase variation in *cipA* (cell invasion protein A), which turned the gene 'on' in every relapse infection isolate population, but does not appear to be important for establishing a primary infection. *cipA* was identified in three independent studies to contribute to *C. jejuni* invasion of host cells in vitro[32,33]; however, this gene had not been shown to play a role in vivo until this work in a human challenge model. Mechanisms of *C. jejuni* cellular invasion are not well defined and no biochemical functions of CipA have been previously identified, although a BLAST (Basic Local Alignment Search Tool, provided by the National Center for Biotechnology Information Online) search revealed *cipA* harbours a domain of unknown function (DUF2972) that is also found genes annotated as glycosyl transferases. To determine the role of CipA during human infection we therefore considered possible glycosylation targets. We find the 'on' variant of *cipA* that was selected for in vivo results in modification of the flagella (Supplementary Fig. 6a). This suggests *cipA* is likely to contribute to cellular invasion and persistence in humans via flagella modification.

Another notable gene associated with relapse infection is *cmeR*, a pleotropic bile-sensing transcriptional regulator that is required for chicken colonization[34]. CmeR functions as a dimer to bind DNA and repress gene expression until *C. jejuni* is exposed to bile in the intestinal environment[35]. CmeR repression is relieved upon bile acid binding, which induces expression of the CmeR regulon including the multidrug efflux pump *cmeABC*[36]. Two unique SNPs that each resulted in truncation of the *cmeR* open reading frame were selected for independently in relapse infection isolates and are in non-phase variable loci (Supplementary Table 4). Similar truncations have been shown to prevent dimerization of CmeR and therefore prevent CmeR repression regardless of environmental bile concentrations[37]. *cmeR* was intact in every primary infection isolate, and, likewise, functional CmeR protein is important for colonization of chickens. However, the independent and functionally conserved relapse variants suggest a *cmeR* 'off' mutation can be advantageous in persisting human infections. This may be because of a resulting constitutive expression of the CmeABC efflux pump that increases resistance to bile acids in the host[38], although losing the ability to sense bile is an unexpected in vivo phenotype for an intestinal pathogen.

pseD is the final named gene variant that is associated with relapse infection. PseD attaches pseudaminic acid to flagellin which contributes to virulence-associated autoagglutination of the

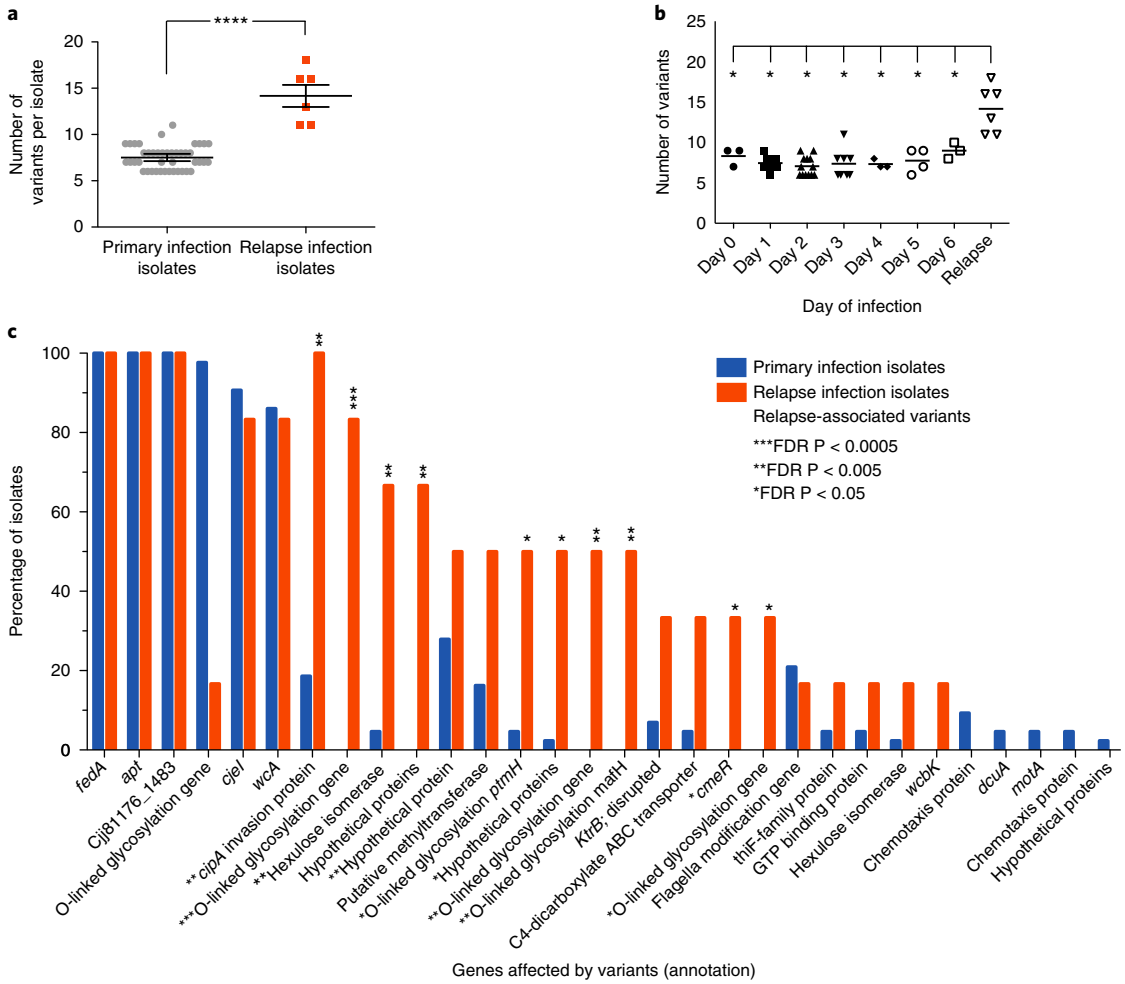

Fig. 3 | Particular genome variants are associated with *C. jejuni* recrudescent infection isolate populations. Primary infection isolates (days 0–6) represent 43 isolate samples while relapse infection isolate populations represent 6 samples. **a**, Bulk genome variation between primary and relapse infection isolates. On average, relapse infection isolates have twice as many genomic mutations compared to primary infection isolates (unpaired two tailed *t*-test with bars representing the mean and standard error of the mean). ****$P < 0.0001$. **b**, Variant accumulation over time. Variants are selected for immediately in human infections (day 0 is the day of inoculation); however, they do not increase over time during the primary infection (one-way analysis of variance, Tukey's multiple comparison test). *$P < 0.0001$. **c**, Genome variants that are associated with relapse infection. After the genome variants were determined for all samples (Fig. 2b), a Fisher exact test was used to determine if variation in particular genes is associated with relapse infection isolate populations. *FDR $P < 0.05$; **FDR $P < 0.005$; ***FDR $P < 0.0005$. Variation of ten genes was statistically associated with relapse infection populations and is noted with a FDR-adjusted one-sided $P < 0.05$. The *y*-axis represents the percentage of samples that had a variant in the gene noted on the *x*-axis. More detailed information is noted in Supplementary Table 4.

bacteria[39]. *pseD* was considered 'on' in every primary infection sample but turned 'off' in a one third of relapse infection samples. The remaining seven relapse-associated gene variants included hypothetical genes, a disrupted aminopeptidase gene, a methyltransferase gene and two additional *O*-linked glycosylation locus genes. Interestingly, an annotated methyltransferase and multiple *O*-linked glycosylation locus genes were identified to be under positive selection in a study of *Burkholderia* infection of cystic fibrosis patients[1], although these genes share low sequence homology with genes in CG8421[1].

One variant is statistically significantly associated with primary infection isolates and not relapse infection isolates (Supplementary Fig. 5, Supplementary Table 11). This is a phase variant in the promoter region of an uncharacterized putative transferase gene, *CJ8421_RS06560* (homologue of Cj1321 in strain NCTC_11168), located in the flagellin glycosylation locus[40] and was strongly selected against in primary infection isolates, but not in relapse infection isolates. In total, seven of the eight flagella modification genes identified in our analysis are associated with either primary or relapse infection isolates, indicating the significance and variability of flagellar modification during human infections (Supplementary Fig 6). Finally, Fig. 4a depicts a SNP tree analysis to visually represent the differences in genomic variations among primary and relapse infection isolates. The closer two samples are on the tree, the more similar their genomic variant profiles are to one another. Primary infection isolates from the same volunteer are more similar to each other than they are to the isolates of other volunteers. However, the relapse infection isolates from different volunteers are more similar to each other than they are to the founding primary infection isolates from the same volunteer. Overall, the relapse-associated variants demonstrate the profound genetic differences that occur in persisting bacteria.

Genome variant selection in *Aotus* primates

The variants selected for during human infection replicated strongly across volunteer isolate populations. This powerfully

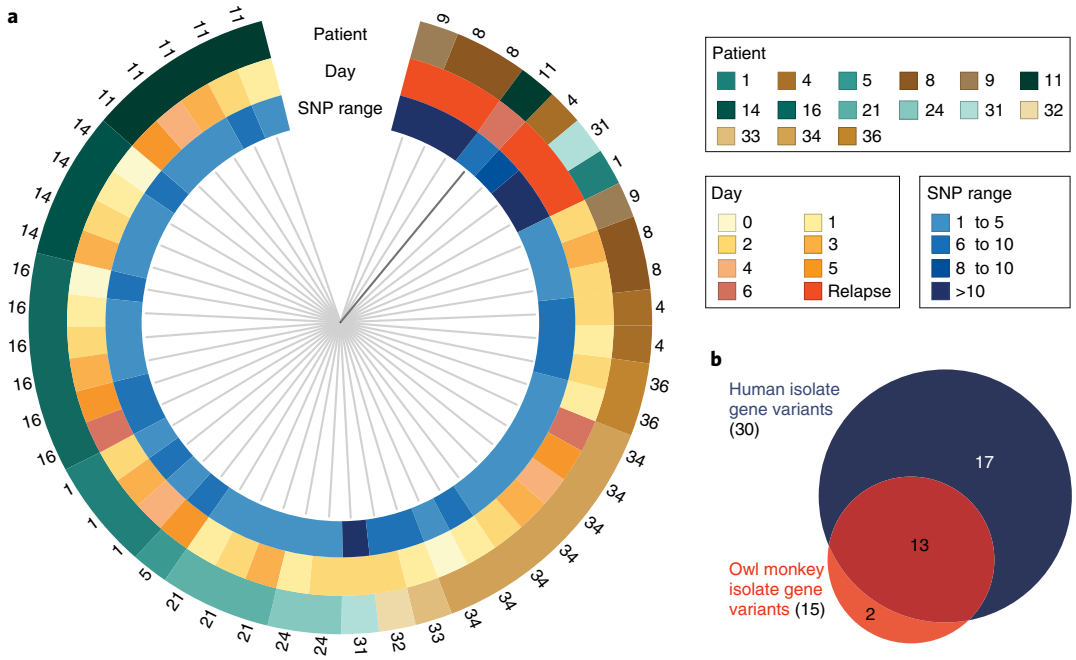

Fig. 4 | Validating *C. jejuni* genomic variant selection in non-human primates. a, A SNP tree representing how similar human infection isolate populations are to one another. Each wedge represents one sequenced isolate population, and the closer two samples are to each other the more similar their genomic variations. Volunteer identification numbers per sample are noted on the outside of the ring, and the day of infection and the number of SNPs detected are denoted by the inner rings. Note that primary infection isolates from the same volunteer are more similar to each other than they are to isolates from other volunteers. However, relapse infection isolates (bright orange) are more similar to each other despite the volunteer they come from. **b**, Conserved gene variant selection across primates. A second, different *C. jejuni* CG8421 inoculum was used to infect owl monkeys, and the genomes of 12 diarrhoeal primary infection isolate populations from five monkeys were sequenced (Supplementary Tables 12 and 13). The Venn diagram shows that the *C. jejuni* genome variants selected for in owl monkey infection closely resemble those of human infection (Supplementary Table 13). This supports the human data and suggests that non-human primates harbour a similar fitness selection environment found in humans. The smaller sample size and no relapse infections contributed to fewer total variants detected in the monkeys.

suggests a conserved selective pressure and adaptive response in vivo. However, over 30 coding sequences were implicated in adaptation, and understanding the fitness contribution of variant combinations within the population is challenging. To validate our data and respect the complexity of population-level adaptation, we performed a similar analysis in a symptomatic non-human primate infection model. We hypothesized adaptations similar to those in human infection would be selected for in a New World monkey, *Aotus nancymaae*. An independently prepared CG8421 liquid inoculum was used to infect *A. nancymaae* and we compared this inoculum genome with 12 diarrhoeal isolate populations taken over 8 days of infection from 5 monkeys (Supplementary Table 12). Despite differences between the human and *Aotus* inoculums (Supplementary Table 12), we found the genetic adaptations selected for in *Aotus* closely resemble those in humans (Fig. 4b, Supplementary Table 13). The three completely conserved variants that were selected for in human infection were already present as a majority in the *Aotus* inoculum, and congruently, were maintained during infection. Two genes were uniquely varied in *Aotus* infection, including a SNV in the promoter region of *CJJ81176_1215*, a gene encoding an uncharacterized, putative NlpA-family lipoprotein (Supplementary Table 13). Interestingly, the second variant is a E70K amino acid substitution in *chuA*, which is also the most upregulated gene during human infection. Therefore, *chuA* can undergo both transcriptional and genetic adaptations during primate infection. Few of the genetically variable genes were also differentially regulated in vivo, yet this is consistent with analyses done in vitro[41] and in vivo[42] that show genes that contribute to fitness are rarely also differentially regulated.

Discussion

Understanding the bacterial pathogen response to the human host revealed genetic mechanisms of success during both establishment of infection and persistent human colonization. Notably, we determined the *cipA* gene had the strongest association with recrudescent, persistent infections, and we went on to identify its role in *C. jejuni* flagellar modification. Nine other variable genes are part of the conserved relapse infection genotype. We suspect this genetic background may enable bacteria to reside in a protected niche within the host during primary infection where they withstand being shed from the host, resist antibiotic treatment and the host immune response and result in recrudescent infection. This genotype would then be the dominant recrudescent population, and a similar pattern was seen in this study. Future therapy design should consider the 'on/off' status of a therapeutic target throughout primary and recrudescent infections, as well as appreciate target expression alterations because of genetic variation of promoter regions or transcription factors such as CmeR.

Tracking pathogen evolution in vitro requires studying multiple cultures grown in parallel, and variants that arise consistently across replicates are considered adaptive. Studying pathogen evolution in vivo can reveal important virulence determinants, although it is difficult to study well-controlled human infections in parallel. Here we used *a C. jejuni* clinical trial to examine bacterial adaptation during human infection and found many genomic variants are well conserved across infected volunteers. We also found human infection exemplifies rapid bacterial adaptation, as variants are selected for in vivo on the day of inoculation. This study characterizes pathogen genetic and transcriptional adaptations across hosts and lifestyles,

and similar collaborative approaches in future clinical studies may increase the possibilities for therapeutic design.

Methods

***C. jejuni*-infected human faeces sample preparation for RNA sequencing.** *C. jejuni* strain CG8421-infected diarrhoea was weighed and added 1:1 in RNA Later reagent as quickly as possible after it was produced and frozen at −80 °C. Samples came from volunteers who were not receiving antibiotics and therefore represent untreated infections. Preserved samples were thawed on ice and total RNA extracted via Trizol-chloroform phase separation. DNA was removed (Turbo DNA-free DNAse kit, Ambion), rRNA depleted (Ribo-Zero human and bacterial rRNA removal kits, Illumina), and libraries built for Illumina Sequencing (Ultra Directional RNA Library Prep Kit for Illumina, New England Biolabs).

In vitro C. jejuni sample preparation for RNA sequencing. *C. jejuni* strain CG8421 was grown at 37 °C in a microaerobic atmosphere (85% nitrogen, 10% carbon dioxide, 5% oxygen) in a Coy Laboratories atmosphere controlled chamber. For RNA sequencing of samples grown on agar plates, Mueller–Hinton agar or 5% sheep blood agar plates were streaked in triplicate from a frozen stock of *C. jejuni* strain CG8421. More than ten colonies from these plates were restreaked on fresh plates and mid-log phase bacteria were harvested and suspended in Trizol reagent for RNA extraction. For RNA sequencing of samples grown in liquid broth, 5 ml aliquots of Mueller−Hinton broth were equilibrated in the microaerobic chamber for 24 hours before being inoculated and were grown with shaking. First, Mueller–Hinton agar plates were streaked in triplicate from a frozen stock of *C. jejuni* strain CG8421. Colonies from these plates were used to inoculate equilibrated broth and grown with shaking to stationary phase. These cultures were then used to inoculate freshly equilibrated Mueller–Hinton broth aliquots, which were grown with shaking until mid-log phase. Bacteria were then harvested via centrifugation and resuspended in Trizol reagent for immediate RNA extraction. Extracted RNA was DNAse treated (Turbo DNA Free DNAse Kit, Ambion), rRNA was removed (Ribo-Zero Bacterial rRNA Removal Kit, Illumina) and the samples were prepared for Illumina Hiseq using the TruSeq Stranded mRNA Library Kit (Illumina).

Illumina RNA sequencing and analysis. For RNA sequencing of samples from infected human faeces, three RNA libraries built from the infected faeces samples of different volunteers were sequenced using Illumina Hiseq Single-End 50 bp reads. Approximately 13 billion reads were obtained in total across all three samples. Using CLC Genomics Workbench software, reads were trimmed to 21 bp and aligned to the CG8421 published reference genome using 100% exact homology match mapping parameters. At least 3 million reads mapped to the reference genome per sample. For in vitro samples, RNA libraries were sequenced and aligned in an identical manner, with at least 15 million reads mapping to the reference genome per sample. To determine differential expression between samples, reads per kilobase per million reads (RPKM) values were determined for each gene, normalized by quintile,and then were used in a Baggerley's test to determine the weighted proportions fold change between groups of biological replicates (all groups were in triplicate) with a FDR-corrected *P* value. Fold changes $\geq|3|$ with a FDR $P \leq 0.05$ were considered significant. Annotation of differentially regulated CG8421 genes was made by considering the annotations of homologous genes in strains 81–176 and 11168 as determined by National Center for Biotechnology Information's online blastp suite. Additional functional annotations were made after considering published literature, using the National Center for Biotechnology Information's PubMed literature search online. Comparison of the CG8421 transcriptome in human faeces to the *C. jejuni* 81–176 transcriptome in the chicken caecum was made by using the published list of expression fold changes per 81–176 gene in the chicken caecum as described in Supplementary Table 2. Both datasets use mid-log phase microaerobic growth in Mueller–Hinton broth as the reference transcriptome. We compared the list of differentially regulated CG8421 genes in human faeces (>|3|-fold, <0.05 FDR *P* value) to the list of differentially regulated 81–176 genes (>|3|-fold, <0.05 adjusted *P* value) in the chicken caecum. Only genes with a homologue in both strains (as determined by similarity of predicted amino acid sequence) were included in the comparison.

Human infection and inoculum preservation. The study protocol was approved by the Naval Medical Research Center and the Western Institutional Review Boards in compliance with all applicable local and Federal regulations governing the protection of human subjects. Samples were obtained from a previously described[15] controlled human infection model (ClinicalTrials.gov Identifier NCT02280044, Navy Medical Research Center IRB NMRC.2014.0013, and Western Institutional Review Board study number WIRB 1147996). Volunteers were healthy adults recruited from the mid-Atlantic area, signed informed consent prior to participating in the study and agreed to all future use of the samples obtained during the study. Volunteers who were admitted to the inpatient facility at Johns Hopkins University Center for Immunization Research for the study had ages ranging from 18 to 50 years (median 30 years), normal stool patterns, were not using antidiarrhoeal or antacid therapy and had no history of clinically significant diseases. Subjects with a history of *Campylobacter* exposure, a personal and/or family history of Guillain–Barré syndrome and/or inflammatory arthritis, were positive for HLA-B27 or whose serum immunoglobulin A titre to CG8421 glycine extract was greater than 1:4000 were excluded from the study. Admitted volunteers were inoculated on day 0 with a *C. jejuni* CG8421 liquid inoculum that was generated using good manufacturing processing and kept on ice. The inoculum is derived from Mueller–Hinton agar plate growths that are suspended in phosphate-buffered saline (PBS), and therefore has complex standing genetic variation (detailed in Supplementary Table 3) and is not derived from a single colony. This resuspension method was used for the *A. nancumaae* inoculum preparation as well. Immediately prior to inoculation, the PBS resuspension human inoculum (target concentration of 5×10^5 colony forming units per ml (c.f.u. ml^{-1}) was diluted in sodium bicarbonate ($NaHCO_3$) buffer to neutralize stomach acids and ingested. After the last patient was inoculated, a portion of the remaining inoculum was used to verify the infection dosage via growth and colony enumeration on *Campylobacter* CVA (cefoperazone, vancomycin and amphotericin B) agar (dosage was 1.7×10^5 c.f.u.) and another portion was frozen at −80 °C to later be used for genomic sequencing.

Human infection isolate collection/preservation. *Campylobacter* isolation from infected faeces was performed as previously described[15]. Briefly, infected faeces were diluted and temporarily incubated in thioglycollate broth, plated on selective *Campylobacter* media and incubated in a microaerobic atmosphere at 37 °C. Colonies on these primary isolation plates were screened by eye and all putative *C. jejuni* colonies were collected and pooled together in glycerol media per sample and frozen at −20 °C. An effort was made to collect at least 10 colonies per sample, and samples with insufficient amounts of *C. jejuni* isolates were identified during analysis of the sample genomes (samples with less than 50× coverage of the genome) and removed from the study.

Genomic sequencing. The frozen inoculum and isolate samples were thawed on ice, genomic DNA was extracted (Easy DNA Kit, Invitrogen) from each sample, followed by processing for Illumina NextSeq Single-Read sequencing. All genome analysis was preformed using CLC Genomics Workbench. Reads were aligned to the CG8421 reference genome for each isolate population. Only isolate population samples with at least 50× coverage of the CG8421 genome were included for genomic variant analysis. These samples averaged >1,000× genome coverage (Supplementary Fig. 2).

Genomic variant calling. Mapped reads were locally realigned and genomic variations compared with the reference genome were identified using a no ploidy assumption low-frequency variant detection method. For the highest quality data we manually removed clear false-positive variant calls, such as SNPs called in extensive pile-ups of single reads or in regions of unreliable coverage. By calling variants that occurred in at least 1% of any sample population, we identified over 600 genomic variants across all samples. This list of variants, including their frequency in the population, per sample, is provided in Supplementary Table 1. To enrich this list for meaningful variants that may impact the human disease state, we aimed to identify genome variants that had major changes in frequency between the inoculum and isolate populations, such as a minority variant in the inoculum becoming a majority variant in an infection isolate population. By defining a variant as a mutation that occurred in at least 25% of a sample population, and identifying variants that differed between the inoculum and an isolate population, we achieved this goal (Supplementary Fig. 1). For example, using this approach, variants that were only called in an isolate population(s) on average had a 12-fold increase in frequency between the inoculum and the infection isolate populations (Supplementary Fig. 3). These variants represented a minority of the inoculum population (~5% on average), but a majority in the isolate population (~60% on average across samples) (Supplementary Fig. 3). These variants were used in the analyses throughout the study and are listed in Supplementary Table 4.

Genomic variant analysis. Our variant calling procedure identified 48 genomic variants with major changes in frequency between the inoculum and least one isolate population. Based on variant location in the published annotated CG8421 genome, these variants are likely to affect ~30 annotated genes. In many cases, different variants across samples had an equivalent predicted affect on the same gene (such as an insertion or deletion induced frameshift at the same location in the same gene) and this is shown for each variant in Supplementary Table 1d. To incorporate these shared variant affects into our analysis, we manually refined the variant lists to reflect the genes affected by each variant (Supplementary Fig. 1, Step 5), so that different variants with the same affects were considered as equivalent. This enabled us to determine if variation in a particular gene was associated with the human disease state (Supplementary Fig. 1, Step 6) such as if a variation in a particular gene is associated with relapse isolate populations compared with primary infection isolate populations. To do this, we preformed Fisher's exact test with a FDR-corrected *P* value in CLC Genomics Workbench to compare variants between sample groups. The Microbial Genomics module of the CLC Genomics Workbench was used to generate the SNP tree seen in Fig. 4a following a previous study[43]. Briefly, the tree visually represents how similar genomic variations are between samples. The closer two samples are to each other, the more similar the

genomic variations. When examining genomic variants between samples, different variant alleles at the same genomic location are scored as different variants. Other statistical tests used to compare variant frequencies were performed in the statistics and graphing software Prism 6 and are noted in the appropriate figure legends.

***Aotus nancymaae* infection, isolate collection and analysis.** The *A. nancymaae* infection protocol was approved by the Naval Medical Research Unit-6 Institutional Animal Care and Use Committee (IACUC approval number NAMRU6-16-03) in compliance with all applicable Federal regulations governing the protection of animals in research. *A. nancymaae* monkeys were infected as described previously[44,45] with a single *C. jejuni* CG8421 liquid inoculum that was harvested from Mueller–Hinton agar plates and resuspended in PBS similarly to harvesting bacteria for the human inoculum. Animals were treated with rantidine and CeraVacx to neutralize stomach acid and were anaesthetised with ketamine intramuscularly. Animals were inoculated with 5×10^{11} bacteria in 5 ml of PBS orogastrically for infection and a portion of the inoculum was frozen at −80 °C for preservation and genomic sequencing. *C. jejuni*-infected diarrhoea was plated on *Campylobacter* selective media as previously described[44,45] and isolates were collected and pooled per sample, processed for sequencing and data analysed in a parallel manner as described for the human infection isolate population samples. After genomic sequencing, 12 diarrhoeal samples from 5 monkeys covering 8 days of infection had high enough *C. jejuni* isolate counts to be included in this study (as determined by CG8421 genome coverage), as indicated in Supplementary Table 12, and were produced by male and female monkeys with a median age of 14 months as noted in Supplementary Table 12. The *Aotus* inoculum was prepared independently of the human inoculum, and, similarly, is not derived from a single colony. This accounts for differences in standing genetic variation between the two inoculums and is detailed in Supplementary Table 12. Therefore, the *Aotus* infection isolate population genomes were compared with the *Aotus* inoculum during genetic analysis of the *Aotus* samples. The samples used in this study were selected from *an A. nancymaae* infection preformed as a randomized controlled immunization and *C. jejuni* CG8421 challenge model. Power calculations were used to determine the appropriate number of animals to be used in that preventative treatment investigation model. To be consistent with our human isolate data, we only used samples if (1) the sample represented symptomatic disease (diarrhoea), (2) the sample was produced by an animal that did not receive therapy before or at the time the sample was produced and (3) genomic sequencing yielded sufficient coverage of the *C. jejuni* CG8421 genome for genetic analysis. Therefore, it was necessary that choosing samples for use in our study be performed in a non-blinded manner.

Cj0617/618 flagellin mobility shift assay. The following genetic studies were performed on strain 81–176 because strain GC8421 is not amenable to genetic analyses. The homopolymeric tract in Cj0617/618 was repaired as previously described[46]. Briefly, the genes were PCR amplified and cloned behind a sigma28 promoter. The G tract was repaired such that the two genes were fused into a single open reading frame, and the codons were modified to introduce silent changes that removed the homopolymeric tract by Quick Change mutagenesis using primers pg.16.18 (5′-CTCCATTTAAACTAATGAGAGGCGGCGGTATT-AGAACGATTTTGTTTGG-3′) and pg.16.19 (5′-CCAAACAAAATCGTTCTA ATACCGCCGCCTCTCATTAGTTTAAATGGAG-3′). The repaired gene and a kanamycin resistance cassette were introduced into the arylsulfatase gene of 81–176. Purified flagellins were separated using ampholytes ranging from 4–6 (Biolyte 4/6; BioRad) as previously described[47]. IEF protein markers were purchased from Serva.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. All genomic and RNA sequencing data used in this study have been deposited in the NCBI sequence read archive under Bioproject PRJNA392448.

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Author contributions

A.A.C. conceived, designed, preformed and interpreted genetic and transcriptomic analyses and wrote the paper. P.G. and M.S.T. established this collaborative project and oversaw this study. B.W.D. and M.S.T. oversaw computational analyses. A.A.C., M.S.T. and P.G. provided critical biological interpretations of the data. J.E.R, C.H., D.S., K.R.T., C.K.P., R.L.G., B.D., J.B., R.M.L., A.C.M., K.J., A.A., D.R.T. and M.S.R. conducted the clinical trial. F.M.P. and J.M.K. collected specimens for transcriptomics. C.P.E. performed experiments relevant to biological interpretation of the data. A.R. and A.J.M. performed the non-human primate experiments. B.W.D., P.G. and M.S.T supervised this work and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Experimental design

1. Sample size

Describe how sample size was determined.

For genomic studies of isolate populations from humans, sample availability, volunteer clinical outcomes, and read coverage of the reference genome, all influenced the number of samples in the study. First, a group of volunteers that had varying clinical outcomes during the human infection trial were determined. These patients included placebo treated and prophylactic treated patients, patients with and without recrudescent infections, and patients with and without severe disease that required early antibiotic intervention. Then available isolate population samples from these volunteers were prepared for genomic sequencing and sequenced. After sequencing and read mapping, the samples with robust coverage of the reference genome were advanced to variant analysis. We defined robust coverage in line with field standards. Samples that had at least 95% of genes covered with at least 25 fold coverage across the entire gene length, and at least 25 average fold coverage across the genome were considered to have robust coverage. Only samples with robust coverage were advanced and included in the manuscript. To our knowledge this work produced the deepest sequencing of C. jejuni isolate populations to date.

For genomic studies of isolate populations from non-human primates, a similar procedure was followed. All isolate populations harvested from days animals had diarrhea were sequenced and those that met the coverage analysis described above advanced to variant analysis and are included in the manuscript.

For transcriptomic studies, availability of RNA-later preserved samples and robust read coverage of the reference genome were the determining factors for sample selection. After PCR and Illumina MiSeq based screenings, 3 infected diarrhea samples from 3 different patients were chosen based on the feasibility of sequencing the samples enough for appropriate read coverage.

2. Data exclusions

Describe any data exclusions.

For genomic samples, exclusion criteria were dependent on genome coverage. Genomic samples with less than 95% of genes with at least 25 fold coverage across their entire length were excluded. As we wanted to be sure the homopolynucleotide tracts in the C. jejuni genome were accurately counted, we produced the deepest genomic sequencing of campy isolates ever published to our knowledge (average fold coverage >1000). Overall, 5 human isolate populations and 5 non-human primate isolate population samples were excluded from the variant analysis due to poor coverage. Overall, the excluded samples had very poor coverage of the genome (approximately < 5 percent of genes with at least 25 fold coverage).

For transcriptomic studies, the feasibility of obtaining robust read coverage of the reference genome excluded many samples. 19 infected diarrhea samples preserved in RNA-later were screened by PCR (to determine relative C. jejuni loads between samples) and/or Ilumina MiSeq (to quantify mappable RNA-seq reads). These screenings identified which samples would require the fewest sequencing reads for appropriate reference sequence coverage to yield robust statistically

significant differential gene expression analysis. Based on these initial screenings alone, three were chosen for the required additional sequencing and are used in the manuscript. All three samples were at least grade 3 stools produced by different volunteers and are considered biological replicates of infected diarrhea populations. Rejected samples would have been cost-prohibitive to sequence to an acceptable coverage.

3. Replication

Describe whether the experimental findings were reliably reproduced.

When repeat analyses/experiments were appropriate to preform they were successful in reproducing the results presented in the manuscript.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Samples were allocated into groups as determined by the clinical outcomes as discussed in the manuscript. Groups of data used in comparison analyses were determined as described in the manuscript. Briefly, we present full data sets first before using appropriate groups of these data in comparisons or figures. For instance, we include all genome variants called in all samples (down to 1 percent frequency of occurrence in the individual sample population) in supplemental data before highlighting the most common variants with the largest change in frequency between pre and post infection in the main text. Similarly, we include the entire statistically significant differentially expressed genes between transcriptomics samples before highlighting special genes of interest.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Those involved with genetic and transcriptional analyses were and are still blind to patient identification information. Those involved with genetic and transcriptional analyses were not blind to clinical outcomes (severe disease, prophylactic vs placebo treatment, recrudescence status) of patients along with their corresponding samples, as these outcomes determined the groups for comparison analyses. There was similarly no blinding of non-human primate isolate populations, as only those from diarrheal samples were relevant for analysis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

▸ Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

CLC Genomics Workbench (Through version 9.5) along with the Microbial Genomics module (Trial version), Prism, and Microsoft Excel (2010 Mac) were used for data analysis when appropriate.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	Material availability is only restricted by the amount of sample collected during the human and non-human primate infection models, minus the amount of material used to prepare nucleic acids for sequencing.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	Not applicable

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	Not applicable
b. Describe the method of cell line authentication used.	Not applicable
c. Report whether the cell lines were tested for mycoplasma contamination.	Not applicable
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	Not applicable

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	Research animals were not directly examined in this study. Bacterial isolate populations were derived from samples produced from an independent study approved by U.S. Navy Medical Research included in the main text disclaimer.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	Human subjects were not directly examined in this study. Bacterial isolate populations were derived from samples produced by volunteers that participated in the ClinicalTrials.gov Identifier NCT02280044 clinical trial and included in the main text disclaimer. These volunteers were considered healthy adults from the mid-Atlantic region of North America.

APPENDIX 3

Harro C et. Al. Does Rifaximin Chemoprophylaxis Prevent Campylobacteriosis in the Human Challenge Model? A poster presentation at the Infectious Diseases Society of America Infectious Diseases Week, San Diego 2015.

Does Rifaximin Chemoprophylaxis Prevent Campylobacteriosis in the Human Challenge Model?

Clayton D. Harro[1], Joanna E. Rimmer[2,3,4], David A. Sack[1], Kawsar R. Talaat[1], Ramiro L. Gutierrez[2], Barbara DeNearing[1], Chad K. Porter[2], Jessica Brubaker[1], Alexander C. Maue[2], Renee M. Laird[2], Frédéric Poly[2], Patricia Guerry[2], Kayla Jaep[2], Ashley Alcala[2], David R. Tribble[5] and Mark S. Riddle[2].

[1]Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, [2]Enteric Diseases Department, Naval Medical Research Center, Silver Spring, MD, [3]School of Immunity and Infection, University of Birmingham, Birmingham, United Kingdom, [4]Academic Department of Military Medicine, Royal Centre for Defence Medicine (Academia and Research), Medical Directorate, Joint Medical Command, ICT Centre, Birmingham Research Park, B15 2SQ Birmingham, UK, [5]Infectious Disease Clinical Research Program (IDCRP), Uniformed Services University of the Health Sciences, Bethesda, MD

Conclusion

This study showed no significant difference in the development of the primary endpoint, Campylobacteriosis, between subjects treated with rifaximin or placebo. However, the difference in mean total loose stool output (mL) is significantly reduced in the rifaximin group, and within this group a reduction in the maximum volume of loose stools in 24 hours was also observed. Therefore, whilst rifaximin does not protect against Campylobacteriosis, this objective data relating to stool volume suggests that fluid loss is reduced, and therefore should be considered when evaluating the efficacy of rifaximin in reducing the severity of the burden of disease in all cause bacterial travelers' diarrhea. Based on the high diarrhea attack rates noted in this human challenge model, the efficacy of rifaximin chemoprophylaxis against campylobacteriosis in a field setting requires further evaluation.

Background

Travelers' diarrhea (TD) is common; the growing concern regarding post-infectious chronic health sequelae raises the need to consider strategies for primary prevention. Studies have demonstrated field efficacy of rifaximin prophylaxis in prevention of travelers' diarrhea where diarrheagenic *Escherichia coli* predominate, as well as in a *Shigella* human challenge model[1]. The efficacy of rifaximin as prophylaxis against other invasive pathogens, such as *Campylobacter jejuni*, remains in question.

Methods

- Design: double-blind, placebo-controlled, randomized (1:1)
- Clinical site: Johns Hopkins Center for Immunization Research
- Primary objective: estimate the efficacy of rifaximin in preventing campylobacteriosis in an experimental *C. jejuni* infection
- Primary endpoint: campylobacteriosis in 144 hours post-challenge
  - Moderate to severe diarrhea (max 24 hr loose stool output)
    - Severe: > 6 loose stools or > 800 g
    - Moderate: 4-5 loose stools or 401–800 g
  - Fever without diarrhea, with an associated symptom
- Prophylactic treatment (Days -1, 0, 1, 2):
  - Rifaximin (550 mg) BID
  - Placebo BID
- Challenge (Day 0): 1.8×10^5 *C. jejuni*, strain CG8421
- Sample size:
  - 30 subjects (1:1) initiated on study treatment
  - 28 subjects (1:1) challenged
- Antibiotic treatment:
  - 144 hours post-challenge (Day 6 or earlier if indicated)
  - Azithromycin 500 mg daily and Ciprofloxacin 500 mg BID for 5 days
- Discharge: Day 10 (earlier if infection cleared & symptoms resolved)
- Outpatient follow-up: Days 14, 21, 28, 35, 56, 84, 180

Results

- Study population: 28 subjects challenged
  - 71% male
  - 93% African-American
  - Median age: 30
- 100% of Subjects had *C. jejuni* CG8421 positive stool
- No difference in campylobacteriosis rate, or the incidence of other clinical signs or symptoms, across study groups (Table 1)
- Lower maximum 24 hour volume and total output by volume of loose stool in the rifaximin group (Table 2, Figures 1 and 2)
- 68% met criteria for early antibiotic treatment
- 18% microbial recrudescence
  - Rifaximin: 13.1%
  - Placebo: 23.1%

Table 2. Loose stool output data, by group

	Placebo (n = 13)	Rifaximin (n = 15)	P-value
Total number loose stools	18 (11, 23)	12 (7, 18)	0.13
Max number loose stools/24 hrs	9 (8, 11)	7 (3, 12)	0.27
Total volume loose stools (mL)	**2565 (1777, 3183)**	**1437 (531, 2213)**	**0.05**
Max volume loose stools/24 hrs (mL)	1063 (946, 1490)	704 (476, 1056)	0.07
Time to first loose stool (hrs)	34.0 (24.5, 49.3)	44.5 (33.1, 56.4)	0.34
Duration of Diarrhea (hrs)	100.4 (83.3, 115.2)	75.2 (71.2, 113.6)	0.36

Table 1. Symptoms experienced, by group (%)

	Placebo (n = 13)	Rifaximin (n = 15)	p-value
Campylobacteriosis	84.6	86.7	1.00
Dysentery	30.8	26.7	1.00
Abdominal Pain/ Cramps	69.2	73.3	1.00
Nausea	38.5	40.0	1.00
Vomiting	0.0	20.0	0.23
Fever	46.2	46.7	1.00

Figure 1. Max volume of loose stool output/24 hrs

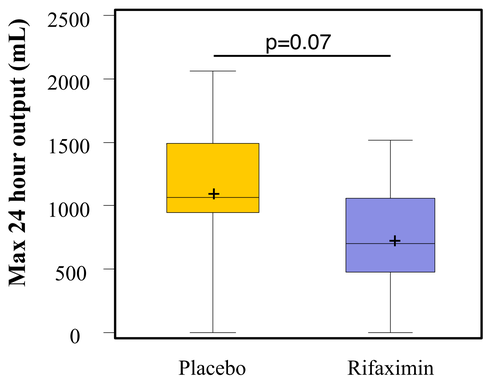
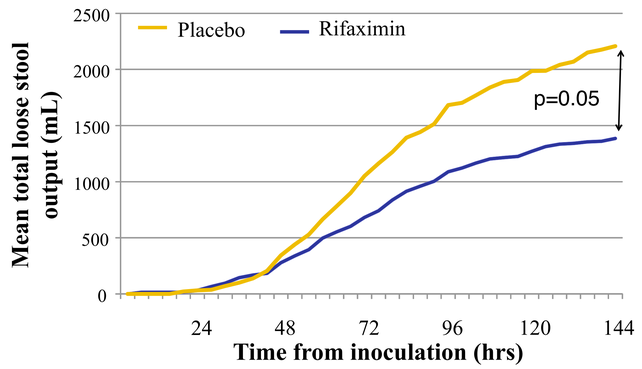


Figure 2. Total volume of loose stool output, up to 144 hrs



Discussion

The results of this study suggest that Rifaximin does not protect against moderate-severe Campylobacter disease in the human challenge model using *C. jejuni* strain CG8421 (despite susceptibility to rifamycins, data not shown).

- Zanger et al. found relatively less efficacy in a TD trial among participants to SE Asia where Campylobacter and Salmonella are known to be common pathogens[2].
- Rifaximin, a bile soluble antibiotic, is known to have less activity in the colon; the site of infection for this pathogen. Alternative forms, rifamycin SV MMX, may have more activity at this site and may be more effective against colonic infections.
- Given the increasing recognition of post-infectious complications of TD, consideration of a safe and effective chemoprophylactic may be of value in reducing the overall burden of disease.
- More studies on safety (including microbiome and ESBL-PE carriage), and efficacy (against acute disease and chronic consequences) are needed.

Acknowledgments and Disclaimers

The views expressed are those of the authors and do not necessarily reflect the official views of the United States Department of the Navy, or the Department of Defense, or the United Kingdom Defence Medical Services. This work was funded by US Navy Advanced Medical Development. Mark Riddle, Ramiro Gutierrez, Chad Porter and Patricia Guerry are military service members or employees of the US government. This work was prepared as part of their official duties. Title 17 USC 105 provides that 'Copyright protection under this title is not available for any work of the US Government.' Title 17 USC 101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person's official duties. Additional academic supervision was provided by Prof. Adam Cunningham, School of Immunity and Infection, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

The product rifaximin was purchased by Johns Hopkins CIR from the pharmaceutical distributer McKesson Corporation. The product is manufactured by Salix Pharmaceuticals.

References

[1] Taylor D.N. et al., *Rifaximin, a Nonabsorbed Oral Antibiotic, Prevents Shigellosis after Experimental Challenge,* Clin Infect Dis. (2006) 42 (9): 1283-1288.

[2] Zanger P. et al., *Effectiveness of rifaximin in prevention of diarrhoea in individuals travelling to south and southeast Asia: a randomised, double-blind, placebo-controlled, phase 3 trial,* Lancet Infect Dis. 2013 Nov;13(11):946-54.

APPENDIX 4

Rimmer J.E et. Al., Development of a Serum Bactericidal Assay to determine functional antibody response following vaccination against *Campylobacter jejuni*. A poster presentation at the Vaccines for Enteric Diseases conference, Edinburgh 2015.

Development of a Serum Bactericidal Assay to determine functional antibody response following vaccination against *Campylobacter jejuni*

Authors: Joanna E. Rimmer[1,2,3], Nina M. S. Schumack[1], Alexander C. Maue[1], Patricia Guerry[1]

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[2]School of Immunity and Infection, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK
[3]Academic Department of Military Medicine, Royal Centre for Defence Medicine (Academia and Research), Medical Directorate, Joint Medical Command, ICT Centre, Birmingham Research Park, B15 2SQ Birmingham, UK

Introduction

Campylobacter jejuni is one of the most frequently isolated diarrhoeal disease pathogens, causing a spectrum of acute and chronic disease.

C. jejuni is encapsulated by a polysaccharide capsule, which can be exploited to develop a capsule-conjugate vaccine. Since other licensed conjugate vaccines have used a Serum Bactericidal Assay (SBA) and fold increases in bactericidal titer as a correlate/surrogate for protection, we evaluated this for *C. jejuni* conjugates.

A SBA was developed to evaluate the functional serum antibody response of both New Zealand White Rabbits vaccinated with the cGMP grade capsule-conjugate *C. jejuni* vaccine, CJCV1, with or without the adjuvant Alhydrogel, as part of a toxicology study; and *Aotus nancymaae* Non Human Primates (NHP) vaccinated with increasing doses of a research grade vaccine; 0.1, 0.5 and 2.5 µg Polysaccharide (PS), or a Phosphate Buffered Saline (PBS) control, (n=5 for each group), then challenged with *C. jejuni,* as previously published (Monteiro et al., 2009, Infect. Immun.77:1128).

Methods

Freshly grown *C. jejuni* cells set to an OD_{600} of 0.1 in dextrose-gelatin-veronal buffer were mixed in 96-well microtiter plates with serially diluted, heat-inactivated sera and baby rabbit complement (BRC), then incubated for 60 minutes under microaerobic conditions at 37° C.

Following enumeration of the surviving bacteria by plate count, the final titer that achieved 50% killing was determined. Control wells contained BRC but no serum.

Assays were performed using sera from New Zealand White Rabbits that had been immunized sub-cutaneously on days 1, 29 and 57 with or without alhydrogel; sera collected on days 0 and 71 were used for the SBA (Figure 1). For NHP that were vaccinated at 6-week intervals with three doses of vaccine, then challenged, sera collected on days 0 and 125 were evaluated (Figures 2 and 3).

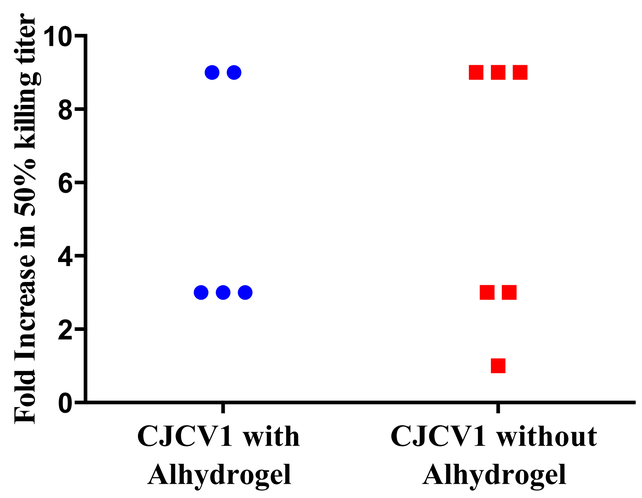
Details of the SBA method were modified between the two sets of experiments.

Conclusions

- **A SBA method that demonstrates an increase in functional bactericidal titers in both immunized New Zealand White Rabbits and *A. nancymaae* NHP has been established.**
- **When considering a correlate/surrogate for protection the difference in the fold increase in 50% killing titers for protected vs. not protected NHPs did not reach statistical significance, perhaps, in part, due to the low numbers of non-protected animals.**
- **Further testing of the SBA method, possibly as part of a human vaccination-challenge model, is required to establish a correlate/surrogate for protection.**

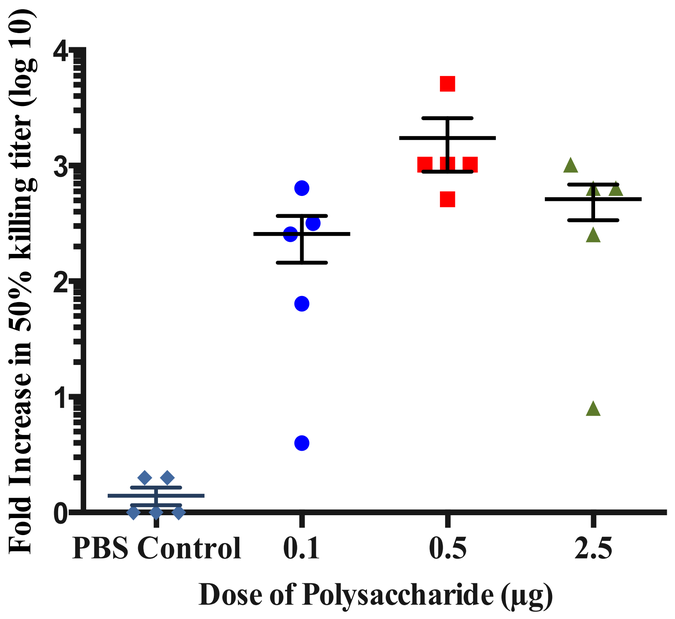
Results

Figure 1: Fold increase in 50% killing titer following vaccination of New Zealand White Rabbits



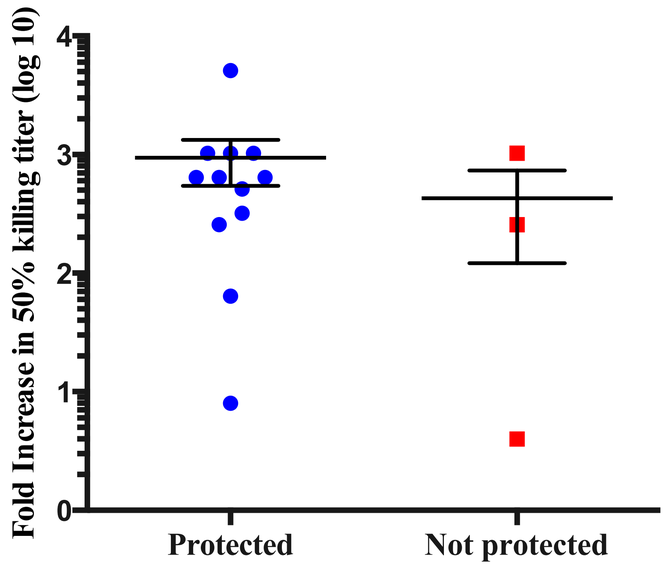
The mean fold increase in titer achieving a 50% killing effect was 5.4-fold if vaccinated using CJCV1 with Alhydrogel, and 5.7-fold if vaccinated using CJCV1 without Alhydrogel.

Figure 2: Fold increase in 50% killing titer following vaccination of NHP using different doses of PS or control



The mean fold increases in the 50% killing titer from pre-vaccination to day-125 using 0.1, 0.5 or 2.5µg of PS were 257, 1,741 and 514-fold respectively, those in the control group had a mean fold increase of 1.4-fold.

Figure 3: Fold increase in 50% killing titer following vaccination of NHP with PS; protected vs. not protected animals when challenged



The mean fold increase in 50% killing titer for vaccinated NHP that were protected following challenge (n=12) was 939-fold, and for those vaccinated but not protected, (n=3) was 428-fold (p=0.43). Those animals that had been vaccinated with the highest dose of PS achieved 100% protection.

Acknowledgments and Disclaimers

- The serum used for the NHP Assays was collected from animals vaccinated and challenged by the Research Team at Naval Medical Research Unit 6, Lima, Peru.
- Additional academic supervision was provided by Prof. Adam Cunningham, School of Immunity and Infection, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

- The views expressed are those of the authors and do not necessarily reflect the official views of the United States Department of the Navy, or the Department of Defense, or the United Kingdom Defence Medical Services.
- This work was funded by the Military Infectious Diseases Research Program, Navy work unit 6000.RAD1.DA3.A0308
- Animal use: The study protocol was reviewed and approved by the NAMRU-6 IACUC (for NHP; protocol no. NMRCD-07-05) and by the Smithers-Avanza IACUC (for rabbits; protocol no. 2056-11033) in compliance with all applicable Federal regulations governing the protection of animals in research.

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APPENDIX 5

Results: Examining the 50% killing titres of the serum of NZWR vaccinated with CJCV1, using the method developed for NHP and human sera, Section 2.8.

For the purpose of these results the initial method is that described in Section 2.7 of the thesis and the revised method is that described in Section 2.8 of the thesis.

The 50% killing titres using the revised assay method were expected to be different to the results from the initial assay method due to the changes made, however, trends in the titre over time, as well as fold changes were expected to be similar and consistent. The trends in titre increase or decrease over time were not reproduced, Figures 1-3. A comparison of the fold changes in 50% killing titre over time for each serum sample was performed, to see if the results in terms of fold-increase or fold-decrease of the two methods were comparable. These comparisons are shown for Groups 2 and 3 in Figures 3 and 4.

The numbers at the top of the graphs represent the rabbit identification number, with the prefix G1, G2 or G3 as group identifiers. The results of the SBAs described in section 3.4 are plotted to show a comparison, and are annotated as 'initial method' in the legend, whilst the new method is the 'revised method'. Where no data is shown for a time-point the 50% killing titre was not achieved. Different colour data points represent replicates of the revised method.

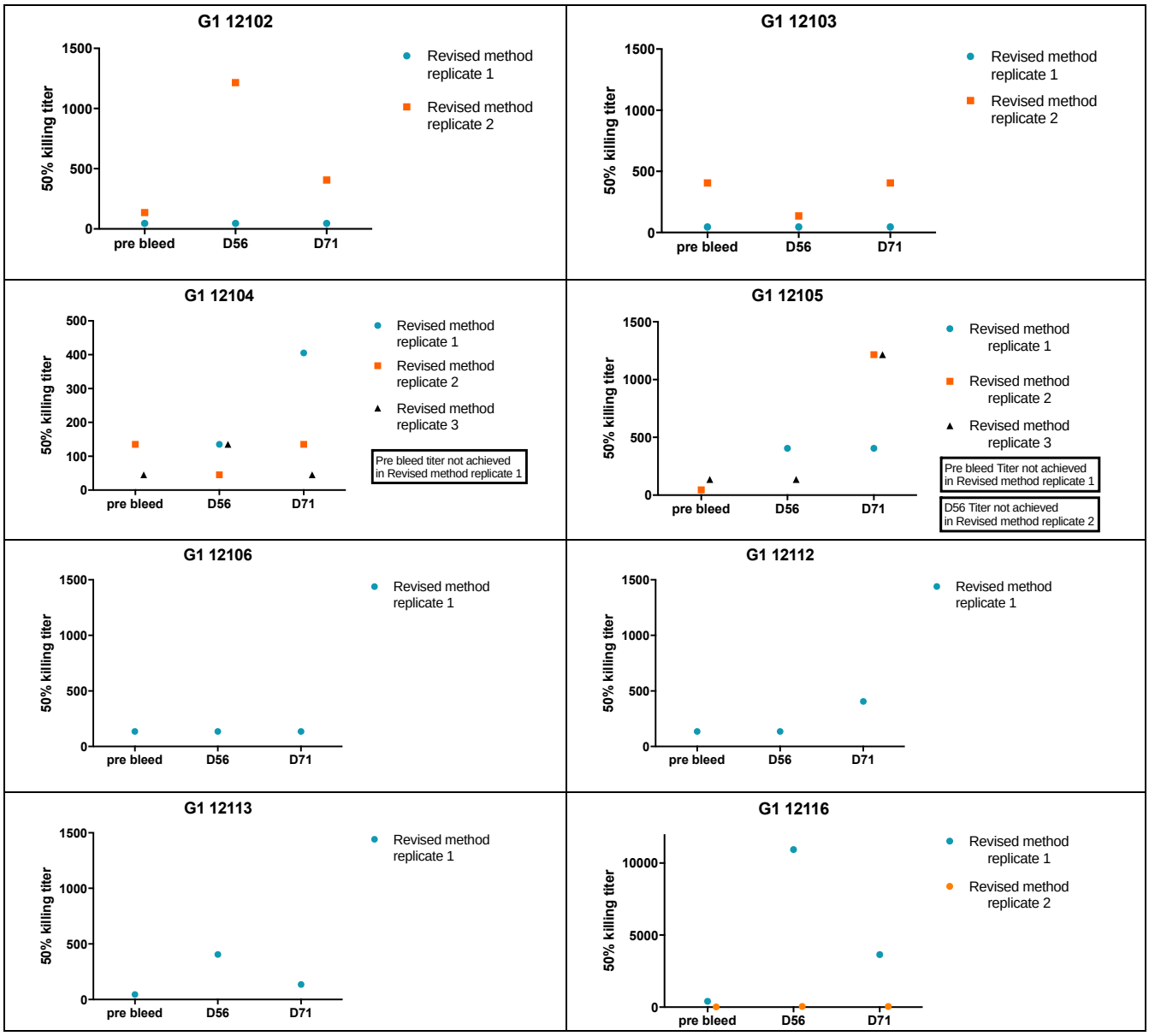

Figure 1 The 50% killing titres were demonstrated despite by Group 1 despite being the PBS control.

Group 1 was not tested using the initial method so there are no data points for this. The results gave measurable titres despite this being the control group which was not expected, therefore either the rabbits had prior exposure to *C. jejuni*, non detected on screening, or the assay falsely detected a killing effect. A further possibility is poor *C. jejuni* growth on the MHP agar plates, used for counting the CFU.

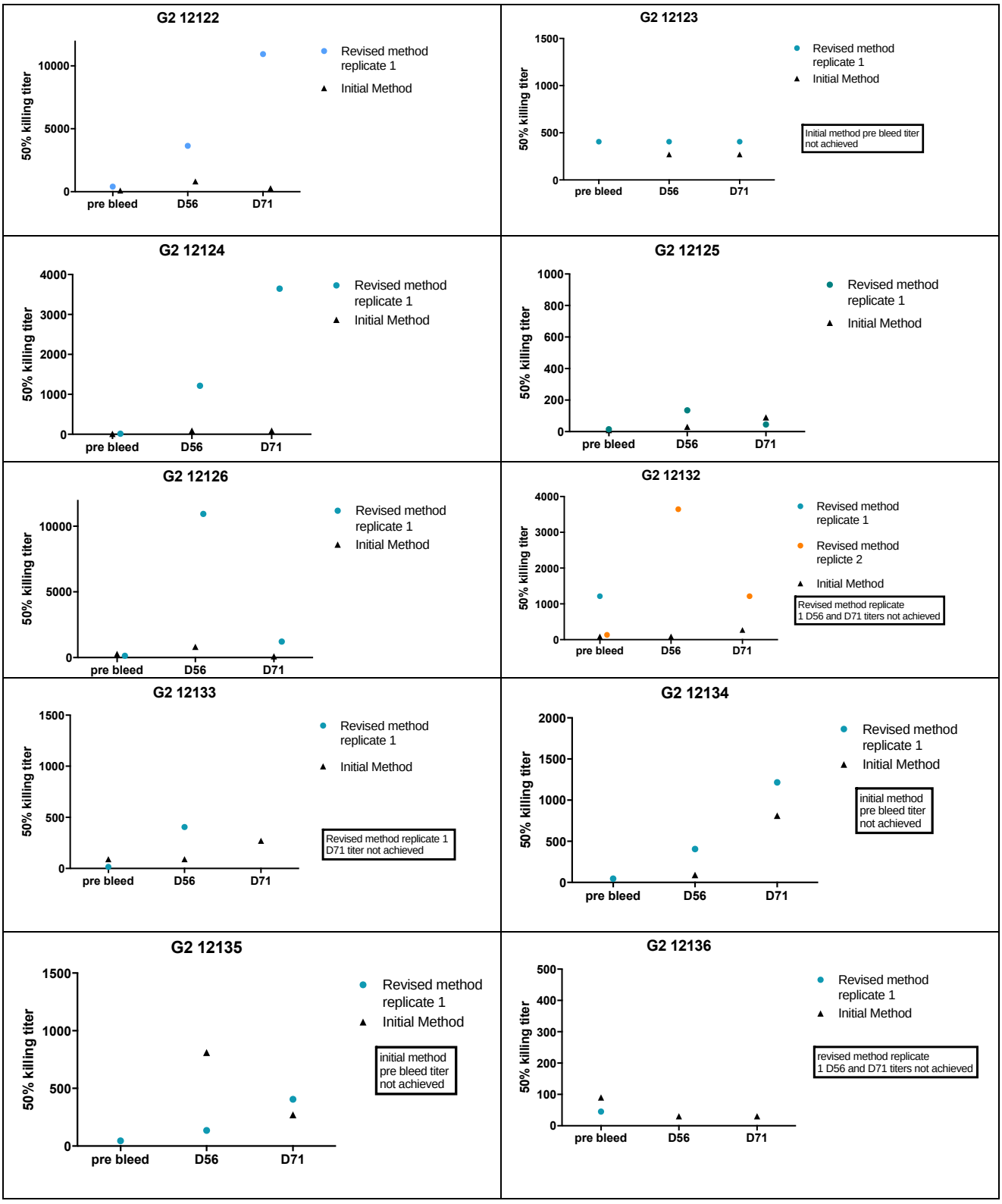

Figure 2 Trends in 50% killing titres for Group 2 vary with method alterations. For rabbits 12122 and 12124 the titres rose over time, this had not been seen when initially tested, for rabbits 12125, 12126 and 12132 the titre rose and fell, which was different to the pattern of their titres when initially tested.

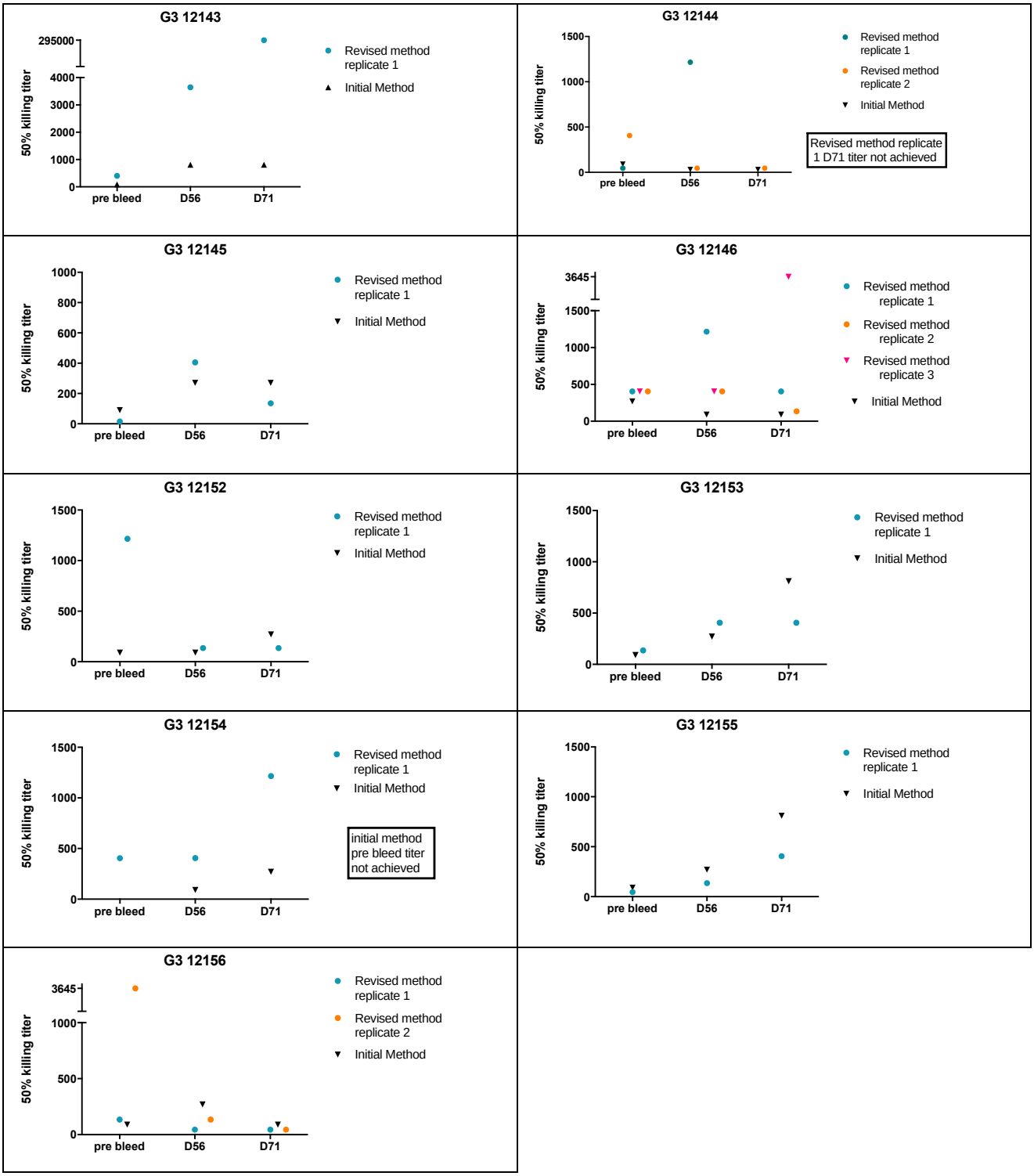

Figure 3 Trends in 50% killing titres for Group 3 vary with method alterations. For rabbits 12145 and 12155 the serum titre patterns over time when tested then re-tested, are similar. However for the other rabbits this is not the case, and when replicates were performed the results were not reproducible.

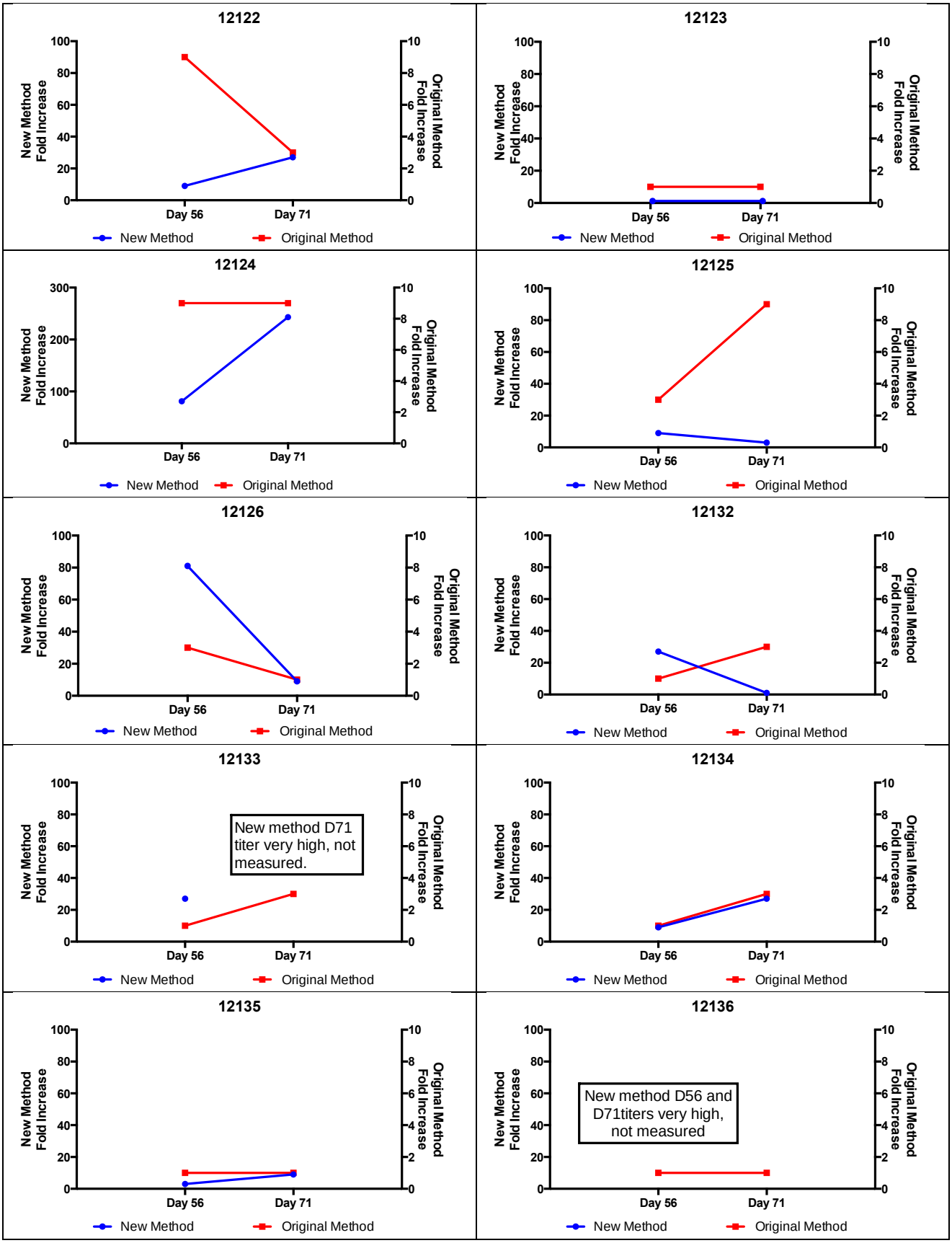

Figure 4 Group 2: Trends in fold-increase in 50% killing titre for Group 2 vary method alterations

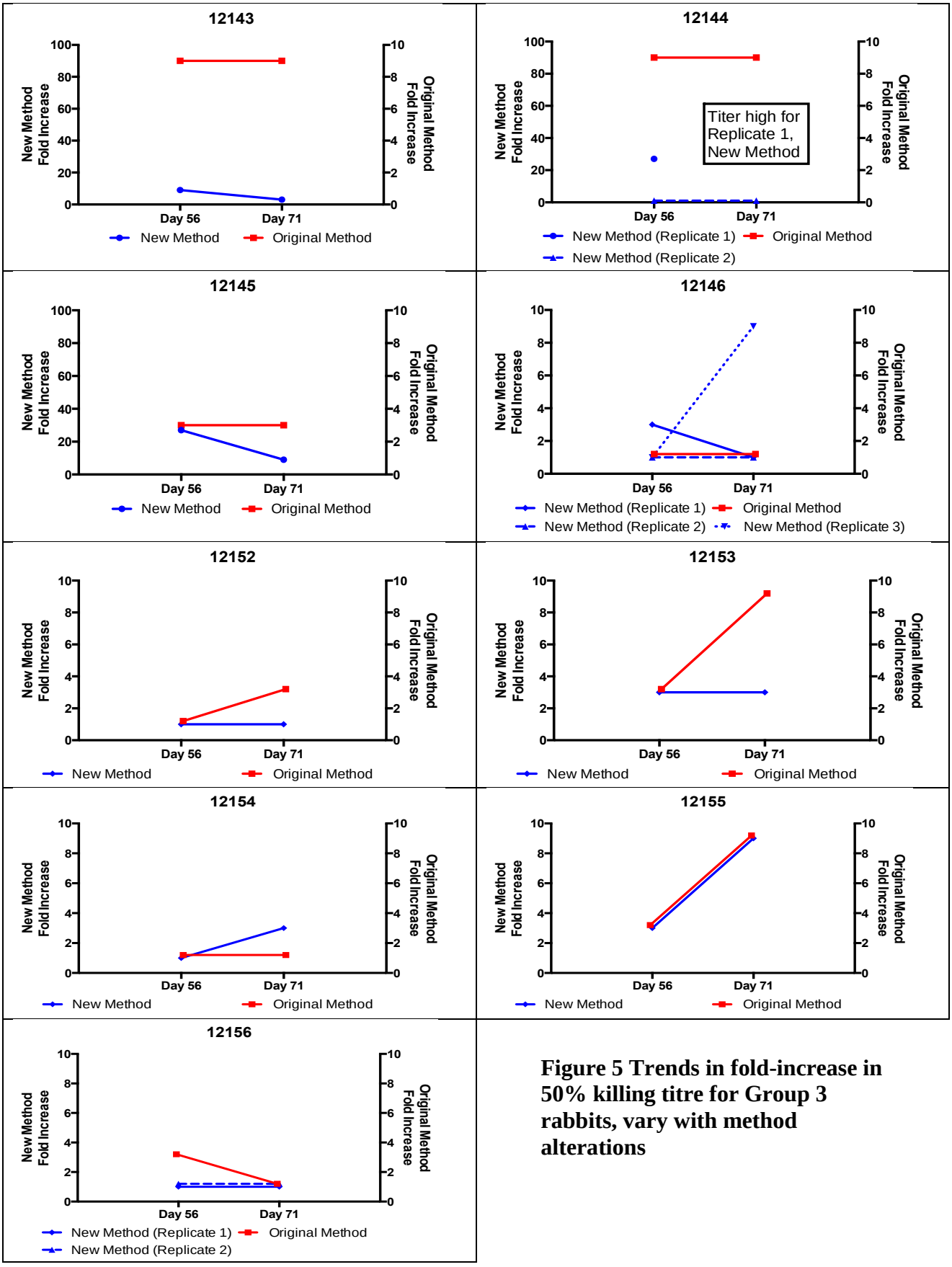

Figure 5 Trends in fold-increase in 50% killing titre for Group 3 rabbits, vary with method alterations

APPENDIX 6

The Study Specific Procedure for the preparation of the *C. jejuni* CG8421 challenge inoculum. This was carried out at the Johns Hopkins School of Public Health.

STUDY SPECIFIC PROCEDURE

Table of Contents

1.0 INTRODUCTION

This procedure describes the preparation of the bacterial inoculum (5. 10^5 cells/ml) for use in the human challenge study titled: Double Blind, Placebo-Controlled Trial Assessing the Efficacy of Rifaximin in Preventing Campylobacteriosis in Subjects Challenged with *Campylobacter jejuni*

2.0 SPECIAL PRECAUTIONS AND ENVIRONMENTAL/SAFETY ISSUES

This protocol involves the use of potentially infectious and hazardous materials. Use appropriate precautions as would normally be required with BSL-2 and comply with Johns Hopkins University (JHU) safety standard operating procedures as well as the SSP for cleaning the biosafety cabinet and incubators prior to use. Specifically these procedures will be carried out by an individual trained in basic microbiologic technology suitable for enteric pathogens as well as trained in this SSP.

BSL-2 procedures will be followed. The individual will wear proper protective equipment including a disposable lab coat and disposable sterile gloves. The work will be done in a certified and cleaned biosafety cabinet. All materials that come into contact with biological agents will be disposed of into the biological waste bags and then incinerated or autoclaved.

3.0 MATERIALS

3.1 Materials for preparation and assessment of challenge inoculum

1. Two vials of GMP grade seed culture *C. jejuni* strain CG8421.

†Affix Seed Lot Vial Label Here

†Affix Seed Lot Vial Label Here

Seed Culture strain Lot # …….

2. Incubator set at 40 +/- 2°C with temperature chart
3. Calibrated micropipette
4. Designated Biological Safety Cabinet (BSC) with current certification
5. Cleaning materials for BSC and incubator

5.1. 10% Bleach solution for cleaning BSC cabinet
5.2. 70% ethanol in disperser bottle for BSC cleaning and Kimwipes for BSC cleaning
5.3. Sterile water (500 mL)

6. Autoclave grade waste container
7. Autoclave waste bags, orange or red
8. Vortex
9. Spectrophotometer (600 nm)
10. Light microscope
11. Laboratory grade Sharpie maker
12. Tamper detecting tape
13. Protective Wear

13.1. gloves, size medium sterile,
13.2. disposable sterile sleeves
13.3. disposable lab coat, 1 each, for all participating personnel

14. For isolation and/or transfer

14.1. Sterile, disposable L spreaders x minimum of 40, fresh package
14.2. Sterile, disposable loops x minimum of 20
14.3. Sterile individually wrapped swabs x 1 box or minimum of 10
14.4. Sterile, disposable transfer pipets x minimum of 10

15. Petri dish turn-table

16. Calibrated 1mL pipette
17. Sterile filter-tip pipette tips for 1000 µL pipette, 1 box unopened
18. Calibrated 200 µl pipette
19. Sterile filter-tip pipette tips for 200 µl pipette , 1 box unopened
20. Pipette-aid
21. Selection of 5mL and 10mL pipette tips
22. Bacteriological Media

22.1. MacConkey blood agar
22.2. Mueller-Hinton agar
22.3. Mueller-Hinton soft agar

23. bulk bottled (≥500ml) sterile PBS, lot number confirmed (USP grade)
24. Campylobacter gas (5% 02, 10% C02, 85% N2)
25. Polyethylene bags
26. Rubber bands
27. Glass microscope slides
28. Glass cover slips
29. Immersion oil (for gram stain)
30. Filter paper (for oxidase test)
31. Difco oxidase reagent
32. Catalase reagent
33. Gram stain Kit
34. BBL CampyPak Plus (BD) (ref#271045)
35. Tube racks, as necessary, to hold all tubes securely in BSC
36. Sterile disposable pipettes (l ml, 5ml, 10ml, 25ml), fresh packages
37. Sterile Falcon centrifuge tubes, 15 ml and 50 ml, fresh packages
38. Spectrophotometer cuvettes
39. Ice
40. Dosage Confirmation (viable plate counts) and Purity Check

40.1. Mueller-Hinton agar
40.2. Campy CVA Agar with 5% sheep's blood
40.3. PBS Solution
40.4. Campylobacter gas (5% 02, 10% C02, 85% N2)
40.5. Polyethylene bags
40.6. Rubber bands

3.2 Materials for transfer of challenge inoculum to Bayview

1. Biohazard container in which to place the inoculum (Bottle F)
2. Syrofoam box with outside cover to carry the inoculum - with Biohazard labels
3. Ice
4. Plastic cups to drink the buffer
5. 120 mL aliquots of bicarbonate (30 aliquots, 1 per subject with 2 spare)
6. 30 mL aliquots of bicarbonate in 50 mL conical tubes (30 aliquots, 1 per subject with 2 spare).

Materials used Supplier and Lot #	
PBS	Gas Pak
MHA CVA Media MHA QC Record Verified: Tech:	Observer:

4.0 METHODS

Day -1 Date ……
Procedures done in safety cabinet in Room 5614

4.1 Initial Inoculation of Plates

1. Determine time to start Day -1 procedures. The clock starts at the time that the vial is inoculated onto the media, and this should occur at 12:00 noon, the day before dosing. This allows for 21 hours incubation, approximately 30 minutes challenge inoculum preparation and 45 minutes transit time between laboratory and the study ward.

2. BSC will be cleaned with disinfectants prior to use using SSP of the Enteric Lab. This will be done at least 2 hours prior to using it. The BSC fan will be turned on at least 30 minutes prior to use.

3. BSC: turn on (time:), Clean and disinfect (time:)

4. Remove 2 *C. jejuni* challenge strain CG8421vials from the -80˚C freezer, place in leak proof container and take to BSC.

5. Remove from outer container and allow to thaw at room temp (about 5 min).

6. Confirm strain and lot numbers of vials, and affix sticker to front page of this form. Also write the information regarding the strain here:

7. Vial 1 - Strain Lot #..... Time removed from freezer……..….

8. Vial 2 - Strain Lot #..... Time removed from freezer……..….

Tech	Observer

9. Using a calibrated pipette place 0.1 mL of the suspension on a Mueller-Hinton (MH) agar plate and spread with a sterile disposable L spreader using the petri dish turn-table. Repeat for a total of 6 plates for each vial, labeling the plates with the vial number.

10. Place three (3) inverted plates from each vial into an anaerobic gaspak box or canister.

11. Activate the Campy gas pack (BBL) following manufactures instructions, insert in jar and seal lid.

12. Repeat steps 9 and 10 above with the six remaining plates (3 for each vial), this is as a backup in case of failure of the first jar or gas pack. Place these plates in a sealed plastic bag containing campygas, in the back-up incubator.

13. Incubate plates in this microaerophilic atmosphere at 40 +/-2°C for 21 (+/-1) h.

14. Temperature of incubator……..

15. Seal incubator with tamper detecting tape (time:)

16. As a purity check, streak a MacConkey plate with the same inoculum and incubate at 37^{o}C (aerobic)

17. Clean and disinfect BSC using SSP of CIR Enteric Lab and turn off blower, outlet, and light for remainder of the day.

Tech	Observer

Day 0 Date ……
Procedures done in safety cabinet in Room 5614

4.2 Initial harvesting of plates

1. The time of dosing minus 2 hours is the optimal Day 0 procedures start time. (for example: If dosing on Day 0 is at 10:15 AM then BSC cleaning, disinfection, and power-up should begin at 8:15 AM).
   BSC: turn on (time:), Clean and disinfect (time:)

Tech	Observer

2. Turn on (time:) spectrophotometer and ensure it is set to read at 600 nm. The spectrophotometer should be on for a minimum of 30 minutes before reading any test samples.

3. Prior preparing inoculum, pre-label tubes and add PBS as follow:
   - Label an empty sterile 50 mL conical tube – Tube "A 1".
   - Label an empty sterile 50 mL conical tube – Tube "A 2".
   - Label an empty sterile 15 mL conical tube – Tube "B 1" and transfer 7.6 ml of sterile PBS to it.
   - Label an empty sterile 15 mL conical tube – Tube "B 2" and transfer 7.6 ml of sterile PBS to it.
   - Label an empty sterile 15 mL conical tube – Tube "B 3" and transfer 7.6 ml of sterile PBS to it.
   - Label an empty sterile 15 mL conical tube – Tube "C".
   - Label two (2) empty sterile 50 mL conical tube – Tube "D" and "E" and transfer 27 mL of sterile PBS to each of the tubes.
   - Label an empty sterile 125 mL bottle "F" and transfer 36 mL of sterile PBS into it.
   - Label two (2) empty sterile 15 mL conical tubes—"Tube F1 for plating" and "Tube F2 for plating".
   - Pre-label four (4) 15ml conical tubes Tube "G1" (-1), Tube "H1" (-2), Tube "I1" (-3) and Tube "J1" (-4) and transfer 9 ml of sterile PBS to each of the tubes.
   - Pre-label four (4) 15ml conical tubes Tube "G2" (-1), Tube "H2" (-2), Tube "I2" (-3) and Tube "J2" (-4) and transfer 9 ml of sterile PBS to each of the tubes.
   -

4. Remove Plates from incubator (time:), Tamper resistant tape verified as intact*:

Tech	Observer

   *If tape was disturbed do not initial, stop preparation, and use backup plates.

5. Incubator temp ……..

6. Time plates first exposed to air:……..

7. Visually examine MH agar plates for growth of Campylobacter and lack of contaminants.

Tech	Observer

8. In BSC add 10 ml of fresh (≤ 1 week), room temperature, sterile PBS to one Mueller-Hinton agar plate with inoculum growth. Use a sterile individually wrapped cotton swab to loosen bacterial growth and then harvest the bacterial suspension using a sterile individually wrapped disposable transfer pipet and transfer to the 50 ml sterile conical tube pre-labeled Tube "A1".

9. Repeat this for a plate from the second vial, and then harvest bacterial suspension using a sterile individually wrapped disposable transfer pipet and transfer to the 50 ml sterile conical tube pre-labeled Tube "A2".

10. Mix tube "A1" by pipetting up and down with a 5 mL, pipet 3-5 times then vortex for 10 seconds and record time (time:).

11. Repeat this for tube "A2", (time:…….).

12. Tubes "A1" and "A2" are maintained at room temperature in the BSC until step 4.8 (inoculum purity/quality check) and transfer to RNA later (step 4.10). Only the tube "A" that was selected for inoculum preparation will undergo steps 4.8 and 4.10.

4.3 Initial measurement of Optical Density

1. Prepare a 1:20 dilution as follows: 0.4ml bacteria + 7.6 ml PBS = 1:20. Using a calibrated micropipette transfer 0.4 ml bacterial suspension from tube "A1" into tube "B1"; and tube "A2" into tube "B2", for optical density determinations.

2. Optical density measures, in duplicate (600 nanometers). Transfer 1 ml of PBS into a clean, plastic cuvette to make a "blank" sample. Mix Tube "B1" on vortex for 3-5 seconds and then transfer 1 ml from Tube "B1" into a clean plastic cuvette. Repeat once for a total of two cuvettes. Confirm that the spectrophotometer is set to zero (0) using the blank sample.

3. Insert first sample into spectrophotometer and record reading. Repeat blank reading. Insert the second sample into the spectrophotometer and record the second reading. The results of the two readings should be within a 10% range of each other, if they are not then restart at the 1:20 dilution point. Determine the average of the two readings.

4. Repeat for tube "B2" using the bacterial suspension in tube "A2".

Tube "B1"

OD #1	OD#2	Average OD

Tube "B2"

OD #1	OD#2	Average OD

Tech	Observer

For the inoculum preparation use the bacterial suspension in tube "A" that gave the highest OD reading in tube "B".

5. If the OD of both tubes "B1" and "B2" are below 0.1 then harvest a corresponding second plate into tube "A", choose the tube "A" and set of plates that previously gave the highest OD. To do this, pipette 5mL of the bacterial suspension from the chosen tube "A" into the second plate and harvest the bacteria using this liquid. After doing a 1:20 dilution in tube "B3", check the OD of the enhanced tube "B3"

Enhanced Tube "B3"

OD #1	OD#2	Average OD

Tech	Observer

6. Set Tubes "B" aside in autoclave waste (time:)

4.4 Prepare Tube C with a target concentration of 10^8/ ml

Assumption: an OD of 0.3 will correspond with a concentration of 10^8/ml. To make this calculation:

a. Calculate *x* where *x* = OD in Tube B…… X 20 (dilution factor)

b. Calculate *y* where $y = 0.3/x$

c. Calculate *z* where $z = y * 10$, *z* is the volume in mL of Campy from **Tube A** to add to PBS to make a **total** of 10 ml in **Tube C**.

d. Value of *x* = _____

e. Value of *y* = _____

f. Value of *z* = _____

Example: If the OD of Tube B is 0.2, the value of *x* is 4, the value of *y* is 0.075, and the value of *z* is 0.75
To made tube C, place 9.25 ml of PBS in the tube and add 0.75 ml of bacteria from Tube A.

g. Mix Tube "A" on vortex for 3-5 seconds and add …… ml (*z* value) of **Tube "A"** to (……..) ml (10-*z* value) of PBS at (time:…….), the total of these will be 10 ml, to make **Tube "C"**, containing 5.10^8 cfu/ml (estimated).

Tech	Observer

4.5 Confirm the OD of Tube C to be 0.3

1. Transfer 1 ml of PBS into a clean, plastic cuvette to make a "blank" sample.

2. Mix **Tube "C"** on vortex for 3-5 seconds and then transfer 1 ml from **Tube "C"** into a clean plastic cuvette. Repeat once for a total of two cuvettes.

3. Confirm that the spectrophotometer is set to zero (0) using the blank sample. Insert first sample into spectrophotometer and record reading. Repeat blank reading. Insert the second sample into the spectrophotometer and record the second reading.

4. Determine the two OD readings. Acceptable range is 0.27 - 0.33 and the two readings should be within 10% of each other. If the readings of the OD are outside the range 0.27 – 0.33 then calculate the average and for every 0.01 that the OD is different from 0.30, (above or below), alter the volume of *C. jejuni* by 10 uL, for example if the average OD is 0.23, repeat the steps to make **tube C** but increase the volume of *C. jejuni* by 70 uL and decrease the amount of PBS by 70 uL, If this does not correct the OD to 0.3 then adjust the volumes of *C. jejuni* and PBS in order to achieve the correct OD and record the volumes used and ODs achieved below.

OD #1	OD #2	Average OD

Additional results if the volumes are altered:

Volume of *C. jejuni*…… Volume of PBS……

OD #1	OD #2	Average OD

Volume of *C. jejuni*…… Volume of PBS……

OD #1	OD #2	Average OD

Volume of *C. jejuni*…… Volume of PBS……

OD #1	OD #2	Average OD

Volume of *C. jejuni*…… Volume of PBS……

OD #1	OD #2	Average OD

Volume of *C. jejuni*…… Volume of PBS……

OD #1	OD #2	Average OD

4.6 Prepare dilution for challenge = 5.10^5 in Bottle F

1. **Dilutions:**

   a. Add 3 ml from Tube C to Tube D which has 27 mL PBS, then vortex 3-5 seconds.

   b. Add 3 ml from Tube D to Tube E which has 27 mL PBS, then vortex 3-5 seconds.

   c. Add 4 ml from Tube E to the pre-labeled (for example of label see below) Bottle F which has 36 mL PBS, then vortex 3-5 seconds.

C. jejuni challenge strain CG8421 Prepared by Johns Hopkins University Enteric Laboratory of the Center for Immunization Research Date _____("Bottle F")

   d. Remove 1.5 mL from Bottle F and place into a 15 mL tube labeled "Tube F1 for plating" and repeat, putting another 1.5 mL into "Tube F2 for plating". Place Tubes F1 and F2 into an ice bucket.

4.7 Transfer of bacterial suspension from room W5614 to Bayview

1. Place **"Bottle F"** in a plastic shipping container on ice using IATA approved packaging. Box should have sufficient ice to maintain low temperature for at least 45 minutes. Pack in a Styrofoam insulated sealed box maintained at room temperature. (**Bottle F** is on ice, but the box is otherwise at room temperature)

2. Transport **Bottle "F"** along with thirty 50 ml conical tubes containing 30 ml bicarbonate buffer, and thirty bottles containing 120 mL bicarbonate buffer, for challenge inoculum administration (see bicarbonate buffer SOP) to Bayview.

3. Departure of **Bottle "F"** and bicarbonate buffer from lab (time: **)**

4. **Note: THIS FORM remains at the CIR laboratory. Remaining information on this page is recorded in the Challenge Dose Dispensation Log as the primary source, and copied here.**

5. Arrival of **Bottle "F"** and bicarbonate buffer at Bayview (time:)

4.8 Inoculum purity/quality check

From tube A, prepare a wet mount preparation and examine by light microscopy and perform Gram stain, oxidase, and catalase testing:

1. Light microscopy: correct motility and morphology,
comments……
……

Time:	Tech	Observer

2. Gram stain (circle one): + - (Result should be gram negative.)

Time:	Tech	Observer

3. Oxidase test (circle one): + - (Result should be positive.)

Time:	Tech	Observer

4. Catalase test (circle one): + - (Result should be positive.)

Time:	Tech	Observer

5. Purity check: Aliquot 100 ul volumes of the final cell suspension onto the center of each of four tryptic soy agar plates supplemented with 5% sheep red blood cells (sBAP) (Remel). Immediately spread the inoculum across the surface of each sBAP with the spreader and turntable. Secure plates with tape and incubate plates, face down, aerobically: two (2) at 37°C and two (2) at 42°C for 24-48h.

Time:	Tech	Observer

6. Motility check: With a sterile inoculating loop, stab the resuspended cell pool in PBS, then stab the center of a MH soft agar plate. Secure plates with tape and gently place, face up into a polyethylene bag. Flush with Campy gas and seal with a rubber band. Incubate plates, face up, in this microaerophilic atmosphere at 37°C for 48 h.

Time:	Tech	Observer

4.9 Validation of the colony counts on the inoculum before and after dosing

1. As the bacterial challenge inoculum is being transported to Bayview, prepare tenfold serial dilutions as follows

2. Vortex Tube **"F1 for plating"** for 3-5 seconds.

3. Using a calibrated pipette, transfer 1 ml from **Tube "F1 for plating"** to Tube G (-1) and vortex tube G (-1) for 3-5 seconds.

4. Change pipette tips. Transfer 1 ml from Tube G (-1) to Tube H (-2). Vortex Tube H (-2) for 3-5 seconds. Continue in the same manner until serial dilutions have been completed through to tube J (-4).

5. Plate 0.1 ml of dilutions H, I and J (-2, -3, and -4) onto 3 Mueller-Hinton agar plates each and spread with disposable L spreader using Petri dish turn-table.

6. Plate 0.1 ml of dilutions H, I and J (-2, -3, and -4) onto 3 Campy CVA agar plates each and spread with disposable L spreader using Petri dish turn-table.

7. Secure plates with tape and gently place, inverted into polyethylene bags. Flush with Campy gas and seal with a rubber band. Incubate no more than 16 plates per bag. Place colony count plates into incubator in Room W5616.

8. Incubate microaerobically in campy gas at 40 +/- 2°C for 40-52 hours:

Time:	Tech	Observer

9. Upon receiving a call from Bayview confirming dosing has been completed, repeat serial dilutions using Tube **"F2 for plating"**

Time:	Tech	Observer

4.10 Addition of RNA later

1. As soon as the inoculum purity/quality checks have been performed and a member of Bayview staff has contacted the lab to confirm that all 30 volunteers have been successfully challenged, add at least 1:1 volume of RNA later to tube "A". The volume of *C. jejuni* must be accurately measured. Add all 10 mL of RNA later to a measured volume of bacterial suspension (must be <10mL). Vortex 3-5 seconds and store at -80°C (+/- 10°C).

Volume of RNA later = ……..(can be all 10 mL) Volume of *C. jejuni*……..

Time:	Tech	Observer

Day 2 Date ……

4.11 Read plates in Room W5616

1. **Plate count from F1 and F2 (before and after challenge). Colony counts on the plates should be counted after 40-52 hrs of incubation.**

2. Remove Plates from incubator at (time:)

3. Temperature record verified: as ……^{o}C

Tech	Observer

4. Recording of number of colonies on the plates

MH Agar	**Tube F1**	**Tube F1**	**Tube F1**	**Tube F2**	**Tube F2**	**Tube F2**
Dilution -2 (Tube H)						
Dilution -3 (Tube I)						
Dilution -4 (Tube J)						

CVA Agar	**Tube F1**	**Tube F1**	**Tube F1**	**Tube F2**	**Tube F2**	**Tube F2**
Dilution -2 (Tube H)						
Dilution -3 (Tube I)						
Dilution -4 (Tube J)						

5. Calculate colony count:

   a. 1 ml per inoculum x 0.1 spread onto the plate x dilution factor. Example: If dilution of -3 is 50 colonies, then 10 x 50 x 10^3 = 5. 10^5. This is the number of bacteria actually given to the volunteers.

   b. Average of the before and after challenge concentrations:

	Before challenge concentration	After challenge concentration
MH Agar		
CVA Agar		

   c. Both agar types are used to allow optimal growth conditions
   d. Calculation of the actual number of CFUs given to volunteers (average of before dosing and after dosing results):……..
   e. Comments relating to the calculation of dose: ……..
   ……
   ……

4.12 Certifications of the Inoculum Preparation:

a. Microbiologist……; Date:……..

b. Pharmacist……..; Date……..

APPENDIX 7

The Study Specific Procedure for the processing of stool samples in order to determine the presence of *C. jejuni* in stool samples. This was carried out at the Johns Hopkins School of Public Health.

TITLE
Processing and identification of fecal specimens for Campylobacter

	Date	Sign/Name	Title
Prepared by:			
Approved by:			
Approved by:			
Authorized by QA:			

PURPOSE
To correctly identify Campylobacter from clinical specimens.

EXECUTOR
Laboratory staff at JHU SPH laboratory

HEAD OF LABORATORY
Dr David Sack

EFFECTIVE DATE
31 October 2014

ENCLOSURE(S)
Nil

REASON FOR UPDATE
N/a

SUPERSEDES DOCUMENT(S)
N/a

Content

1. Introduction

This SSP describes the procedure for identifying the presence of Campylobacter in human stools.

2. Special Precautions and Environmental/Safety Issues.

This protocol involves the use of potentially infectious and hazardous materials. Use appropriate precautions as would normally be required with patient's samples and comply with John Hopkins University safety standard procedures. Specifically, these procedures will be carried out by individuals trained in Standard Blood borne Safety Training. Treat all subjects' samples as if known to be infectious for HBV, HIV, and other blood borne pathogens. Wear proper protective equipment, lab coat and gloves. Dispose all materials that come in contact with biological agents into the biological waste bags.

3. Material and Equipment

3.1 Test samples/controls

Stool samples delivered from Bayview
Control strains (from ATCC):

- *E. coli* (25922)
- *C. jejuni* (33291)
- *Pseudomonas aeruginosa* (27853)

3.2 Materials

Item	Item Number	Source
CVA Medium	01270	Remel
Campy Thioglycollate Broth medium	K128 R060370	Hardy Diagnostics Fisher/Remel
Campy Gas (N2 85%, CO2 10%, O2 5%)		AirGas
PE bags	22-260170	BelArt Scienceware
Rubber bands	common	
MacConkey agar	212123	Difco Laboratories
Glycerol	BP229-1	Fisher
Mueller-Hinton agar	275730	Difco Laboratories
Bacto Agar	214010	Difco Laboratories
Oxidase reagent	261181	BD
Catalase reagent	300195	Gibson BioReagents
Filter Paper (Whatman No.1 or equivalent)		
Sterile 1.8 ml round-bottomed cryovials	377267	Nunc
Sterile Inoculating loops (10µL)	22-363-597	FisherBrand
Gram Stain reagents	B12539	BD
Microscope supplies (slides, coverslips, oil, etc.)	Common	
Sterile cotton tip swabs	23-400-125	FisherBrand
50 mL conical sterile tube		
PBS		
Balance		

4. Procedure

4.1 Analysis Procedure

Note: This protocol involves the use of infectious and hazardous materials. Use appropriate precautions as would normally be required with BL-2. Specimens will arrive as fecal specimens on swabs in tubes of Thioglycollate broth, either in a 1 mL or 9 mL tube. Prior to day 6, the lab will receive both a 1 mL and a 9 mL tub; starting mid-day on day 6, only a 1 mL tube will be received. After antibiotics have started, the lab will process only the 1 mL tube. No specimen shall be refused, however, results obtained from specimens that were delivered at inappropriate temperatures (e.g. too warm, or frozen), unsealed, or otherwise unsuitable will be documented and reported to the principal investigator.

1. **A: Pre-antibiotic: For processing stool samples,** use the swab (transported in thioglycollate broth) to inoculate two (2) CVA agar plates onto the first quadrant, followed by streaking with a sterile inoculating loop across the remaining three quadrants. This procedure will be performed on swabs from 1 mL and 9 mL thioglycollate broth tubes. The CVA plates will be placed in an airtight PE bag and incubated in a microaerobic environment generated by filling the PE bag with

Campylobacter gas, sealing with a rubber band for 48 hours (40-52 hours) at 40°C (37-42°C).

B Post-antibiotic: The lab will process only the 1 mL thioglycollate broth tube per specimen. Place 200 µL of stool/Thioglycollate solution onto four (4) CVA agar plates and spread using a spreader. In addition, use the swab to inoculate one quadrant of a separate plate and, using a loop, streak across the remaining three quadrants. The CVA plates will be placed in an airtight PE bag and incubated in a microaerobic environment generated by filling the PE bag with *Campylobacter* gas, sealing with a rubber band for 48 hours (40-52 hours) at 40˚C (37-42˚C).

2. After approximately 48 hours (range of 40-52 hours) of incubation, remove the CVA plates from the microaerobic environment. Check for growth and typical Campylobacter morphology: Look for suspicious colonies that appear as small, mucoid, grayish, flat colonies with irregular edges and no hemolytic patterns. They may also appear as round, convex, entire edge, glistening colonies 1-2mm in diameter. Colonies may be pinpoint in size or spreading over large area of the plate.

3. If presence of *C. jejuni* is suspected, perform oxidase test (positive), catalase (positive), and gram stains (gram-negative rods, seagull-shaped bacillus). Contamination of *Pseudomonas aeruginosa* may occur but they usually prefer to grow at 25°C to 35°C aerobically; whereas *C. jejuni* requires a microaerobic atmosphere to grow, and a temperature range of 37°C to 42°C.

   a. Oxidase-positive and Catalase-positive colonies are typical of *C.jejuni* and may be reported as presumptive *C. jejuni* if growing on Campy CVA agar at 37 - 42°C, in a Campy-Biobag.
   b. If the Gram stain is hard to read, re-culture the suspect colonies on MacConkey agar and incubate aerobically at 37°C. Campylobacter spp. will not grow on MacConkey agar at 37°C.
   c. **Gram Staining Procedure**: Please refer to the Gram staining SSP, SSP 203, for procedural information.
   d. **Catalase testing**: Refer to the SSP204 for Catalase test.
   e. **Oxidase testing**: Refer to the SSP205 for Oxidase.

5. Data Reporting.

Record the result of the test on Record of Specimen Results for Study Campylobacter Microbiology

6. Quality Assurance.

From each commercial Lot of plates, a plate should be incubated at the same temperature and gas content as its intended use to ensure sterility. CVA will be tested with ATCC quality control bacteria. *E. coli* (25922), *C. jejuni* (33291), *Pseudomonas aeruginosa* (27853)

7. References

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Hendrickson, D., and M. Krenz. 1991. Reagents and stains, pp.1289-1314. In Balows, A., Hausler, W., Herrmann, K., Isenberg, H., and H. Shadomy (eds), Manual of Clinical Microbiology. ASM, Washington, D.C.

Nachamkin, I. 1999. Campylobacter and Arcobacter, pp.716-726,. In. Murray, R., Baron, E., Pfaller, M, Tenover, F, and R. Yolken (ed), Manual of Clinical Microbiology, 7th ed, ASM Press, Washington, D.C.

Nachamkin, I, Allos, B. and T. Ho. 1998. Campylobacter species and Guillain-Barre syndrome. Clin. Microbiol. Rev. 11:555-567.

Nachamkin, I. Microbiologic approaches for studying Campylobacter species in patients with Guillain-Barre syndrome. 1997. J. Infect. Dis. 176(Suppl 2): S106-14.

APPENDIX 8

Inclusion and Exclusion Criteria for the Clinical Trial Double Blind, Placebo-Controlled Trial Assessing the Efficacy of Rifaximin in Preventing Campylobacteriosis in Subjects Challenged with *Campylobacter jejuni*

Inclusion Criteria:

1. Male or female between 18 and 50 years of age, inclusive
2. General good health, without significant medical illness, abnormal physical examination findings or clinically significant laboratory abnormalities, as determined by the PI (may consult with the Research Monitor on a case-by-case basis)
3. Demonstrate comprehension of the protocol procedures and knowledge of Campylobacter illness by passing a written examination (pass grade ≥ 70%)
4. Willing to participate after informed consent obtained
5. Available for all planned follow-up visits and remain available for clinic visits (for examination, blood draws and stool collection) and monitoring for 90 days post-challenge and by phone for 180 days post-challenge
6. If the subject is female, she is eligible to enter if she is of:

a. Non-childbearing potential (i.e., physiologically incapable of becoming pregnant, including any female who is post-menopausal. For purposes of this study, postmenopausal is defined as one year without menses); or must have documentation of having undergone tubal ligation or hysterectomy. OR

b. Childbearing potential; has a negative serum pregnancy test at screening and a negative urine pregnancy test on admission (Study Day -1), and agrees to the use of an efficacious hormonal or barrier method of birth control during the study, abstinence is acceptable

Exclusion Criteria:

General health/issues

1. Presence of a significant medical condition (e.g., psychiatric conditions; gastrointestinal disease, such as peptic ulcer, symptoms or evidence of active gastritis/dyspepsia, inflammatory bowel disease, irritable bowel syndrome (as defined by the Rome III criteria or

medical diagnosis); alcohol or illicit drug abuse/dependency) or laboratory abnormalities, which in the opinion of the investigator preclude participation in the study

2. Evidence of IgA deficiency (serum IgA < 7 mg/dL or below the limit of detection of assay)

3. Positive serology results for HIV, HBsAg, or HCV antibodies

4. Positive urine toxicology screen

5. Significant abnormalities in screening laboratory haematology or serum chemistry, as determined by PI or PI in consultation with the Research Monitor and sponsor

6. Use of any medication known to affect the immune function (e.g., corticosteroids and others) within 30 days preceding receipt of the challenge inoculum or planned to be used during the active study period

7. Nursing mother on the day of admittance to the inpatient unit

Study-specific exclusionary conditions based on potential increased risk or complicating outcome ascertainment

8. Personal or documented family history of Guillain-Barré syndrome or neuromuscular disease; or an inflammatory arthritis such as reactive arthritis, Reiter's syndrome, ankylosing spondylitis, or rheumatoid arthritis (not including osteoarthritis or vague history of arthritis relatively late in adulthood)

9. Evidence of neurological abnormalities

10. Evidence of inflammatory arthritis on exam

11. HLA-B27 positive (flow cytometry)

12. Allergy or prior intolerance to any of the following antibiotics: rifaximin, azithromycin or fluoroquinolones

13. Fewer than 3 stools per week or more than 3 stools per day as the usual frequency

14. Loose or liquid stools other than on an occasional basis

15. Regular use of laxatives or antacids (regular defined as at least weekly)

16. A fever in the 2 weeks prior to time of challenge

17. Use of antibiotics during the 7 days before bacterial dosing

18. Use of proton pump inhibitors, H2 blockers, or other antacids within 48 hours of dosing

19. Use of any other investigational product within 30 days preceding the receipt of the challenge inoculum, or until study completion

20. History of diarrhoea in the 2 weeks prior to planned inpatient phase

21. Have household contacts who are < 2 years old or > 80 years old or infirm or immunocompromised

22. Employment as a health care worker with direct patient care, in a day-care centre (for children or the elderly), or direct food handler; includes individuals who work directly with food in commercial establishments

23. Blood donation of one pint or more within the past 30 days

24. History of *Clostridium difficile*-associated diarrhoea

Prior Campylobacter exposure

25. History of microbiologically confirmed *Campylobacter* infection

26. Serological immunological evidence of prior Campylobacter exposure defined as homologous strain Campylobacter-specific anti-glycine extract IgA endpoint titre > 1:2,000, giving a net OD of 0.15-0.29

27. History of diarrhoea during or within 1 week of travel to countries with high *Campylobacter* rates, within the past two years

28. History of vaccination for or ingestion of *Campylobacter*, including participants in previous human experimental *Campylobacter* experiments within 3 years

29. Other dietary or environmental exposures that may place the subject at high risk for prior Campylobacter exposure (to be determined on a case-by-case basis by the PI).