CAMPYLOBACTER JEJUNI COLONIZATION OF BROILER CHICKENS

A Thesis submitted to the College of

Graduate Study and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Veterinary Microbiology

in the College of Graduate Studies and Research

University of Saskatchewan

Saskatoon, Saskatchewan

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To my family,

whose encouragement and support made it possible to complete my graduate studies

ABSTRACT

The pathogenesis of *C. jejuni* in broiler chickens is still poorly understood despite the importance of poultry meat as a source of infection in humans. The overall objective of this project was to understand the role of flagella and *Campylobacter* invasion antigens in mucosal and systemic colonization, and to evaluate the vaccine potential of *C. jejuni* paralyzed flagella mutants. As a first step to track *C. jejuni in vivo*, a Green Fluorescent Protein (GFP) reporter system that is constitutively expressed was constructed. The system was transformed into different *C. jejuni* strains and isolates, and their mucosal and systemic spreading was studied over the period of 7 days. *C. jejuni* NCTC11168V1 and V26 share the same background but differ in their ability to colonize chickens. *C. jejuni* 81-176 and K2-55 share the same genetic background but K2-55 has an insertion mutation in *pflA* gene that produced paralyzed flagella. Although the K2-55 flagella remained intact structurally, it did not secret *Campylobacter* invasion antigens (Cia). The reporter system was stable in all of these strains both *in vitro* and *in vivo*. Fluorescent bacteria were visualized successfully using fluorescent and confocal microscopes. *C. jejuni* NCTC11168V1 and 81-176 were detected in the intestinal tract and in the liver and spleen of more than 30% of the challenged birds, while V26 and K2-55 were only detected in the intestinal tract. *C. jejuni* 81-176 and K2-55 did not spread systemically to the spleen and liver of BALB/c mice challenged using the same approach, although they colonized the ceca.

A live attenuated vaccine based on *C. jejuni* K2-55 protected broiler chickens from *C. jejuni* 81-176 challenge in chickens following streptomycin treatment of drinking water. The same vaccine had no significant protection against a heterolgous *C. jejuni* NCTC11168V1 strain challenge. The vaccine was a poor stimulator of secretory IgA.

Macrophage-like HD11 cells inflammatory response to the presence of *C. jejuni* K2-55 was not significantly different from their response to wild-type 81-176 when measured by qRT-PCR. The lack of Cia secretion and motility had no effect on expression of IL-1β, IL-2, IL-6, IL-8, IL, IL-10, IL-12β, or TLR5. A *flgK* mutant expressing the flagella up to the hook had a significantly lower expression of these genes.

Acknowledgments

IN THE NAME OF ALLAH, MOST GRACIOUS, MOST MERCIFUL

I would like to express my deepest gratitude to Dr. Andy Potter, for his invaluable advice, support, understanding, and guidance during my studies.

I am also grateful to members of Advisory Committee members, Dr. Brenda Allan, Dr. George Khachatourians, Dr. Lorne Babiuk, and Dr. Vikram Misra for their help, guidance, and insight.

I give all my gratitude and thanks to my parents, Tawfiq and Ibtihaj, who's my debt to them, is beyond measure. I also thank my brothers Osama, Monjed, and Amal for their unconditional support and encouragement.

I am also indebt to Yasmine, my fiancé-to be, for her help, understanding, and support in the times of need.

Many thanks to Dr. William Miller for providing the AF292555 plasmid, Dr. Patricia Guerry for providing the K2-37 and K2-55 strains, Dr. Debu Biswas for his help with secretion protein assays, Neil Rawlyk for technical help, and Dr. Hugh Townsend for his help with statistical analysis.

I thank VIDO graduate students, especially Audrey Chu, Taseen Desin, and Dr. Oudessa Dego for the good times and lengthy discussions, both scientific and social. I also thank Vaccine Development Group for their feedback during my research.

I also would like to thank VIDO Animal Care personnel for care and handling of the animals.

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List of Abbreviations

ABC transporter	ATP-binding Cassette
BA	Blood Agar
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
bp	Base pairs
CadF	*Campylobacter* adhesion to Fibronectin
CDT	Cytolethal Distending Toxin
CFU	Colony Forming Unit
CFU/g	Colony Forming Unit per gram
CKC	Chicken kidney cells
Cia	Campylobacter invasion antigens
CO_2	Carbon dioxide
DNA	Deoxyribonucleic Acid
ddH_2O	Double-distilled Water
DEPC-treated	Deionized, diethylpyrocarbonate-treated
EDTA	Ethylenediaminetetmcetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
EMEM	Eagle minimum essential medium
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FBS	Fetal Bovine Serum
FDA	Federal Drug Administration
FACS	Fluorescence Activated Cell Sorting

FITC	Fluorescein Isothiocyanate
fla^{-}	unflagellated
fla^{+}	flagellated
FSP	Flagella Secreted Proteins
Fur	Ferric uptake regulator
GBS	Guillain-Barré Syndrome
GFP	Green Fluorescent Protein
GIT	Gastrointestinal tract
ID50	Infectious Dose 50
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
LB-KM	Luria-Bertani supplemented with Kanamycin
LBP	Lipid Binding Protein
LF	Limited-Flora
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LT	Labile Toxin
MAP	Mitogen Activated Protein

MEM	Minimal Essential Medium
MHA	Mueller-Hinton agar
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
MOMP	Major Outer Membrane Protein
mRNA	Messenger Ribonucleic Acid
NF-κB	Nuclear factor-κB
nm	Nanometer
nu/nu	Null/Null
OCT	Optimal cutting temperature
OMP	Outer Membrane Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pflA	Paralyzed flagella gene
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RacR	Reduced ability to colonize
RILT	Rabbit ileal loop
RITARD	Removable Intestinal Tie Adult Rabbit Diarrhea
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
Rs-GFP	Red-shifted Green Fluorescent Protein
SCID	Severe Combined Immunodeficiency
SD	Standard Deviation
SDS-PAGE	Sodium Dodycel Sulfate Polyacrylamide Gel Electrophoresis

SEM	Standard Error of Mean
sIgA	Secretory IgA
SMR	Streptomycin-Resistant
SOC	Super Optimal broth with Catabolite
SOD	Superoxide dismutase
T3SS	Type Three Secretion System
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor-α
Tween 20	Polyethylenesorbitan monolaurate 20
USA	United States of America
UV	Ultra Violet
VBNC	Viable But Not Culturable
VIDO	Vaccine and Infectious Disease Organization
v/v	volume/volume
VT	Verotoxin
w/o	Without
w/v	Weight per volume
WT	Wild-type

Chapter I: Literature review

1.1. *Campylobacter*

1.1.1 *Campylobacter* biology:

The name *Campylobacter* is derived from two Latin words, campylo and bacter, which together means curved-rod. This reflects the microscopic appearance of *Campylobacter* as a Gram-negative bacterium that is spiral in shape, 0.2-0.5 µm wide and 0.5-5µm long, and the initial classification as a *Vibrio* was based on its shape. *Campylobacter* could appear spherical or coccoid if it is in the state of viable but non-cultureable form (VBNC). Transition to this form is the result of various stress conditions the bacteria might encounter including old bacterial culture that is depleted of nutrients. The presence of uni- or bi-polar flagella makes *Campylobacter* motile. The flagellum is 2.6-3.9 µm in length and ~21 nm in width (Guerry, 2007). The motility of *Campylobacter* is a great asset in colonization of the intestinal tract of its host.

Campylobacter is microaerophilic, growing best in 3-15% oxygen (O_2), and capnophilic as the best carbon dioxide (CO_2) concentration for their growth is 3-5%. The genome size of *C. jejuni* is 1.6-1.7 Mbp with a GC ratio of ~30% (Parkhill et al., 2000). A distinct feature that might be involved in *C. jejuni* pathogenesis and immune evasion is the hypervariable regions in the genome sequence (Parkhill et al., 2000). Some *C. jejuni* strains harbor extra-chromosomal elements including plasmids and bacteriophages (Bacon et al., 2000)

1.1.2 *Campylobacter jejuni* morphology:

Campylobacter jejuni is generally described as spiral in shape, but a growing culture of *C. jejuni* would have different shapes that predominate over the course of time (Griffiths, 1993; Tangwatcharin et al., 2006), making *C. jejuni* pleomorphic. When the culture is in the exponential phase, most of *C. jejuni* cells assume the typical short spiral morphology, but as the culture reaches the mid-stationary phase, cells are almost twice the length of exponential phase cells (Griffiths, 1993). As the culture ages further and enters the late-stationary phase, cells that are coccoid in shape become predominant, and the remaining spiral cells are 3-4 times the length of *C. jejuni* cells in the exponential phase (Griffiths, 1993).

1.1.3. Epidemiology of *Campylobacter*:

Campylobacter jejuni is estimated to be responsible for 90% of campylobacteriosis cases in humans, and *C. coli* for the remaining 10% (Janssen et al., 2008), therefore most of the data available represent *C. jejuni* infections. The estimated number of campylobacteriosis cases varies from a country to a country with a range of 12.7 cases/100,000 in the USA to a high of 396 cases/100,000 in New Zealand (Baker et al., 2007). These numbers are an estimate based on the number of cases reported and confirmed by laboratory tests then corrected to project an estimation of unreported cases. This estimation is very important since most cases of *C. jejuni* gastroenteritis are self-limiting and usually unreported.

C. jejuni infection is generally sporadic and in contrast to infections caused by other food-borne pathogens such as *Salmonella* and *E. coli* O157, outbreaks are rarely reported. The reported outbreaks are usually associated with drinking contaminated/ untreated water (Kuusi et al., 2005) and consumption of raw milk (Teunis et al., 2005).

1.2. *C. jejuni* and poultry:

Although *C. jejuni* has been isolated from all food-producing animals and pets, poultry products contaminated with *C. jejuni* remain the main source for human infection (Jorgensen et al., 2002). Studies have identified the consumption of poultry or poultry products as a risk factor for *C. jejuni* infections. This includes raw or undercooked chicken (Hopkins et al., 1984; Istre et al., 1984), cooked chicken (Harris et al., 1986), and chicken consumed or prepared in a restaurant or commercial production facility (Effler et al., 2001). Direct evidence for the role poultry plays came from two studies. The first study was conducted in Belgium after the dioxin crisis during the early summer of 1999, when the carcinogenic substance was found in the feed of poultry. A ban was issued and all poultry products were withdrawn from the market. During the period of poultry product ban there was a 40% reduction in the number of reported *C. jejuni* infections (Vellinga and Van Loock, 2002).

The second evidence comes from Iceland, which had a low incidence of *C. jejuni* infections prior to 1996 (Stern et al., 2003). At this time, consumers had access to frozen poultry only, but after 1996 fresh poultry products became available. The incidence of *C. jejuni* confirmed cases increased from only 38 cases per 100,000 to reach a peak of 116

cases per 100,000 in 1999. Upon investigation, it was found that 62% of broiler carcasses were contaminated with *C. jejuni* (Stern et al., 2003). As a preventive measure, birds from poultry flocks testing positive were frozen and only birds from *C. jejuni*-free flocks were sold fresh. As a result, fresh chicken carcasses available for consumers which were contaminated with *C. jejuni* dropped to 16%. The corresponding drop in human cases was significant with only 33 per 100,000 cases in 2000 (Stern et al., 2003).

These two pieces of evidence suggest strongly that poultry products are responsible for a significant proportion of human infections. The data collected through a *Campylobacter* screening project in The Netherlands further supports the hypothesis that poultry is a risk factor for *Campylobacter* infection. This project estimates that 20-40% of laboratory confirmed *C. jejuni* cases are linked to poultry consumption or handling (Janssen et al., 2008). The source of *C. jejuni* in the remaining cases (up to 50% of confirmed cases) is largely unknown, leaving the possibility of another major risk factor that might be involved or the combination of other minor factors unanswered.

1.2.1. Sources of *C. jejuni* on the poultry farm:

To develop an efficient control strategy, an understanding of the sources of *C. jejuni* colonization in broiler chickens is needed. There are many studies that have investigated the source(s) of *C. jejuni* on the farm (Barrios et al., 2006; Gregory et al., 1997; Hald et al., 2008; Van Overbeke et al., 2006). These studies are very important in understanding the sources of *C. jejuni* infection but they have relied mainly on surveys and questionnaires rather than making use of genetic tools necessary for identifying the source of the infection and clonal relationship between the different isolates on the farm.

Given the role broiler chickens play in transmission of *C. jejuni* to humans, most of the available studies have focused on this large segment of the poultry industry, making available information from other segments such as layers and turkeys limited. In this section, the various potential sources for *C. jejuni* infection of the poultry flock will be examined.

It is generally accepted that the main source of *C. jejuni* transmission from the environment to poultry flocks is horizontal transmission. Drinking water, houseflies and beetles, general farm practices such as human movement and flock thinning, the presence of multiple broiler houses on the same farm, pets and wild life in the immediate vicinity

of the farm has been identified as some of the main risk factors according to a systematic review conduct by Adkin and colleagues that analyzed a 159 papers that investigated more than 1200 flocks from United States and Europe (Adkin et al., 2006). However, it is important to note that the presence of *C. jejuni* in the rearing environment of a poultry flock does not indicate that the flock will necessarily be colonized (Stern et al., 2001).

1.2.1.1. Water:

Water chlorination is a common practice in the poultry industry. The chlorine dose used for water treatment is lethal for *Campylobacter* and contamination of water is usually detected only after the flock is shedding (Jacobs-Reitsma, 1995; Shane et al., 1985). This suggests a minor role for water in introducing *C. jejuni* into the farm, but a potential role for spreading the bacteria within the same poultry house once several birds start shedding.

1.2.1.2. Insects:

Houseflies and darkling beetles have been implicated in *C. jejuni* transmission. Consuming a single adult or larva darkling beetle infected with *C. jejuni* is enough to infect 90% of chickens under experimental conditions (Strother et al., 2005), illustrating the impact this pest can have as a vector not only for *Campylobacter*, but *Salmonella* as well (Skov et al., 2004).

Fly traffic in and out of a broiler house has been shown to be a risk factor for transmission of *C. jejuni*. About 8% of the flies caught outside poultry houses in Denmark contained *C. jejuni* (Hald et al., 2004), with some flies carrying the same strain colonizing the chickens (Hald et al., 2004). When 20 broiler houses were under strict fly control using extra and double fly screens during the summer season of 2007 (Hald et al., 2007), only 15.4% of the flocks were positive, compared to the control group with regular fly screens that was 51.4% positive for *C. jejuni* (Hald et al., 2007).

1.2.1.3. General farm practices and human movement:

Poor hygiene-related practices, retaining used litter in the same poultry house, and flock thinning (depopulation schedule) are clearly a potential source of infection. *Campylobacter* has been isolated from the water of farms' footbath, farmer's boots, external clothes, and equipment (Jacobs-Reitsma, 1997; Stern et al., 2001). A study ranking the sources of *Campylobacter* infection in the United Kingdom has considered

flock thinning as the major source of infection (Adkin et al., 2006). Thinning is a common practice in the UK, as it lowers the density of the birds and allows the producer to supply the market demand for certain bird sizes. It requires personnel to enter the farm with their equipment, which have been found to be usually contaminated with *C. jejuni* (Newell, 2001), thus contaminating *Campylobacter*-free flocks, (Hald et al., 2001), and causing *Campylobacter* positive birds to be sent to market.

Biosecurity practices vary from farm to farm and this affects the probability of a farm being infected. The best example is the high percentage of colonized organic or free-range flocks compared to conventional reared flocks (Van Overbeke et al., 2006). In organic broiler production, birds must have access to open space, which probably leads to added fly and other insect exposure, which are highly correlated with *C. jejuni* transmission (Hald et al., 2004; Hald et al., 2008). Broiler chickens raised using organic guidelines had a significantly higher *C. jejuni* in their feces than broiler chickens from the same hatchery, which were raised within the same geographical area using conventional methods (Van Overbeke et al., 2006).

1.2.1.4. Presence of animals other than poultry on the farm:

The presence of other animals on the farm or on its immediate surroundings has been identified as a risk factor for flock contamination in several studies (Bouwknegt et al., 2004; Kapperud et al., 1993; van de Giessen et al., 1992). The presence of cattle has the greatest impact on contamination of broiler flocks with *Campylobacter* when compared to other domestic animals (Gregory et al., 1997). When genotyping of *flaA* was carried out, *Campylobacter* isolated from chickens had the same *flaA* genotype as those isolated from cattle on the same farm were shedding (Stern et al., 1997). In another study, the same *C. jejuni* isolate found in cattle was detected in flies, rodents, the water of the farm, and the feces of sparrows from surrounding urban areas (Adhikari et al., 2004). In Alberta, when 46 bovine and 41human *C. jejuni* isolates obtained from the same geographical area within a short period of time were compared using comparative genomic hybridization, a high degree of similarity was found, leading to the division of these isolates into 13 clusters that mostly contained both cattle and human isolates (Hannon et al., 2008).

Wildlife might play a role in spreading *C. jejuni* as the droppings of wild birds near several farms contained *C. jejuni*, but the strains were usually different from the ones found colonizing the broilers on these farms (Craven et al., 2000).

Although these studies clearly show that *Campylobacter* is widely spread on individual farms, it does not indicate which was infected first, the chickens or the other animals, but it is an indication of the potential contamination that might be caused by the presence of these animals and insects, and the role they might play in spread of the organism.

1.3. *Campylobacter* interaction with avian and human hosts:

"If it was the responsibility of laboratory scientists to determine if *Campylobacter* spp. could become a food-borne pathogen, it would be deemed the least likely candidate. However, not for the first time, bacteria have proved scientists wrong, and *Campylobacter* spp. continue to be a serious public health threat."

-*Murphy et al*. (Murphy et al., 2006)

The main source for human infections is handling and consumption of contaminated poultry meat. *Campylobacter* colonizes chickens with no apparent symptoms. No single source is solely responsible for the contamination of chicken flocks with *Campylobacter*. Once in contact with the host, *Campylobacter* has to overcome several host defense mechanisms to establish an infection, and it uses the same mechanisms both in human and avian hosts but with different outcomes. It is not well understood why the outcomes of the interaction are different. The lack of a reproducible animal model that exactly mimics the human disease remains the major obstacle in understanding the pathogenesis of human disease (Young et al., 2007). On the other hand, the tools available for studying the pathogenesis of *C. jejuni* in chickens are rather limited.

C. jejuni posses a limited repertoire of typical virulence genes that are common to other enteric bacteria. These include the flagella and its secreted proteins (Guerry, 2007), cytolethal distending toxin (CDT) (Johnson and Lior, 1988), and capsule (Bacon et al., 2001). *C. jejuni* has several attributes and genes that help the bacteria to survive in both

the host and the environment, including adhesins, acid resistance, heat and cold stress resistance (Butzler, 2004).

In the following section, the mechanisms employed by *C. jejuni* during its course of interaction with the host will be discussed, and the outcome of interaction with an avian or a human host will be compared.

1.3.1. Infection:

The clinical presentation of *C. jejuni* infection in humans is acute gastroenteritis with a varying degree of fever, diarrhea and abdominal cramps. In some cases, fresh blood, pus, or mucus may appear in the stool sample (Butzler, 2004). Diarrhea caused by *C. jejuni* ceases after 3 days, but it is not unusual for the abdominal cramps to occur after the diarrhea had stopped. The minimum infective dose that has been established in clinical studies is 500-800 bacterial cells (Black et al., 1988; Robinson, 1981), and the incubation period is typically 3-5 days, but incubation for as long as 10 days had been reported. Although most cases of *C. jejuni* infection do not require hospitalization and are usually self-resolved, a condition known as Guillain–Barré syndrome (GBS) is the most serious long term disorder caused by *C. jejuni* (Rhodes and Tattersfield, 1982; Yuki, 2001). Guillain–Barré syndrome is an autoimmune disease caused by the antibodies generated in an immune response to *C. jejuni* lipooligosaccharide (LOS) (Yuki, 2001). These antibodies do not distinguish between LOS and human GM1 ganglioside; so long after *C. jejuni* infection has been cleared the antibodies will destroy the peripheral nerve tissue, leading to paralysis (Godschalk et al., 2007; Kuijf et al., 2007). The evidence of the role *C. jejuni* play in GBS is discussed in the Animal Models section below. Depending on the severity of the condition, GBS patients may be hospitalized, especially if the respiratory muscles are involved. If managed properly, most patients recover with good medical care (Douglas and Winer, 2006). Not all *C. jejuni* serotypes are associated with GBS, as molecular mimicry is limited to a number of serotypes such as HS:19 (Kuroki et al., 1993). The presence of these serotypes or others that induce antibodies that cross-react with gagnliosides does not necessarily lead to development of GBS (Nachamkin et al., 1999), indicating a multifactorial process in which both host and *C. jejuni* play a role. Other immune-mediated diseases associated with *C. jejuni* infection

include reactive arthritis (Berden et al., 1979; van de Putte et al., 1980) and urethral inflammation known as Reiter syndrome (Rothe and Kerdel, 1991).

Chickens display no symptoms upon infection with *C. jejuni*, although up to 10^{10} colony forming units (CFU) might be present per gram of feces. The ability to colonize chickens varies greatly from strain to strain, but under experimental conditions as little as 40 CFU of chicken-passaged *C. jejuni* were enough to cause maximal colonization (Cawthraw et al., 1996). Horizontal transfer from other poultry and the environment is probably the main source for infection (Wagenaar et al., 2006). Under field conditions, *C. jejuni* is not detected in newly hatched chicks, but after 2-3 weeks of placement in a poultry barn it is readily detected (Newell and Fearnley, 2003). Once a few birds are infected on the farm, the high levels of *C. jejuni* shed in the feces of these birds, and the coprophagic nature of chickens accelerates the spread of the bacteria throughout the flock.

1.3.2. Acid resistance:

To establish infection, *C. jejuni* has to survive the acidity of the stomach in humans, and crop and gizzard in chickens. To date, no single gene has been found responsible for acid resistance; upregulation in expression profiles of several genes that are involved in production of heat shock proteins, and response to oxidative stress of *C. jejuni* recovered from the stomachs of orally inoculated neonatal piglets suggests that they play a role in resistance of *C. jejuni* to acid (Adhikari et al., 2004).

1.3.3. Interaction with intestinal surfaces:

C. jejuni colonizes the small intestine in the initial stages of the infection in humans, then establishes itself in the colon (Black et al., 1988). In chickens, the preferred site for colonization is the cecum, but *C. jejuni* has been isolated from other parts of the gastrointestinal tract, including small and large intestine and cloaca. The cecum of the chicken and the human colon are similar in the abundance of lactic acid and short-chain fatty acids (Clench, 1995), which allow *C. jejuni* to thrive.

Mucus is the main defensive barrier of the gastrointestinal tract. *C. jejuni* overcome the mucus layer in both humans and chickens using its corkscrew shape and darting motility *C. jejuni* (Guerry, 2007). The shorter than usual *O*-sidechain of *C. jejuni*'s LOS may also reduce nonspecific binding to glycopeptides of the intestinal

mucin layer (McSweegan et al., 1987; McSweegan and Walker, 1986). Avian mucus might be an important factor in the asymptomatic nature of the infection in chickens. Adding avian mucus to human primary intestinal cells significantly reduced the number of internalized cells compared to cells covered with mucus from human origin (Byrne et al., 2007). Once *C. jejuni* has overcome the mucus barrier, it will be in direct contact with the intestinal epithelial cells.

1.3.4. Body temperature:

One of the main differences between humans and chickens is core body temperature. Poultry generally maintain a range of 41° to 45 °C. A temperature-sensitive two-component regulatory system named RacR-RacS was found to be vital for colonization of wild-type in chickens and growth of *C. jejuni* at 42 °C (Bras et al., 1999). When *C. jejuni* cultures were transferred from 37° to 42 °C, almost 20% of genes were either up- or down-regulated (Stintzi, 2003), including genes involved in membrane structure modification and protein transport.

1.3.5. Adherence:

Bacterial adhesion to the host cell surface occurs mainly by adhesin-receptor interaction. One of the common adhesion mechanisms enteric bacteria utilize to adhere to intestinal epithelial cells are pilli. *C. jejuni* has type II and IV secretory mechanisms, which are the main requirement for pilus synthesis, but its genome does not contain any pilus genes, and the only pilus-like structure reported using electron microscopy (Dolg et al., 1996) was proven to be an artifact caused by the media used (Gaynor et al., 2001). Several proposed *C. jejuni* adhesins have been studied extensively *in vitro* and *in vivo*. The fact that heat-killed *C. jejuni* binds to cultured cells at the same efficiency that live bacteria bind (Konkel et al., 1992a) suggests that the crucial adhesins are constitutively synthesized. The outer-membrane protein (OMP) CadF binds specifically to fibronectin (Fn)(Konkel et al., 1997). The 37 kDa protein was found to be conserved among 38 out of 40 *C. jejuni* isolates tested (Konkel et al., 1999a). In human colonic T84 cells, CadF binds to fibronectin on the basolateral surface (Konkel et al., 2005), and the binding domain was identified to be residues of phenylalanine-arginine-leucine-serine using enzyme linked immunosorbent assay (ELISA) (Konkel et al., 2005). *C. jejuni* binding to INT407 cells was inhibited when pre-incubated with the identified binding domain

(Konkel et al., 2005). The presence of CadF was found to be essential for both invasion *in vitro* and colonization of day-old chicks (Ziprin et al., 1999).

Two lipoproteins are suggested to play a role in *C. jejuni* adhesion, JlpA and CapA. The lipoprotein JlpA contains a signal peptide and an N-terminus lipoprotein processing site. The lipoprotein is crucial for binding to HEp-2 cells, as *jlpA* deletion lead to significant reduction in adhesion, and incubation of the *C. jejuni* with anti-JlpA antibodies reduced adhesion in a dose dependent relationship (Jin et al., 2001). Pro-inflammatory factors, nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinase were triggered in HEp-2 cells when incubated with JlpA (Jin et al., 2003).

The surface localization of CapA was determined using immunogold electron microscope (Ashgar et al., 2007). The autotransporter is important for *C. jejuni* binding to Caco-2 cells (Ashgar et al., 2007), as *capA* mutant had significantly lower cell adherence and invasion abilities. A *capA* mutant was undetectable two weeks after the challenge of day-old chicks, while chickens challenged with wild-type remained colonized (Ashgar et al., 2007).

In conclusion, the experiments outlined above demonstrate that *C. jejuni* has multiple adhesins that play an important role of binding to human cell lines, and in colonization of chickens' intestinal tract.

1.3.6. Translocation, invasion, and intracellular fate:

Several studies have shown clearly that *C. jejuni* resides in intestinal epithelial cells during the course of infection. Since epithelial cells are non-phagocytic by nature, *C. jejuni* must be able to induce its own uptake. In this section, *C. jejuni* translocation, invasion, and intracellular fate in cells associated with intestinal epithelium will be discussed.

Van Spreeuwel and colleagues were able to detect *C. jejuni* in the biopsies of 22 human patients that were confirmed positive by culture using a combination of immunohistochemistry and electron microscopy (van Spreeuwel et al., 1985). The invasive bacteria resided mainly in colonic submucosa without any major disruption in the mucosa. This demonstrates the ability of *C. jejuni* to cross the epithelial layer and reside in the submucosa.

Macaca mulatta monkeys orally challenged with 2.7 X 10^{10} CFU of *C. jejuni* showed clinical signs of campylobacteriosis including diarrhea (Russell et al., 1993). When colonic samples were inspected using electron microscopy, *C. jejuni* were found to be located both extracellularly in the mucosa and submucosa, and intracellularly, either in a membrane-bound vacuole, or free in the cytoplasm. The researchers also noticed structural damage to the epithelial cells, some of which had undergone apoptosis (Russell et al., 1993).

Enterocytes obtained from the small intestine of newly born piglets have been used as a primary cell line to study the invasiveness of *C. jejuni* clinical isolates. *C. jejuni* invaded the cells at a higher rate than an *E. coli* control strain as judged by recovery rates of bacterial cells using bacterial culture (Babakhani and Joens, 1993).

Taken together, these three experiments demonstrate that *C. jejuni* has the potential to invade epithelial cells *in vivo* or in primary cell lines. However, little is known about the factors that induce bacterial internalization and the mechanisms that are used by the cell to take up the bacteria.

The ability of *C. jejuni* to translocate was studied in polarized Caco-2 as these cells more closely resemble the human intestine to a much better extent than other available cell lines (Everest et al., 1992). When 33 different *C. jejuni* isolates were studied, *C. jejuni* from clinical isolates were able to translocate at a much higher frequency (86%) than non-clinical isolates (48%) (Everest et al., 1992), indicating a strain-to-strain variability.

It is not clear which mechanism is preferably used by *C. jejuni* to translocate across the epithelial cell barrier. Translocation of *C. jejuni* is inhibited at 20 °C (the temperature that inhibits the host endocytic pathway) (Konkel et al., 1992b), and scanning electron microscope images show cellular pseudopods extending towards and enveloping the bacteria (Biswas et al., 2000), suggesting that *C. jejuni* can translocate using a trans-cellular route. *C. jejuni* also disrupted the epithelial barrier and induced the production of pro-inflammatory cytokine response in polarized T84 colonic cells (Chen et al., 2006),indicating the possibility of a para-cellular route as well.

Additional *in vivo* strategies may be utilized by *C. jejuni* to translocate across the epithelium. Microfold cells (M cells), which are found in follicle-associated epithelium

and specialize in antigen sampling, have been shown to taken up *C. jejuni* in rabbits (Walker et al., 1988). The mechanism *C. jejuni* preferably uses to cross the intestinal epithelial barrier in humans remains unknown (Young et al., 2007).

Several studies have investigated the role that some of *C. jejuni* components may play to induce internalization, but most of these factors have been studied based on the reduction in the ability of their corresponding mutants to colonize. Most of these mutations have a limited impact on *C. jejuni*'s invasion ability, which indicates that these factors are indirectly involved in colonization. In *Salmonella enterica* serovar Typhimurium, dozens of different mutations had an indirect impact on invasion ability of the bacteria by affecting functions that support the invasion but are not directly involved in it, including some Type three secretion system (T3SS) effector molecules (Coburn et al., 2007). Applying these findings to *C. jejuni*, a limited number of factors have an impact large enough to be considered detrimental to internalization. An example of the factors directly related to invasion is the flagellum, which is discussed in more detail in the following section. An example of factors indirectly related to invasion is the *C. jejuni* capsule, which is critical for attachment to cell lines (Bacon et al., 2001). Capsule mutants have reduced invasion ability, which might be a side effect for the reduction of attachment ability of *C. jejuni* (Bacon et al., 2001; Watson and Galan, 2008).

The systemic spread of *C. jejuni* in humans beyond the intestinal tissues is limited. Based on current literature, septicemia is uncommon during campylobacteriosis in humans, occurring in only 0.4% of the cases (Allos and Blaser, 1995).

In chickens, *C. jejuni* is believed to mainly reside in the lumen and mucus layer of the cecum rather than invading cells (Beery et al., 1988). The bacteria comes in close contact with epithelial cells with no apparent adhesion or invasion (Beery et al., 1988). This is intriguing since *C. jejuni* was found in the internal organs of broiler chickens (Dhillon et al., 2006) and broiler breeders (Cox et al., 2006). Clearly, *C. jejuni* has to come in close proximity and somehow be able to translocate across the intestinal epithelial barrier to be able to spread systemically; however, there is no direct evidence of which mechanism is being used for this purpose.

Many bacterial pathogens incorporate intracellular survival mechanisms as a part of their pathogenesis in an attempt to manipulate the host to their advantage. Some, like

Listeria monocytogenes, will break out from the phagocytic vacuole where they reside and replicate in the cytosol (Drevets and Bronze, 2008). Others, like *Mycobacterium tuberculosis*, will prevent phago-lysosomal fusion which is a critical step in killing invading bacteria (Pieters, 2008). Finally, some will live in membrane-bound compartment, such as *Legionella pneumophila*, which resides in a compartment known as the *Legionella*-containing vacuole (LCV) inside the macrophage. This vacuole provides a safe niche where it can replicate safely until large numbers of bacteria burst out of the vacuole and spread the bacteria through repetitive cycles of invasion and replication (Shin and Roy, 2008).

C. jejuni is capable of invading host cells, but most studies concluded that its intracellular fate is affected by the strain to strain variability. Using different cell lines and different culture methods for recovery of the bacteria, it is generally thought that *C. jejuni* is not viable after 24 hours of internalization. Using Fluorescent Cell Sorter Analysis (FACS) and staining for viability, Watson and Galan demonstrated that *C. jejuni* remains viable although it is not recoverable using routine laboratory culturing methodologies (Watson and Galan, 2008). When invasion/survival assays were conducted by growing *C. jejuni* under oxygen-limiting conditions, bacterial counts were not significantly reduced after 24 hours of internalization, indicating that *C. jejuni* can survive in intestinal epithelial cells for a period of 24 hours. It however, undergoes metabolic changes that require growth conditions that are different from those provided by the methods used routinely in the laboratory (Watson and Galan, 2008). This physiological shift probably reflects *C. jejuni* response to the low concentration of oxygen intracellularly (Watson and Galan, 2008).

Where *C. jejuni* resides once it is internalized is crucial for intracellular survival. An early report has suggested that *C. jejuni* 81-176 resides within a membrane-bound vacuole after internalization into Caco-2 cells (Russell and Blake, 1994). A more recent report shows that *C. jejuni* deviates from the regular canonical endocytic pathway in non-phagocytic cells to reside in a unique compartment; therefore, avoiding phago-lysosome fusion (Watson and Galan, 2008). The fate of *C. jejuni* is very different in bone marrow-derived macrophages, as it is delivered directly to lysosomes and killed (Watson and Galan, 2008). In human monocytic cells, *C. jejuni* strain 81-176 is capable of survival

and even replication to increase recovered bacterial counts by 3 logs (Hickey et al., 2005). *C. jejuni* survives well in chicken epithelial cell lines including primary cecal epithelial crypt cells (Van Deun et al., 2007) and chicken kidney cells (CKC) (Smith et al., 2005). *C. jejuni* ability to survive in avian macrophages varies from strain to strain, but *C. jejuni* is generally killed within 24 hours and could be killed within 6 hours when cultured with chicken peritoneal macrophages (Myszewski and Stern, 1991)

1.4. *Campylobacter* flagellum:

The flagellum is a locomotory organelle that propels *C. jejuni* in a direction dictated by chemical signals received from the environment (Guerry, 2007). It plays a vital role in *C. jejuni* colonization, transmission from host to host, and secretion of various proteins. The presence of *C. jejuni* flagellum has consistently been linked to virulence; that is why it is probably the most studied structure of the organism.

There are at least 8 proteins that share significant structure and sequence similarities between Type Three Secretion System (T3SS) and the flagellum (Van Gijsegem et al., 1995). Several studies suggest that the flagellum and T3SS share a common evolutionary ancestor (Kubori et al., 1998; Marlovits et al., 2004). However, it is not clear which is more ancient, as some studies suggest that the flagellum is more ancient (Liu and Ochman, 2007), while there is a considerable evidence in the literature that indicates that T3SS is more ancient (Journet et al., 2005).

In the following section, flagellar and T3SS structure, function, and the role they play during pathogenesis will be discussed.

1.4.1 Flagellar structure:

Each flagellum is composed of a basal body, motor complex, a switch for the motor complex, hook, junction proteins that bind the hook to the long filament, and a filament cap. The motor complex generates motility by switching the flagellum from one side to the other. The filament is a helical, long, thin structure that extends outside the bacterial cell surface. Flagellar subunits (or building blocks) are called flagellin. The *C. jejuni* flagellum is composed of two flagellin subunits, FlaA, and FlaB, which makes it more complex than *E. coli*'s which is a monomer. Both FlaA and FlaB are synthesized at the same time, but *flaA* is expressed much more than *flaB* (Nuijten et al., 1991).

Regulation of *flaA* expression is controlled by σ^{28} (Nuijten et al., 1991), while *flaB* expression is regulated by σ^{54} (Hendrixson et al., 2001). There are two reports with different outcomes regarding the *flaA*$^{-}$*flaB*$^{+}$ mutant. In one report, the mutant had a truncated flagellum (Wassenaar et al., 1991), while in another, the mutant had a full-length flagellum and was as motile as the wild-type, suggesting that a shift might occur in the expression to have one gene predominantly expressed at a given time (Wassenaar et al., 1994).

1.4.2. Structural and functional relationship of Type Three Secretion System (T3SS) and flagellum:

The *C. jejuni* flagellum extends from the cytoplasm to the cell exterior. The major extracellular component of a flagellum is a rotating filament that is connected to a basal body that provides rotary motor function via a small curved structure known as the hook. All the external proteins that are involved in the formation of the hook and the filament have to be exported sequentially (Macnab, 2003). *C. jejuni* employs a flagellar T3SS pathway that exports these structural proteins, and secretes virulence factors that are not a component of the flagellum extracellularly (Konkel et al., 1999b). *Y. enterocolitica* secretes a phospholipase (YplA) that contributes to its survival in host tissues during infection mainly through its flagella, and also through the other T3SS it harbors (Schmiel et al., 1998). *Salmonella enterica* serovar Typhimurium secretes two effectors SptP and SopE occasionally through the flagella T3SS rather than SPI1 secretion system (Lee and Galan, 2004). The secretion of these effectors through more than one T3SS reveals that these systems are not only structurally related, but they share a common protein targeting mechanism (Warren and Young, 2005).

1.4.3. Proteins secreted by flagellar export apparatus:

C. jejuni produces three sets of proteins that are exported via flagellar T3SS and play a role in pathogenesis. These include *Campylobacter* Invasion Antigens (Cia), Flagella Secreted Proteins (Fsp), and FlaC. Overall, *C. jejuni* secretion mechanisms are poorly understood compared to those of other enteric bacteria. *Campylobacter* invasion antigens consist of at least 8 different proteins (CiaA-H) (Konkel et al., 1999b). The only *Campylobacter* invasion antigen with a known corresponding gene is CiaB, a 73-kDa

protein, is important in internalization of *C. jejuni* into INT407 cells (Rivera-Amill and Konkel, 1999) and colonization of chicken's ceca (Ziprin et al., 2001), as a *ciaB* mutant has a reduced ability to invade INT407 cells (Rivera-Amill et al., 2001) and reduced ability to colonize the chicken's ceca (Ziprin et al., 2001). *Campylobacter* invasion antigens secretion requires a stimulatory signal that can be induced *in vitro* by growing *C. jejuni* in the presence of INT407 cell line (Konkel et al., 1999b), adding bile salts or serum-free media (Rivera-Amill et al., 2001; Rivera-Amill and Konkel, 1999), chicken mucus (Biswas et al., 2007b), and chicken or bovine serum (Biswas et al., 2007b). Secretion of these proteins requires the presence of one of the two flagellar filaments, FlaA or FlaB (Konkel et al., 2004).

A recent experiment conducted by Pacheco and Konkel proved that CiaB could be secreted through a heterologous T3SS (Pacheco and Konkel, 2008). The researchers transformed *Yersinia enterocolitica* with a vector that expresses *ciaB*. The bacteria had 3 different T3SS's, including a flagellar T3SS that could be separately induced under certain culture conditions. Under conditions that induced the flagellar T3SS, CiaB was detected in the supernatant of the bacterial culture using polyclonal anti-CiaB serum. A *Y. enterocolitica* flagella mutant did not secrete the protein, but the cell lysate contained the protein, indicating that the protein was produced but not secreted. This is strong evidence that CiaB is secreted through the flagella, and that the flagella acts as T3SS since a heterologous T3SS could secrete the protein (Pacheco and Konkel, 2008).

Flagella secreted protein A (FspA) is another *C. jejuni* flagella secreted protein (Poly et al., 2007). When 41 *C. jejuni* isolates were tested, FspA was detected in all of them, and two variants (FspA1 and FspA2) that share only 33% similarity were found (Poly et al., 2007). In contrast to Cia proteins, secretion of FspA requires minimal flagellar structure (expression of a flagellar structure up to the hook protein), and occurs simply when the bacteria is grown in broth (Poly et al., 2007). The exact role that FspA plays during infection is unknown, but when INT407 cells were incubated with recombinant FspA2, the protein bound to the cells and induced apoptosis (Poly et al., 2007). When recombinant FspA1 was given as an intranasal vaccine to BALB/c mice, 57.8% were protected against intranasal challenge with a homologous *C. jejuni* 81-176 strain (Baqar et al., 2008). A much less characterized protein, FlaC, is secreted through

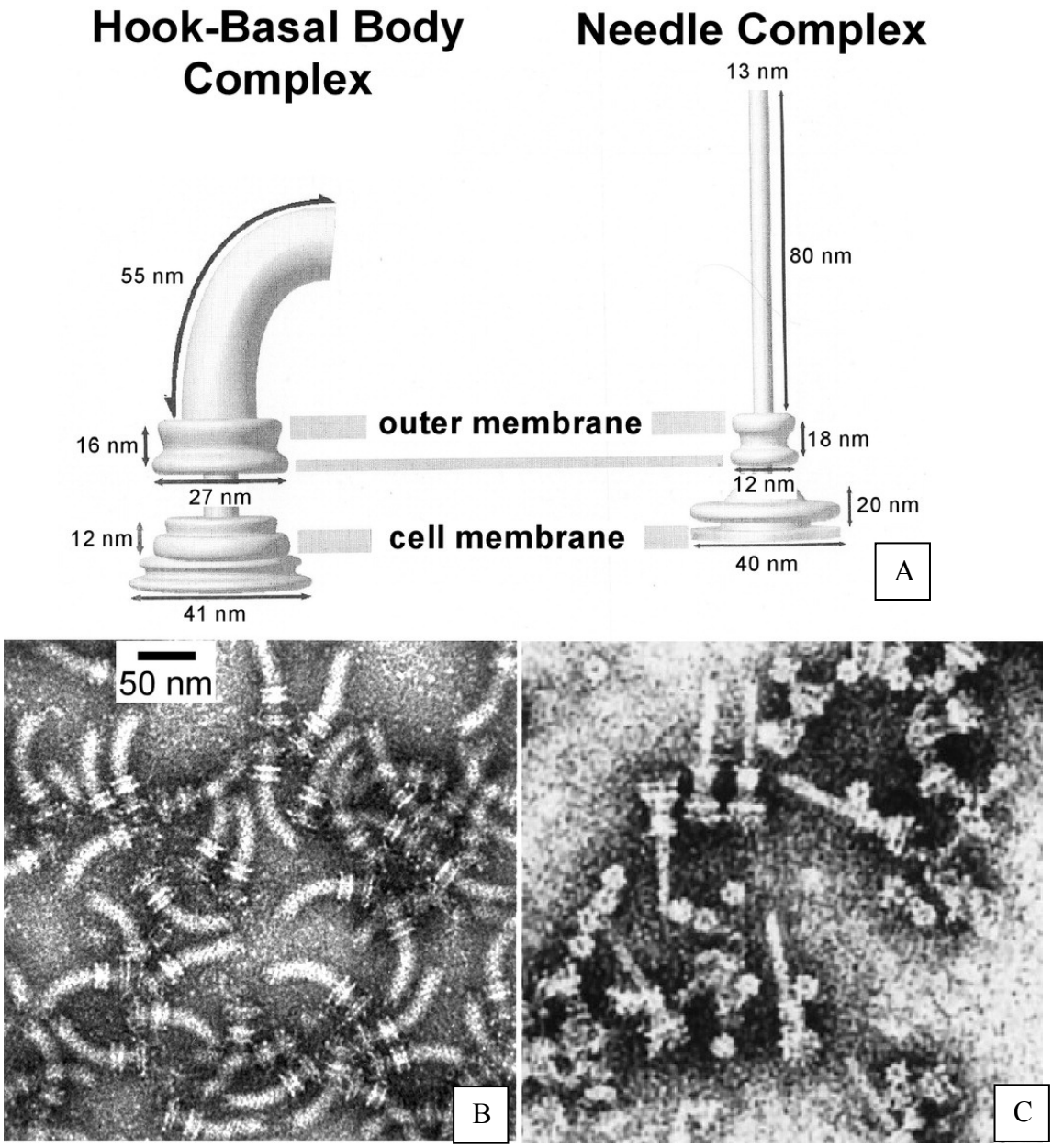

Fig 1.1: Comparison of bacterial flagella and T3SS structures. (A) The upper schematic drawing illustrates the main similarities between the flagellum and T3SS, which includes the outer membrane and cell membrane rings, the hook in the flagellum and the rod of T3SS needle that extend from bacterial surface (Macnab, 2003). (B) Main flagellar components as seen by EM (Jones et al., 1990). (C) Electron microscope image of needle complexes of T3SS. The needle is 80 nm long and 13 nm wide, and it is a stiff, straight tube, in contrast to the flagellum, which is bent (Kubori et al., 1998). Figs A, B, and C are taken directly from (Macnab, 2003).

the flagellar secretion apparatus as well (Song et al., 2004). The FlaC protein is 70% similar to FlaA over a stretch of 40 amino acids, but is much smaller in size.

The secretion of FlaC requires a functional flagellar T3SS. For FlaC to be secreted extracellularly, the expression of the flagellum up to the hook is required (Poly et al., 2007). A *fliG* mutant (null-flagellar mutant) expressed but did not secret FlaC (Poly et al., 2007). A *flaC* insertion mutation produces a fully motile mutant but with reduced ability to invade HEp-2 cells (Song et al., 2004). The role FlaC might play in pathogenesis is unknown.

In conclusion, proteins secreted through *Campylobacter* flagellar export apparatus stimulate host cell signaling, internalization of bacteria, apoptosis, and are critical to colonization of the ceca of chickens. The role of *Campylobacter* invasion antigens, flagella secreted proteins, and FlaC are summarized in Table 1.1. To date, direct evidence for the injection of these proteins into the host cell is lacking.

1.4.4. Role in pathogenesis:

Both flagella and T3SS play an important role in establishing the bacterial colonization in the host. Type III secretion system is critical for delivery of the effector molecules into the eukaryotic cell. The flagellum provides motility, which is a well established virulence factor for *C. jejuni* and this is critical in cell invasion (Carrillo et al., 2004; Yao et al., 1994). Mutations that abolish motility or the complete flagellar structure almost completely inhibit internalization. At the same time, flagellum serves as an export apparatus for flagellar secreted proteins, which are involved in internalization into host cells as discussed above (Konkel et al., 1999c).

Two other unique features of *C. jejuni* flagellum help in evading the immune system. A mutation in the epitope which interacts with Toll-like receptor 5 (TLR5) that prevents activation of TLR5 signaling pathway (Watson and Galan, 2005). This gives the bacteria a major advantage early in the infection as it delays induction of the innate immune system. Secondly, the glycosylation of flagellar filament creates an antigenically diverse population of *C. jejuni* that eludes the adaptive immune system as discussed below.

	Campylobacter Invasion Antigens	Flagella Secreted Proteins	Flagellin
Proteins secreted	At least 8 proteins Cia A-H	FspA, with two variants: FspA1 and FspA2	FlaA, FlaB, and FlaC
Size	12.8-108 kDa CiaB 73 kDa	15.5 kDa	26 kDa
Required flagellar structure	Fully functional flagellum (either FlaA or B)	Minimal flagellar structure (up to the hook)	Minimal flagellar structure (up to the hook)
Function	- Modify cell behavior - Enhance invasion of host cell	- Induce apoptosis of INT407 cells - protection of mice vaccinated intranasally with FspA2	FlaA, B: flagellum building blocks FlaC: might play a role in invasion
Stimulatory signal	FBS, bile salts, or presence of eukaryotic cells	Normal growth conditions in broth stimulate secretion	Normal growth conditions in broth stimulate secretion
Effect of mutation	-Reduced INT407 invasion -Reduced cecal colonization	No observed effects on adherence or invasion of INT407	FlaC: - Reduced HEp-2 cells invasion - No effect on motility and morphology

Table 1.1: Proteins secreted by *C. jejuni* flagellar T3SS. Three groups of proteins secreted through flagellar T3SS have been identified. Flagellin (the main structural unit of flagellar filament), *Campylobacter* invasion antigens (Cia), and Flagella Secreted Proteins (FSP).
FBS: Fetal bovine serum, Cia: *Campylobacter* invasion antigens, Fsp: Flagella secreted protein, Fla: flagellin.

1.5. Immune response to *C. jejuni* infection:

Given the widespread presence of *C. jejuni* in fresh poultry and other food products, the number of campylobacteriosis cases would be expected to be higher than what is reported (Janssen et al., 2008). The role played by the host to combat infection is as critical as the virulence attributes of the infecting *C. jejuni* strain in determining the clinical outcome. In the following sections the role of innate and adaptive immune response in the light of present literature is discussed.

1.5.1 Innate immune response to *C. jejuni* infection:

The innate immune system represents the first line of defense against pathogens. *C. jejuni* has an array of mechanisms to delay or avoid detection by immune system. Two unique features of the *C. jejuni* flagellum are directly involved in immune evasion. The first is an alteration in the structure of the area recognized by TLR5 that evades flagellum-mediated stimulation of TLR5 (Watson and Galan, 2005). The other feature is the heavy glycosylation of the flagella, which is thought to play a role in immune evasion (Szymanski et al., 1999). *C. jejuni* is a poor Toll-like receptor 9 stimulator, and this is due to its AT-rich genome that lacks the unmethylated CpG dinucleotides necessary for TLR9 activation (Dalpke et al., 2006). Chickens contain TLR orthologues, but their stimulation by *C. jejuni* has not been studied yet.

C. jejuni induces the expression of several pro-inflammatory cytokines in both humans and chickens. Production of IL-8 results in the activation of several cellular immune mechanisms. *C. jejuni* induces IL-8 production in both human epithelial cells and chicken intestinal primary cell lines (Borrmann et al., 2007). Another pro-inflammatory cytokine, IL-1β, is induced by *C. jejuni* in human and chicken cells (Jones et al., 2003). Production of IL-6, iNOS, and nitric oxide are stimulated in HD11 cells and CKC (Smith et al., 2005). Incubation of either live or heat-killed *C. jejuni* with HeLa cells activated NF- κB (Jones et al., 2003). The activation process is not immediate, and takes a minimum of 16 hours. The activation of NF- κB by *C. jejuni* in human cells is not surprising, as gastroenteritis is the main clinical manifestation of campylobacteriosis, and many of the pro-inflammatory genes mentioned above are activated by NF- κB. Dendritic cells produce IL-1β, IL-6, IL-8, IL-10, and a high level of IL-12 after internalization of

live or heat-killed *C. jejuni* (Hu et al., 2006). The high levels of IL-12 indicates a Th1 polarized immune response in humans (Hu et al., 2006). Although cytokines are induced during *C. jejuni* infection, the disease outcome in chickens is very different. It is possible that the response in chickens is local or brief, preventing any lesion formation or further recruitment of inflammatory cells. Another possibility is that the immune response to *C. jejuni* is somehow redirected towards tolerance. This is supported by the inability of most live or killed vaccines to protect chickens from colonization, but when attenuated *Salmonella* expressing *C. jejuni* antigen CjaA was used as a vaccine, the CFU/g of feces in the ceca of challenged birds were lowered by more than 6 logs, indicating a potent and protective immune response (Wyszynska et al., 2004).

1.5.2. Adaptive immune response to *C. jejuni* infection in humans:

Antibodies are detected by the second week after infection in humans. These antibodies are directed against several major *C. jejuni* antigens, including LOS (Mills and Bradbury, 1984) , major outer-membrane protein (MOMP) (Kervella et al., 1992), and flagellin (Cawthraw et al., 1994). Levels of IgM, IgA and IgG peak within 8-14 days post-infection (Blaser and Duncan, 1984). Activity of IgM and IgA is relatively short lived, as IgA returns to a basal level within 20 days, and IgM gradually decreases within two months to a basal level (Cawthraw et al., 2002). In contrast, IgG activity declines over the period of one year (Cawthraw et al., 2002). Three main observations suggest that antibodies might provide some protection. The first evidence is that human volunteers re-challenged with *C. jejuni* had relatively milder clinical symptoms using a homologous challenge (Black et al., 1988). The second evidence is that breast milk with high maternal anti-*Campylobacter* IgA has been shown to be related to protection in young children against *C. jejuni* infection (Renom et al., 1992). Finally, reports of asymptomatic carriers in developing countries where people are constantly exposed to *C. jejuni* suggest that antibody formation might be protective (Blaser et al., 1985).

1.5.3. Adaptive immune response to *C. jejuni* infection in chickens:

As discussed in the innate immune response section, a *C. jejuni* infection does not induce any inflammatory signs in the gut or any pathology regardless of the age at the time of infection despite its large numbers per gram of feces. In contrast to humans, an

antibody response takes a longer time to develop and IgM, IgG, and IgA antibody activity generally increases in the serum after two weeks of a challenge (Rice et al., 1997; Sahin et al., 2003). Secretory IgA level takes a longer time to increase as it is usually detected only after 3 weeks of an oral challenge (Myszewski and Stern, 1990; Rice et al., 1997).

Broiler breeders infected with *C. jejuni* deposit large amounts of anti-*Campylobacter* antibodies into their eggs (Sahin et al., 2002; Sahin et al., 2001). When they hatch, day-old chicks contain maternal antibodies that can recognize several outer-membrane proteins (Sahin et al., 2001). These maternal antibodies have bactericidal activity *in vitro* (Sahin et al., 2001), and might play a role in delaying colonization of day-old chicks. Colonization of chicks hatched from infected breeders was delayed by 2-4 days compared to chicks from non-infected ones (Sahin et al., 2003).

1.6. Preventing *C. jejuni* carriage by broiler chickens:

Several methods have been proposed to reduce or eliminate *C. jejuni* in the final poultry product available to the consumer. Epidemiological modeling predicts that the maximum reduction in human infections with *C. jejuni* could be achieved by preventing or lowering the infection of chickens on the poultry farm (de Zoete et al., 2007). The ubiquitous nature of *C. jejuni*, as discussed above, makes control by biosecurity alone very difficult. Since the biosecurity barrier could be easily breached, it is important to have other measures that protect chickens from being colonized. A limited number of preventive measures have been explored, including vaccination, competitive exclusion, and breeding birds that have a *Campylobacter*-resistant background.

1.6.1. *Campylobacter*-resistant chickens:

Naturally *Campylobacter*-resistant chickens have been reported in several inbred lines (Boyd et al., 2005). If a genetic locus that is strongly correlated with persistent reduction in *Campylobacter* colonization could be identified, chickens might be selected for this trait. However, it is a common observation during experimental infection of broiler chickens that the cecal colonization levels are different from one bird to the other and from one experiment to the next. More in-depth studies are needed to determine the efficacy and the feasibility of this approach, especially if the genetic trait encoding for resistance interferes with weight gain or feed conversion efficacy.

1.6.2. Competitive exclusion:

Competitive exclusion is the protective effect of non-pathogenic bacterial flora in limiting pathogenic bacteria colonization. Competitive exclusion has been used in the poultry industry to control foodborne pathogens such as *Salmonella* with an acceptable level of success. Several attempts to use competitive exclusion with *Campylobacter* had different outcomes. Mead and colleagues tested two different mixtures, defined aerobically grown bacteria from the ceca of *Campylobacter*-free laying hens, and an undefined anaerobic mixture grown from the same source (Mead et al., 1996). The anaerobic bacteria mixture significantly reduced colonization with *C. jejuni* after oral challenge. Another study by Hakkinen and Schneitz showed promising results when a commercial competitive exclusion product that is designed to target *Salmonella* also protected against *Campylobacter* colonization in broiler chickens (Hakkinen and Schneitz, 1999). Despite the success of these experiments, other competitive exclusion products did not protect against *Campylobacter* colonization (Aho et al., 1992). The exact mechanism of protection against *Campylobacter* is not clear, but bacteriocins produced by other bacteria might be the main factor in protection (Stern et al., 2005). When the bacteriocin of *Paenibacillus polymyxa* was purified, microencapsulated in polyvinylpyrrolidone, and added to the feed of broiler chickens it prevented colonization by *Campylobacter* as the challenged group had no detectable *Campylobacter* in their cecal contents (Stern et al., 2005). The limited number of studies and the unknown impact of bacteriocins on chicken's microflora are a limiting factor in recommending this approach, for the time being, as a solution to *Campylobacter* colonization of broiler chickens.

1.7. Vaccination:

A protective vaccine against *C. jejuni* in broiler chickens must be safe, cost effective, and produce protective immunity quickly and as early as possible given the short life span of broiler chickens. As well, it should protect against several strains, as the same flock will likely be colonized by more than one strain, as discussed earlier. Epidemiological modeling predicts that a reduction by 3 logs in the level of *C. jejuni* shedding by broilers should significantly reduce human infection (de Zoete et al., 2007).

1.7.1. Role of antibodies in protecting against *C. jejuni* infection:

As discussed above in the adaptive immune response section, chickens hatched from a breeder infected with *C. jejuni* will start shedding 2-4 days after chickens hatched from non-infected breeders (Sahin et al., 2003). Chickens that had no maternal antibodies are easier to infect as the required dose for infection was 50% less than chickens with maternal antibodies. As proof of principle, this demonstrates that maternal antibodies play a role in protecting hatched chicks for the first few days in their life, and it confirms that an adaptive immune response induced by a vaccine might be protective.

1.7.2. *C. jejuni* experimental vaccines tested to date:

To date, there is no licensed vaccine against *C. jejuni* in broiler chickens. The vaccines that have been studied or are being developed are whole-cell killed or live attenuated vaccines, flagella-based vaccine, and subunit vaccine.

1.7.2.1 Whole-cell vaccines:

Whole-cell vaccines are an attractive choice for vaccination since they are relatively cheap and easy to produce, and are generally considered safe to use (Walker, 2005). Unfortunately, whole-cell vaccines have had limited success in protecting broiler chickens against colonization with *C. jejuni*. This is contrary to the results obtained with WCV used against other enteric pathogens (Walker, 1999, 2005).

Rice et. al used formalin inactivated *C. jejuni* whole-cell vaccine to vaccinate day-old broiler chickens (Rice et al., 1997). Each dose contained $1x10^9$ CFU and chickens were vaccinated multiple times. When chickens were challenged using a seeder model (a small group of infected chickens housed with non-infected ones), the maximum reduction in colonization in the vaccinated group compared to those non-vaccinated was 1.5 logs. The addition of *E. coli* heat-labile toxin (LT), which acts as a mucosal adjuvant, did not enhance the immune response (Rice et al., 1997). Billiary IgA levels did not increase after vaccination, but using Western blots, IgA from vaccinated chickens detected more *C. jejuni* antigens than that from non-vaccinated chickens.

A whole-cell vaccine made of $1x10^{10}$ formalin killed CFU in Freund's complete adjuvant administered subcutaneously elicited a high antibody titer (Glunder, 1995), but there was no significant difference between the shedding of vaccinated and non-vaccinated chickens challenged using a homologous strain (de Zoete et al., 2007). A high

antibody titer was also obtained using heat-killed *C. jejuni in ovo* (Noor et al., 1995). Embryos were vaccinated at day 16 of incubation, and 7 days post-hatch. Although IgG antibody titers decreased after the booster dose, which might have resulted from the neutralizing effect of the vaccine antigen, IgA titers in bile and intestinal scrapings had increased significantly (Noor et al., 1995). Immunized chickens were not challenged, therefore it is unknown whether these high antibody titers were protective or not.

1.7.2.2. Subunit-based vaccines:

Flagella/flagellin and outer membrane proteins (OMPs) were tested as vaccine candidates against *C. jejuni* infection based on their predominant immunogenicity. It is assumed that an immunogenic antigen would lead to an immune response that is protective for chickens.

A vaccine made of purified flagellin or flagellin combined with heat killed *C. jejuni* administered twice intraperitoneally at days 16 and 29 of age was able to reduce the intestinal colonization with *C. jejuni* (Widders et al., 1996). The combination of flagellin and heat-killed *C. jejuni* resulted in a higher IgG and IgM in serum and a higher IgG in the intestine than flagellin alone.

Khoury and Meinersmann developed a recombinant flagellin-based vaccine (Khoury and Meinersmann, 1995). The *flaA* gene was cloned in-frame with the B subunit of *E. coli* heat labile toxin (LT-B). The protein fusion was expressed in *E. coli*, purified and administered intramuscularly and orally in different doses. Not all the birds responded to vaccination, with only 75% containing FlaA antibodies in their intestinal secretion. The researchers vaccinated a group of birds twice at 2 and 4 weeks of age and challenged them orally at 3 weeks of age with 2 X 10^8 CFU. The vaccinated group had 27.6% of the birds colonized by the end of the experiment, while 49.3% of control birds remained colonized.

Immunization with plasmid DNA that contain the *flaA* gene gave results that are within the same range of other flagella-based vaccines (de Zoete et al., 2007). Although vaccinating the birds intramuscularly twice on day 2 and 18 did not induce any detectable antibodies, birds challenged had approximately a 2 log reduction of *C. jejuni* carriage in the intestinal tract when compared to controls.

An attenuated *S. enterica* serovar Typhimurium strain expressing the CjaA protein was able to reduce *C. jejuni* colonization of chickens by ~ 6 logs compared to control. Both CjaA and CjaC are putative ABC transporter proteins that are strongly immunogenic (Raczko et al., 2004). When 30 clinical isolates of *C. jejuni* were tested using hybridization, the genes *cjaA* and *cjaC* were found in all of them (Pawelec et al., 2000). The researchers selected CjaA to be constitutively expressed in *Salmonella* and vaccinated 20 chickens twice at 2 and 14 days of age. Serum IgG and mucosal IgA levels against both CjaA and *Salmonella* increased as detected by ELISA. Chickens were challenged at 4 weeks of age and had significant reduction with only three of the 20 chickens shedding *C. jejuni* that was above the detection level ($1x10^3$ CFU/g) of the selective media used over the period of 12 days. The experiment lacked a third group that should have received a *Salmonella* strain with the plasmid only which expressed no CjaA protein. This would have determined precisely the role of *Salmonella* induced immune response in protection against *C. jejuni*. This vaccine has a great potential as it provide a significant reduction in *C. jejuni* cecal counts of the vaccinated birds and induces an immune response against *Salmonella*.

Although a flagellin-based vaccine induced an immune response, the majority of antibodies generated are not directed towards surface epitopes but rather the unexposed flagellar structure (Widders et al., 1998). The post-transcriptional modifications of *C. jejuni* flagella (Doig et al., 1996) may influence the efficiency of a flagellin-based vaccine as at least 16 flagellar pseudaminic acid (PseAm) residues varies in amount and form. Antibodies formed against these residues on one flagellum may not necessarily recognize PseAm residues on another (Logan et al., 2002).

The dampened immune response induced by whole-cell vaccines or live-attenuated *C. jejuni* vaccine might be the result of either limited exposure to the immune system and antigen presenting cells, as some live-attenuated strains are cleared quickly, or the absence of certain structures that might be necessary for a more rigorous immune response but are not produced under the vaccine growing conditions, such as *Campylobacter* invasion antigens and other flagella-secreted proteins. Even with natural infection with *C. jejuni*, the chicken's immune system takes a relatively long time to respond and produce an adaptive immune response as discussed above. To enhance the

efficacy of a vaccine, a stronger and a faster immune response would be useful. This was achieved using the attenuated *Salmonella* vaccine (Wyszynska et al., 2004). Other types of recombinant vaccines, including genetically engineered pox virus and adenovirus, might be as efficient in exposing the antigens to the chicken's immune system and therefore induce a better immune response, as these vaccines have protected against an array of poultry disease including Newcastle disease (Perozo et al., 2008a) and infectious bursal disease (Perozo et al., 2008b).

1.8. Eliminating *Campylobacter* from infected broiler flocks:

An alternative approach to preventing the infection is to eliminate *C. jejuni* from the infected flock. The use of antibiotics is not an option as the life span of a broiler chicken is a maximum of 42 days, which means that most antibiotic treatments should stop 3 weeks earlier (day 21), therefore preventing their use. Also, the emergence of a *C. jejuni* strain that has antimicrobial resistance is a major public health concern.

The use of bacteriophage as a treatment for *C. jejuni* infection resulted a limited reduction in bacterial counts in the cecum. When a mixture of two bacteriophages was given to broiler chickens colonized by *C. jejuni* for a period of 6 days, an initial reduction in bacterial counts in cecal content by 3 logs compared to an untreated control group was observed (Wagenaar et al., 2005). After 5 days of treatment, bacterial counts increased, reducing the difference to only one log. The colonization level remained the same throughout the experiment. It is not obvious why the counts initially were lower, but it is possible that *C. jejuni* resistant to the bacteriophage became more dominant in the population. Bacteriophage treatment is still in an early phase of testing, and it has several limitations such as the rapid resistance that develops against the phages used and the governmental regulatory restrictions that might require the elimination of the phage from the final poultry product (Rees and Dodd, 2006)

1.9. Antimicrobial resistance in *C. jejuni*:

Antibiotics are infrequently used for treatment of campylobacteriosis in humans as the disease is typically self-limiting and only occasionally requires hospitalization. In some severe cases antibiotic treatment is unavoidable. The drug of choice for *C. jejuni*

infections is erythromycin (member of the macrolide family), followed by ciprofloxacin (member of the fluoroquinolone family) (Nachamkin et al., 2002).

A dramatic increase in *C. jejuni* resistance to fluoroquinolones since the mid-1990s was observed in the United States of America (Nachamkin et al., 2002). During the period of 1982-1992, *C. jejuni* isolates from 142 patients had no fluoroquinolone resistance, and only 2% erythromycin resistance. Fluoroquinolone resistance in 47 *C. jejuni* isolates from patients in 2001 increased to 40.5% compared to isolates from the 1980's. A similar increase has been documented for *C. jejuni* isolates from the late 1990's. Erythromycin resistance remained low, with an increase in resistance in only 4% of the total isolates. The increase in resistance in human *C. jejuni* fluoroquinolone isolates was linked to the licensing of fluoroquinolone use in poultry production (Jimenez et al., 1994; Khachatourians, 1998). Enrofloxacin, a fluoroquinolone, was licensed in the early 1990s in Europe and Asia, and soon after a high incidence of resistant *C. jejuni* isolates from animal sources was reported. In Spain, 99% *C. jejuni* isolated from broiler and pig farms were resistant to fluoroquinolones, while 72% of human isolates were resistant at the same time period (Saenz et al., 2000). The same trend was observed in Taiwan, where 92% of *C. jejuni* isolated from chickens were resistant to fluoroquinolones, and 52% of human isolates were resistant (Li et al., 1998). The antimicrobial resistance pattern in Australia was different to the situation in Europe and Asia, as fluoroquinolones were never licensed for use in poultry (Hart et al., 2004; Unicomb et al., 2003). The numbers of resistant isolates from humans and animals remained low, and no increase in resistance has been observed (Unicomb et al., 2003).

Streptomycin, which belongs to the aminoglycoside class of drugs, is a bactericidal antibiotic that prevents protein synthesis initiation by binding to the 16S rRNA subunit. Aminoglycoside resistance in general, specifically streptomycin, is not common, as studies have found resistance in 0-2.76% in various *C. jejuni* isolates (Velazquez et al., 1995). *C. jejuni* isolates from rinses of poultry carcasses in the USA were sensitive to aminoglycosides, making this group of antibiotics a good marker for molecular techniques. Resistant *C. jejuni* isolates modify the antibiotic, so that it is unable to interact with the ribosome, through the enzyme aminoglycoside adenyltransferase (AAD) (Pinto-Alphandary et al., 1990). Two types of this enzyme have

been found in *C. jejuni* and they both provide streptomycin resistance; AAD (3") which is encoded by *aadA*, and AAD(6) which is encoded by *aadE* gene. The majority of the resistant strains encodes *aadA*, but some streptomycin-sensitive strains encodes an *aadA* homologue on a plasmid but with no apparent resistance (Nirdnoy et al., 2005).

1.10. Animal Models Other Than Poultry for Studying *C. jejuni* infection:

A considerable amount of *C. jejuni* pathogenesis and colonization research was performed using chickens as a model. This model is of particular importance as poultry products are considered to be the main source for *C. jejuni* infections as discussed above, and recognizing how an immune response is generated in a chicken and the factors that are crucial for *C. jejuni* colonization of the intestinal tract of a chicken is important in reducing the carriage of *C. jejuni* in chickens, therefore reducing the chance of infection in humans (Doyle and Erickson, 2006). However, the lack of clinical disease in chickens makes this model unsuitable for studying disease pathogenesis. The wide spectrum of symptoms that results from *C. jejuni* infection in humans makes having several animal models necessary. The infection could occur with no apparent symptoms, mild or severe diarrhea, or could result a chronic sequel such as Guillain-Barré syndrome (Wassenaar and Blaser, 1999). In this section, mouse, pig, ferret, and rabbit animal models will be discussed.

1.10.1 Mouse

Mice are used often in studying many aspects of human diseases including immune responses, pathogenesis, and the factors that might affect colonization (Bacon et al., 2000). Under natural conditions, *C. jejuni* is not normally isolated from the intestinal tract of laboratory mice, but using various experimental settings colonization could be induced. Kazmi *et al*. injected infant mice intraperitoneally with *C. jejuni* (Kazmi et al., 1987), causing a self-limiting diarrhea. The value of this model is very limited, as the infection is introduced through injection rather than ingestion, and the mice are too young to have a proper immune response. Adult athymic (nu/nu) gonobiotic mice also developed transit diarrhea 7-9 days after oral challenge in addition to splenomegaly 1-2 weeks after colonization (Yrios and Balish, 1986). Chang and Miller were able to develop a mouse model with reproducible colonization results using wild-type C3H mice

that contained pre-defined microflora (Limited-flora) that included *Firmicutes*, *Lactobacillus*, and *Acinetobacter* spp. (Chang and Miller, 2006). After oral gavage with a *C. jejuni* culture, these mice remained colonized with a high level of *C. jejuni* (up to 10^8 CFU/gram of feces). Using a dose response titration, the researchers determined that the ID_{50} was 2 X 10^2 CFU. Mice had a limited inflammatory reaction in their intestine and cleared the infection a few weeks after the challenge. When limited-flora mice that are immuno-deficient (SCID) were challenged with *C. jejuni* orally, they developed severe typhlocolitis in the colon and cecum. Gastritis also occurred on day 28 after the challenge. Inflammatory cells involved were mainly granulocytes with some mononuclear cells. This model might be of limited value in studying pathogenesis as the lesions are far more severe than typical *C. jejuni* infections in humans, but it might serve well in identifying genes involved in virulence or colonization. MacKichan and colleagues used SCID mice with limited-flora to show that the two-component regulatory system of *C. jejuni* (*dccR*-*dccS*) is crucial in *C. jejuni* colonization of the gastrointestinal tract (MacKichan et al., 2004), as the *dccR* mutant was not able to stimulate any inflammatory response by mice.

The intranasal route of infection with *C. jejuni* is highly artificial in humans or animals, but BALB/c mice that received *C. jejuni* intranasally developed a systemic infection and had increased mortality among the challenged group (72% of the mice died) (Baqar et al., 1996). When killed *C. jejuni* was administered as a vaccine intranasally, it induced a strong humeral immune response and mice survived a challenge with a homologous strain that was lethal in the non-vaccinated group (Baqar et al., 1996). The researchers got different mortality ratios using different *C. jejuni* strains, making intranasal challenge more suitable for evaluation of *C. jejuni* virulence rather than studying pathogenesis and immune response.

Nuclear factor-κB (Nf-κB) deficient mice were used to study pathogenesis of *C. jejuni* and the potential role for NF-κB to the resistance of the infection (Fox et al., 2004). These mice were found to be susceptible to *Helicobacter hepaticus*, an enterohepatic bacterium that is closely related to *C. jejuni* (Fox et al., 2004). *H. hepaticus* pathogenesis is caused partially by a toxin that has a pathway similar to the cytolethal destending toxin of *C. jejuni* (Chien et al., 2000). Mice that received *C. jejuni* 81-176 were colonized at a

high level (75-100%) and developed gastritis and proximal duodenitis, but the bacteria did not spread beyond the GIT to any of the internal organs. In the same experiment, a *C. jejuni cdtB* mutant was administered orally to NF-κB deficient mice. The mutant colonized at the same level as the wild-type and caused the same lesions as the wild-type with the exception of typhlocolitis lesions. Also, the IgG (adaptive immune response) and IgG2a (innate immune response indicator) levels were significantly lower than in the control mice. This is not surprising, as these mice cannot produce IL-1, IL-6, IL-12, and TNF-α, which are crucial for innate and adaptive immune responses (Fox et al., 2004).

1.10.2. Rabbit:

Rabbits were used in studying *C. jejuni* colonization and vaccine development (Caldwell et al., 1983; Walker et al., 1988). A removable Intestinal Tie Adult Rabbit Diarrhea model (RITARD) was first used in 1983 by Caldwell et al. in an attempt to mimic typical campylobacteriosis in humans. The cecum was ligated, and a slip knot was placed at the terminal ileum, and 1 X 10^9 CFU of bacterial culture was injected into the mid-small bowel. The loose ends of the knot were left protruding through the incision, and four hours later the slip knot was released. Rabbits received two different strains, the first caused diarrhea in 52% and death of 41% of the rabbits. The other strain caused diarrhea in 73% and death of 65% of the rabbits in the group. None of the rabbits in the control group that did not receive *C. jejuni* died (Caldwell et al., 1983). The clinical symptoms caused by challenging rabbits were similar to the results obtained when the same two strains were given to human volunteers (Black et al., 1988), indicating that this model might be useful in predicting the virulence of each strain in humans. The model requires some surgical skills and extra time to perform surgery on each and every animal in the experiment, making it a more demanding challenge model and therefore a less attractive choice than others.

Rabbits were also used to demonstrate the role of *C. jejuni* LOS molecular mimicry in Guillain-Barré syndrome (GBS). The observation that the sera of patients with GBS had developed antibodies to minor gangliosides GM1b and GalNAc-GD1a and to *C. jejuni* lead to the assumption that GBS is caused by molecular mimicry of *C. jejuni* to the gangliosides (Ho et al., 1999). Rabbits were used to study GBS by both injection with gangliosides (Yuki, 2001) and injection with *C. jejuni* LOS (Yuki et al., 2004).

Using a mixture of gangliosides extracted from bovine brain to inject rabbits, Yuki was able to induce high anti-GM1 IgG antibodies and flaccid limb weakness (Yuki, 2001). Upon histopathological examination, peripheral neurons were degenerated without cellular infiltration but rather IgG deposits. To prove the connection with *C. jejuni*, rabbits were injected with *C. jejuni* LOS that had an epitope similar in structure to GM1 (Yuki et al., 2004). Rabbits quickly developed high levels of anti-GM1 IgG and developed flaccid limb weakness. Researchers used *E. coli* and *Salmonella* LOS as controls, and none of the control animals developed GBS or GBS-like symptoms (Yuki et al., 2004). Through the use of rabbits, the four criteria of Koch's postulates were fulfilled (Ang et al., 2004):

- Presence of an epidemiological link between GBS and *C. jejuni*
- Identified the role of anti-GM1 IgG antibodies
- Identified *C. jejuni* molecules that mimic gangliosides
- Reproduced the disease in an animal model (rabbit)

1.10.3. Nonhuman primates:

Nonhuman primates have been found to be infected with *Campylobacter* in several studies over different parts of the world. Bryant and colleagues found *C. jejuni* in 46% of Patas monkeys with chronic diarrhea (Bryant et al., 1983). The monkeys experienced muco-hemorrhagic diarrhea, and when treated with streptomycin or tetracycline for 10 days the diarrhea ceased. After one month, an outbreak of diarrhea occurred among the colony, and *C. jejuni* was identified as the causative agent. It is possible that the treatment did not completely clear the infection, and given the feco-oral habits of monkeys, re-infection may have occurred (Bryant et al., 1983). In another colony of infant *Macaca nemestrina* monkeys, multiple strains of *C. jejuni* were found in animals with and without diarrhea (Russell et al., 1990). Using DNA hybridization and polyacrylamide gel electrophoresis protein profiles, the researchers were able to show that an infection with one strain lasted for 3-4 weeks (Russell et al., 1990). Fitzgeorge *et al.* infected Rhesus monkeys with a human strain of *C. jejuni* (Fitzgeorge et al., 1981). The animals shed the bacterium for a long period of time, but only had a brief period of bacteraemia, diarrhea and inappetence. When the animals were re-challenged with the

same strain, no clinical symptoms were observed, and shedding lasted for only 3 days post-challenge (Fitzgeorge et al., 1981). Russell *et al.* challenged *Campylobacter*-free monkeys with 3 X 10^{11} CFU of strain *C. jejuni* 81-176 (Russell et al., 1989). Bloody feces and fluid diarrhea were observed. A strong antibody response to *C. jejuni* occurred as a result of the challenge. Re-challenging the monkeys with both homologous and heterologous strains resulted only a mild diarrhea, indicating a partially protective immunity (Russell et al., 1989).

1.10.4. Ferret:

Ferrets are commonly colonized by *C. jejuni*. In a study by Fox *et al.* 61% of 168 ferrets sampled were positive for *C. jejuni* (Fox et al., 1983). *C. jejuni* has been isolated from weanling ferrets (kits) suffering from mucoid diarrhea (Bell and Manning, 1990). When ferrets were orally challenged with 10^8-10^{10} CFU *C. jejuni* isolated from a diarrhea outbreak in minks or humans; their feces became mucoid or they developed diarrhea (Bell and Manning, 1990). When the strain isolated from mink was administered intravenous the animals developed watery diarrhea. The diarrhea in both cases was self-limiting, and was resolved in 3-8 days. Animals developed antibodies as detected by an agglutination test and cleared the infection in a period of 7 days. When some of the ferrets were re-challenged with a homologous strain, no diarrhea developed but the animals were colonized by *C. jejuni*. The researchers inoculated animals 5 times either orally or intra-rectally over the period of 5 weeks and they were able to induce immunity to both disease and infection. Kits born to dams with high antibody titer were colonized with *C. jejuni* upon infection, but they did not develop diarrhea. Bacteremia developed in ferrets challenged orally on days 2-5, but animals developing bacteraemia had similar clinical signs to the animals with no clinical signs.

Burr and colleagues were able to prevent development of diarrhea in 80% of challenged ferrets by vaccinating them orally 4 times with a *C. jejuni* formalin-fixed whole cell vaccine (Burr et al., 2005). The vaccination did not prevent colonization, but provided protection against disease caused by both homologous and heterologous strains. The addition of leukotoxin as an adjuvant or increasing of the number bacteria in the vaccine did not improve the protection.

The ferret model of infection results in clinical symptoms that resembles those associated with campylobacteriosis in humans, but the infective dose that has been reported is high, and the duration of bacterial shedding is short, limiting the ability to use this model for vaccine testing.

Animal models described above are summarized in Table 1.2.

	Chicken	***Mouse***	***Ferret***	***Non-human primates***	**Rabbit**
Minimal infectious dose	> 40 cfu	> $2x10^2$ CFU	N/A	N/A	N/A
Infection Symptoms	None	varies from no symptoms to diarrhea	self-limiting diarrhea, moderate enteritis	self-limiting diarrhea, some spleenomegaly	flaccid paralysis
Genetic background (Inbred/ outbred)	outbred	inbred	outbred	outbred	outbred
Main application	-Colonization -Vaccine	-Pathogenesis, -Role of specific genes in disease development	-pathogenesis -onset of clinical symptoms	-pathogenesis -clinical symptoms	-GBS pathogenesis -Enteritis
Critical findings	Role of: flagella, motility, secreted proteins, and adhesins in colonization	-Contrasting the role of innate and adaptive immunity in limiting infection -Gut microflora effect on colonization	Role of maternal antibodies in protection against diarrhea	Role of adaptive immunity in protection against future *C. jejuni* infections	The role of *C. jejuni* in GBS

Table 1.2: Various animal models used for studying *C. jejuni*. This table highlights animal models used in studying *C. jejuni* pathogenesis and autoimmune diseases induced by *C. jejuni*.

N/A: Not available; GBS: Guillain-Barré syndrome.

1.11. Green Fluorescent Protein:

Green fluorescent protein is found naturally in the *Aequorea victoria* jellyfish. Shimomura and colleagues discovered GFP while working on another chemiluminescent protein, aequorin (Shimomura et al., 1961). They found that the solution containing GFP was greenish in sunlight, yellowish under tungsten light bulb, and bright green under UV (Shimomura et al., 1961). Further analysis of GFP showed that the emission spectrum peaked at 508 nm wavelength (Johnson et al., 1961). Green fluorescent protein is a 27 kDa monomer made up of 238 amino acid residues in a single polypeptide chain, and it emits its green light by converting the blue chemiluminescent of the Ca^{2+}- sensitive photoprotein, aequorin, into a green light (Chalfie et al., 1994). The active chromophore in GFP is a cyclic tripeptide that becomes fluorescent only after it is embedded within the GFP peptide (Niwa et al., 1996). Wild-type GFP (WT-GFP) absorbs UV with a maximum peak of absorbance at 395 nm and minor peak of 470 nm (Chalfie, 1995). Its maximum emission of green light is at 508 nm with a shoulder of 540 nm (Chalfie, 1995). Green fluorescent protein undergoes photochemical transformation when illuminated. This transformation is what enables scientists to use GFP in the various assays. The outcome of photochemical transformation as a result of illumination for a period of time is either an irreversible photobleaching, change of fluorescent excitation from 488 nm to 406 nm, or conversion of excitation maximum from 395 nm to 475 nm (Elowitz et al., 1997). When a single chromosomal copy of *gfp* gene was used, no fluorescent signal was detected (Errampalli et al., 1999). The number of molecules that are required to visualize a tissue cell is around 10,000 GFP molecules (Errampalli et al., 1999). However, the commercially available variants of GFP, including the red-shifted GFP (RsGFP) are estimated to have a signal that is 20-35 times more intense and photobleach at a slower level at the same excitation peak (Cormack et al., 1996).These variants were generated by introducing a point mutation to the *gfp* sequence (Cormack et al., 1996)

Green fluorescent protein is resistant to a variety of conditions that are frequently used in experimental procedures, including heat, various pH conditions, and several commonly used chemicals. Increasing the temperature from 15 °C to 65 °C only slightly affected the excitation peak of the WT-GFP, and the protein retained 50% of its

fluorescence at 78 °C (Ward and Bokman, 1982). Green fluorescent protein is resistant to alkaline pH, but it could be affected by low pH, as some quenching might occur at pH 5.5 (Patterson et al., 1997). This quenching might reduce illumination, but it does not eliminate it. Green fluorescent protein was proved to resist organic solvents, detergents, and most common proteases (Ward and Bokman, 1982). As well, GFP is resistant to 8M urea solution and to 1% SDS (Chalfie, 1995), which are commonly used in laboratory procedures.

1.11.1. GFP applications:

Green fluorescent protein has become one of the most commonly used tools in research. GFP have been employed by environmental scientist to study the dynamics of *gfp*-tagged bacteria in soil, water, and biofilms (Errampalli et al., 1999). Moller et al. studied the distribution of *gfp*-tagged bacteria in mixed population biofilms (Moller et al., 1998). The experiment was conducted in a flow chamber attached to a scanning confocal microscope that allowed for continuous monitoring of the growing biofilm. The researchers found that *Pseudomonas putida* colonized the outer parts of the biofilm, while *Acinetobacter sp* colonized the inner part only (Moller et al., 1998).

One of the most important applications for GFP is studying the host-pathogen relationship. Green fluorescent protein has been used to study internalized bacteria, genes expressed *in vivo*, and bacterial spread throughout the host. Cho and Kim were able to verify that an environmental isolate of *S. typhi* has a viable but nonculturable (VBNC) state using a *S. typhi* with a *gfp* gene integrated into the chromosome (Cho and Kim, 1999). By comparing the counts of plate-grown *S. typhi* to the counts of fluorescent bacteria in their sample the researchers were able to prove that many viable *S. typhi* will not grow using routine bacterial culture conditions, and therefore even if the bacterium is nonculturable, it is still viable (Cho and Kim, 1999).

Mixter and colleagues used *C. jejuni* transformed with a *gfp*-bearing plasmid to track the bacteria inside the host (Mixter et al., 2003). The fluorescent bacteria were injected into the peritoneum (IP) of BALB/c mice, and after 4 hours the intraperitonial fluids were collected. The fluids were analyzed using flow cytometry analysis, which revealed that most of the bacteria were associated with granulocytes. Using various sets of markers, researchers were able to demonstrate that *C. jejuni* associated mainly with

neutrophils and macrophages (Mixter et al., 2003). Although this experiment used IP injection, which is not the natural route of infection, it still showed that tracking of fluorescent *C. jejuni* inside the host is possible.

Thone et al. used *Salmonella enterica* serovar Typhimurium expressing GFP to study the localization of bacteria in spleenocytes (Thone et al., 2007). The researchers had to screen over a 100 different GFP constructs to find a construct that was expressed strongly enough to be detected by flow cytometry and that emitted a wavelength differentially from the host cell autofluorescence. Using this construct they were able to demonstrate that only a small subset of spleenocytes were highly infected with the majority of the bacteria, while other spleenocytes contained only a few bacterial cells (Thone et al., 2007).

Green fluorescent protein -expressing bacteria are bright enough that they can be visible from outside an animal that is infected with the bacteria. Zhao *et al.* gavaged nude CD-1mice with 1 X 10^{11} CFU of *E. coli* harboring a plasmid that expresses *gfp* (Zhao et al., 2001). Using a light box illuminated by blue light fiber optics, fluorescent bacteria were visible during their replication and spread throughout the intestinal tract. In the same experiment, mice were injected via the IP route and the *E. coli* spread over time was easily tracked without the need to sacrifice the mice to be examined (Zhao et al., 2001).

Miller and colleagues constructed a shuttle vector that encoded the *gfp* gene that was fused to a consensus *Campylobacter* promoter sequence (Miller et al., 2000). *C. jejuni* was transformed with the *gfp* plasmid and tested on a variety of surfaces including plants and chicken skin, and it was also used to infect Caco-2 cells.

From the set of experiments discussed in this section, it can be concluded that GFP has a variety of applications in biological sciences and especially bacterial pathogenesis and interaction with host cells.

1.12. Summary:

C. jejuni continues to be a global challenge that can affect humans of all ages. Poultry, especially broiler chickens, are the main source of human infections. The control of infection in broiler chickens should dramatically affect the level of campylobacteriosis in humans. Based on current literature, vaccination remains the most feasible approach for control of *C. jejuni* spread in chickens.

Although the immune system is able to limit the disease outcome, the details of cells and signalling molecules involved in combating *C. jejuni* infections are yet to be determined.

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Chapter II: Hypothesis, Objectives, and Specific Aims of This Study

2.1. Hypothesis:

It is our hypothesis that flagella are vital to colonization of the chicken intestine by *C. jejuni*, and this is mediated by proteins secreted by the flagellar export apparatus which is involved directly in colonization.

2.2. Objective:

The pathogenesis of *C. jejuni* in broiler chickens is poorly understood. The overall objective of this project is to understand the role of flagella and *Campylobacter* invasion antigens in mucosal and systemic colonization, and to evaluate the vaccine potential of *C. jejuni* paralyzed flagella mutants.

2.3. Specific aims:

Based on the hypothesis and objectives stated above, the specific aims of this study are:

1. Determine the mucosal and systemic sites(s) of colonization of *C. jejuni* using green fluorescent protein (GFP).
2. Evaluate the ability of *C. jejuni* mutants to colonize broiler chickens and BALB/c mice.
3. Test the vaccine potential of a paralyzed flagella *C. jejuni* mutant.
4. Evaluate chicken macrophage inflammatory response to *C. jejuni* flagella and secreted protein mutants.

Chapter III: General Materials and Methods

3.1. Introduction:

The purpose of this chapter is to describe the bacterial strains and laboratory methods that were common to several experiments and therefore avoid repetition.

3.2. *Campylobacter jejuni* strains:

Strain *C. jejuni* NCTC11168V1 has a NCTC11168 genetic background and was originally isolated from a case of human enteritis in 1977 (Ahmed et al., 2002). The genome of this strain was fully sequenced in 2000 (Parkhill et al., 2000). This strain does not contain any plasmids.

Strain *C. jejuni* NCTC11168V26 has the same genetic background as NCTC11168V1but has been passaged for an undetermined number of times over the years resulting a variation in gene profile expression, lowered *in vitro* invasion, decreased ability to colonize chickens, and a limited ability to spread from an infected chicken to non-infected chicken (Carrillo et al., 2004).

Strain *C. jejuni* NCTC11168*flgK* has an NCTC11168 background and an insertion mutation in *flgK* gene, which encodes the hook-flagellin junction (Fernando et al., 2007). This mutation allows the expression of flagellar structure up to the hook, resulting in a truncated flagellum, and prevents secretion of *Campylobacter* invasion antigens (Fernando et al., 2007).

Strain *C. jejuni* 81-176 has a well studied genetic background and was originally isolated in 1981 from 9-year-old girl's feces who consumed raw milk on a field trip to a diary farm (Black et al., 1988). Human volunteers receiving *C. jejuni* 81-176 passaged in the lab have suffered from gastroenteritis (Black et al., 1988). The genome of this strain has been sequenced and published (Hofreuter et al., 2006). It contains two plasmids, pVir and pTet (Bacon et al., 2000). This strain has been proved to be virulent in ferrets (Yao et al., 1997) and Rhesus macaques (Islam et al., 2006). Colostrum-deprived piglets infected with *C. jejuni* 81-176 developed a bloody diarrhea that was virulent enough to force

Naikare and colleagues to euthanize the animals after 3 days of challenge (Naikare et al., 2006). Strains *C. jejuni* K2-37 and K2-55 have an 81-176 genetic background, but contain an insertion mutation in *pflA* gene, that allows them to express full length flagella that are paralyzed (Yao et al., 1994), resulting in non-motile strains.

Bacterial strains used in the set of experiments described in this thesis are listed in Table 3.1.

3.3. *Campylobacter* storage and growth conditions:

The source for *C. jejuni* strains NCTC11168V1, NCTC11168V26, and 81-176 was minimally-passaged freezer stock courtesy of Dr. Brenda Allan (VIDO, Saskatchewan). For each strain used, 10 µl from the frozen stock was spread on 5% Mueller-Hinton agar supplemented with 5% sheep blood (MHA-Blood) (PML Microbiological, Richmond, BC) and grown for 24-48 hours under microaerophilic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) at 42 °C. The growing colonies were re-streaked on MHA-Blood using a sterile cotton swab and grown for 16-18 hours to produce confluent growth. The bacteria were harvested and resuspended using Mueller-Hinton broth (MHB). Sterile glycerol was added to make up 25% of the final solution. The *C. jejuni* stock was stored at -70 °C until used.

C. jejuni strains were routinely cultured on Mueller-Hinton agar (MHA), (Difco), or MHA-B under microaerophilic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) at 42 °C unless otherwise stated.

3.4. *C. jejuni* cultured on bi-phasic media:

For several laboratory experiments, *C. jejuni* was grown in bi-phasic culture that offers the nutrients and dynamics of a broth culture and the support and attachment from agar. *C. jejuni* was grown from frozen stock as described above. Each plate was harvested using 5 ml MHB and transferred into 5 ml sterile test tube. Bacterial harvest was well-suspended by pipetting several times, and 250-500 µl were transferred to an MHA plate covered with 2.5-3ml MHB.

Name	Description	Reference
***C. jejuni* strains**		
NCTC11168V1	A variant of the sequenced NCTC11168, colonizes chickens efficiently although it is isolated from a human enteritis case.	(Ahmed et al., 2002; Parkhill et al., 2000).
NCTC11168V26	A variant of NCTC11168 that colonizes chickens poorly and has a limited expression of flagella and other genes.	(Carrillo et al., 2004).
NCTC11168*flgk*	Has an insertion mutation in *flgK* gene, expresses the flagellar structure up to the hook. Does not secret *Campylobacter* invasion antigens	(Fernando et al., 2007).
81-176	Isolated from a human source and caused gastroenteritis in human volunteers. The genome has been sequenced.	(Black et al., 1988; Hofreuter et al., 2006).
K2-37	Has an 81-176 genetic background, but contains an insertion mutation in *pflA* gene, expresses full length flagella but lacks motility.	(Yao et al., 1994).
K2-55	Another 81-176 *pflA* mutant that expresses full length flagella but lacks motility.	
***E. coli* strains**		
DH5α	Laboratory strain used for hosting plasmids and as a helper strain in tri-parental transformation.	(Miller et al., 2000).
EC2576	DH5α genetic background carrying plasmid AF2925555 encoding *gfp* gene	(Miller et al., 2000).
EC1690	DH5α genetic background carrying plasmid pHG101	This study
***Salmonella* strains**		
Salmonella enterica serovar Typhimurium strain SL1344	A well studied strain that has been used in a number of studies and vaccine development. Used in this study as a positive control for TLR5	(Gulig and Curtiss, 1987)

Table 3.1: Bacterial strains used in this study. This table lists *C. jejuni, E. coli,* and *Salmonella* strains used in this study, with a brief description of their main characteristics.

The mixture was well-suspended by pipetting 3-5 times and the plate was incubated upright under microaerophilic conditions at 42 °C for 14-16 hours.

3.5. Karmali selective medium:

Karmali selective medium is based on a formula described by Karmali et. al. (Karmali et al., 1986) The medium is blood-free, and it is developed specifically for isolation of *C. jejuni* and *C. coli*. The agar base contains activated charcoal, Columbia agar base, and a small amount of haemin.To prepare 500 ml of Karmali agar plates 21.5 g of *Campylobacter* agar base (Karmali) from Oxoid (Cat # CM0935) (Nepean, ON) were dissolved in ddH_2O, brought to boil, and sterilized by autoclaving at 121 °C for 15 min. The medium was allowed to cool down to ~50 °C before the addition of 1 vial of *Campylobacter* selective supplement SR0167E (Oxoid) reconstituted in 2 ml of 1:1 ethanol: sterile distilled water, which contains 50 mg of sodium pyruvate, 16 mg of cefoperazone, 10 mg of vancomycin, and 50 mg of cycloheximide. Medium was mixed well and then poured into Petri dishes and allowed to cool and solidify and were stored at 4 °C until needed.

3.6. Determination the number of *C. jejuni* colony-forming units (CFU) per gram of cecal contents:

Both ceca were collected aseptically from each chicken and placed in a sterile Petri dish (VWR) labeled with bird and group numbers. Each cecum was opened from the blind end and the cecal contents were emptied into a pre-weighed disposable 25 ml plastic tube with a flat bottom. The amount of cecal contents collected ranged from 0.2-0.4 g. To each sample X10 the weight of fridge-cold normal saline was added and the contents were vortexed until well-suspended. From each sample dilutions of 10^1-10^6 were made in normal saline. From each dilution 25 µl were plated on 1/3 of the surface area of a Karmali selective agar plate yielding a total of two plates per bird. Plates were incubated at 42 °C for 48 hours under microaerophilic conditions. Generally dilutions with 5-200 colonies were counted unless less than 5 colonies grew in 10^1 dilution.

3.7. Polymerase Chain Reaction (PCR) primers:

Primers were designed and analyzed using Clone Manager Professional Suite for optimization of annealing temperature, GC content, exclusion of self-complementarily and primer dimers. The specificity of the designed oligonucleotides was tested by BLAST searches for short, nearly exact matches at http://www.ncbi.nlm.nih.gov/BLAST/. All primers used were purchased from Invitrogen Canada (Burlington, ON). Before initial usage, primers were resuspended to a concentration of 10µM/µl. A working solution of 1 µM/µl was made and used in PCR and qRT-PCR reactions. All resuspended primers were stored at -20 °C after each use.

3.8. Broiler Chickens:

All broiler chickens used in the various studies were purchased from a local commercial source (Lilydale hatcheries, Wynyard, SK), and were delivered to VIDO Animal Care facility on the morning of day of hatch. All experiments were carried out according to the guidelines of Canadian Council on Animal Care and University of Saskatchewan Committee on Animal Care and Supply.

3.9. Preparation of *C. jejuni* samples for Electron Microscopy:

An electron microscope (EM) was used to obtain images of *C. jejuni* paralyzed flagella mutant to confirm the presence of full length flagella and the absence of any visible structural changes. Overnight cultures of *C. jejuni* 81-176, K2-37, and K2-55 were harvested and subcultured using bi-phasic media. To minimize viable but nonculturable (VBNC) form of *C. jejuni*, the bacteria were grown for no more than 14 hours and 5 ml of MHB were used instead of 3. Bacterial cells were harvested using 5 ml normal saline and collected into 14 ml sterile test tubes which were kept on ice. The samples were processed immediately. A few drops of bacterial broth were placed on the surface of a sterile Petri dish. A copper grid covered with Formvar film and coated with carbon (SPI-Chem, West Chester, PA) was placed on top of the bacteria suspension and allowed to float freely for 3-5 minutes to allow for the bacteria to adhere. The grids were stained with 1% (w/v) ammonium molybdate for 1 min and were allowed to dry before examination with a Philips-410 electron microscope.

3.10. Preparation of tissue samples for EM:

Freshly collected tissues from liver, spleen, and ceca were immediately placed in 1.5% glutaraldehyde (SEGMA, Oakville, Ontario) to avoid drying out. Samples were cut into small pieces using a surgical carbon steel blade (VWR, Mississauga, ON), with each piece less than 1 mm thick allowing for complete penetration by fixative. Samples were fixed using 1.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 3 hours at 4 °C, and washed 3 times (once every hour) following fixation using 0.1M sodium cacodylate buffer. Postfixing of samples was achieved by incubation in 1% OsO_4 in 1.25% sodium bicarbonate buffer for 1 hour at room temperature. Samples were dehydrated and stained at the same time with a series of steps that started with 50% ethanol for 5 min, 70% ethanol saturated with uranyl acetate for 1 hour, 70% ethanol only for 5 min, 95% ethanol for 5 min, and finally 3 washes with 100% ethanol for 5 min each. Samples were submitted to the EM laboratory (Veterinary Biomedical Sciences Department, Western College of Veterinary Medicine) for molding and trimming. Samples were examined using a Philips-410 electron microscope.

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Chapter IV: Colonization of *Campylobacter jejuni* in Broiler Chickens

4.1. Introduction

Poultry-borne transmission of *C. jejuni* remains a major public health concern in Canada (Thomas et al., 2008a), The United States of America (Cardinale et al., 2003), and The European Union (Wagenaar et al., 2006). *C. jejuni* infections are not limited to the developed world, as campylobacteriosis is widespread in developing countries with a significant contribution to mortality in infants and diarrhea in adults (Coker et al., 2002). The number of confirmed *C. jejuni* cases has increased steadily since the establishment of laboratory techniques to isolate the bacteria from clinical samples (WHO, 2001) and reached an estimated 400 million infections each year worldwide (Girard et al., 2006). The main clinical manifestation of *C. jejuni* is a varying degree of diarrhea and abdominal cramps that usually ceases after 3 days, but it is not unusual for the abdominal cramps to occur after the diarrhea had stopped. Although most cases of *C. jejuni* infection do not require hospitalization and are usually self-resolved, an autoimmune disease known as Guillain–Barré syndrome (GBS) is the most serious sequel caused by *C. jejuni* (Rhodes and Tattersfield, 1982; Yuki, 2001). The disease manifests itself as descending or ascending paralysis and depending on the severity of the condition, GBS patients might be hospitalized, especially if the respiratory muscles are involved. If managed properly, most patients recover under good medical supervision (Douglas and Winer, 2006).

Broiler chickens display no clinical signs when they are colonized with *C. jejuni* despite the heavy carriage of the bacteria in their ceca (up to 10^{10} CFU per gram of cecal content). The ability of the organism to colonize chickens varies greatly from strain to strain, with as little as 40 CFU of chicken-passaged *C. jejuni* able to cause maximal colonization under experimental conditions (Cawthraw et al., 1996). Other studies have reported 5 X 10^4 CFU are required for two-week old broiler chickens to be colonized (Ringoir et al., 2007). Under field conditions, *C. jejuni* is not detected in newly hatched chicks, and is only detected after 2-3 weeks of placement in a poultry barn (Newell and Fearnley, 2003). Horizontal transfer from other poultry and the environment is probably the main source for infection (Wagenaar et al., 2006).

Epidemiological studies have projected that reducing the shedding of *C. jejuni* by broiler chickens will reduce the occurrence of disease in humans, and the most effective approach is vaccination (Wagenaar et al., 2006). A basic understanding of *C. jejuni* interaction with its host is required to identify the best vaccine candidates and mechanisms of immunity required for protection. Studies conducted in chickens have mainly focused on colonization of the intestinal tract with limited investigation of systemic spread. These studies have identified some of the antigens that are involved in intestinal tract colonization process including CadF (Ziprin et al., 2001) and flagellin (Fernando et al., 2007). It has been established that *C. jejuni* is mainly associated with intestinal mucus layer, does not adhere to the intestinal cells, and have a limited systemic spread (Beery et al., 1988). Cox *et al*. found that *C. jejuni* spreads quickly to several lymphoid organs but was not detected by culture after 1 day of infection (Cox et al., 2006).

Negative results using standard procedures for isolation of *Campylobacter* from clinical samples does not necessarily prove that the sample was *Campylobacter* free, as these procedures have a minimal detection level that could be as high as 10^2 CFU/ml of intestinal content (Beery et al., 1988). The physiological changes that *C. jejuni* undergoes might be significant in the ability to recover the bacteria, as Watson and Galan were able to recover *C. jejuni* from samples that were negative using standard laboratory microaerophilic conditions by incubating the culture under oxygen-limiting conditions (Watson and Galan, 2008). Researchers postulate that the intracellular conditions *C. jejuni* encounters during its infection might lead to this physiological shift of the bacteria.

The objective of experiments described in this chapter was to study the mucosal and systemic sites of *C. jejuni* colonization. To achieve this objective, an alternative method for standard laboratory culture to study *C. jejuni* pathogenesis in broiler chicken was introduced. Transformation of a plasmid expressing Green Fluorescent Protein (GFP) into *C. jejuni* enabled *in vivo* visualization of the bacteria in various tissues under the fluorescent microscope.

4.2. Materials and Methods:

4.2.1. Bacterial strains:

C. jejuni NCTC1168V1and *C. jejuni* NCTC1168V26 have been described in General Material and Methods (chapter III). Two *E. coli* strains were used in this study: DH5α, a laboratory strain that does not contain any plasmids and strain EC2576 (DH5α background) which contains plasmid AF2925555 that expresses the *gfp* reporter system (described below).

4.2.2. Bacterial growth conditions:

E. coli strains were stored in Luria-Bertani (LB) broth containing 25% glycerol at -70℃. Prior to use, 50 µl of frozen stock were inoculated into the appropiate broth and grown overnight with shaking at 37 °C. To determine bacterial counts in the overnight culture, duplicate 1ml aliqouts of the broth were diluted 10-fold from 10^{-1} to 10^{-6} and plated on LB agar and incubated overnight at 37 °C. Colony counts on plates with 30-300 colonies were used to calculate the bacterial concentration.

4.2.3. Plasmids:

Plasmid AF292555 was a generous gift from Dr. William W. Miller (U.S. Department of Agriculture, Albany, California) (Miller et al., 2000). This plasmid contained the *Campylobacter* plasmid mobility element (*mob*), *Campylobacter* plasmid origin of replication, ColE1 origin of replication, and *Campylobacter* kanamycin resistance gene (antibiotic marker), and promoter site that were used in constructing the *gfp* reporter system (pHG101).

4.2.4. Plasmid purification and storage:

Plasmids were purified using Qiagen QIAprep Miniprep kit (Cat# 27104 Qiagen Inc, Mississauga, ON) according to the manufacturer instructions using an Eppendorf 5417C tabletop centrifuge. Plasmid DNA was eluted in 50 µl of RNase-DNase-free water distilled water. To determine DNA purity and integrity, a 2 µl sample was analyzed using 1% agarose gel electrophoresis. DNA concentration in the sample was determined using spectrophotometric analyses (UltraSpec 3000, Pharmacia Biotech, Sweden). Samples were diluted 1:100, and for samples with OD_{260}/OD_{280} ranging between 1.8-2.0 and DNA concentration was determined using the formula OD_{260} = OD X 50 ng/µl X dilution factor. Purified DNA was stored at -70 °C until used.

4.2.5. Polymerase Chain Reaction (PCR):

The PCR mixture consisted of 170 µl of sterile water, 5 µl of each of the four dNTP's (Fermentas, Burlington, ON), 10X reaction buffer (supplied with dNTP's and contains 750 mM Tris-HCl (pH 8.8 at 25 °C), 200 mM $(NH_4)_2SO_4$ and 0.1% (v/v) Tween 20), and 5U of Taq polymerase (Fermentas). Each individual reaction contained 43 µl of reaction mixture, 2.5 µl of each primer (at 10 pmoles/µl), and 2 µl DNA were dispensed into a sterile PCR tube. Tubes were placed in Applied BioSystems 2720 thermal cycler (Applied BioSystems , Streetsville, ON), and parameters were set as described in General Materials and Methods chapter. Primers were designed and prepared as described in General Materials and Methods chapter and are listed in Table 4.1.

4.2.6. DNA digestion and ligation:

DNA digestion enzymes were purchased from New England BioLabs (Pickering, ON). Up to 1 µg of DNA was digested per reaction. The reaction mixture contained 7.5 µl of dd H_2O, 1 µl of DNA, 1 µl of reaction buffer (supplied with the enzyme), and up to 1 µl of digesting enzyme. Reactions were incubated at 37 °C for 2-4 hours. Cleavage of the DNA substrate with two enzymes simultaneously was also performed using the same reaction conditions, but including two compatible enzymes.

DNA was ligated using Rapid DNA ligation Kit (Fermentas). Each ligation mixture contained 3:1 ratio of vector:insert DNA, rapid ligation buffer, water and T4 DNA ligase. The mixture was spun and allowed to sit at room temperature for 5 minutes. For each transformation 2 µl were used, and the rest of ligation mixture was stored at -20 °C for future use.

4.2.7. Preparation of chemically competent cells and heat-shock transformation:

A stock of frozen chemically competent *E. coli* DH5α cells was prepared by plating a loopful of the bacteria on an LB plate which was incubated overnight at 37 °C. A single colony was then picked and inoculated in 2 ml of LB broth overnight with shaking at 200 rpm/minute. The culture was transferred into 500 ml 2X YT broth in a 2 liter flask and incubated at 37 °C with shaking until a 0.5 absorbance at 600 nm wavelength ($O.D._{600} = 0.5$) was obtained. The culture was transferred into pre-chilled 250 ml sterile centrifuge tubes and were placed on ice for 10 minutes. Cells were pelleted

at 2000 X *g* at 4 °C for 10 minutes. The supernatant was removed completely and 80 ml of sterile RF-I solution (100 mM KCl, 50 mM $MnCl_2$, 30 mM Potassium acetate, 10 mM calcium chloride and 15% glycerol) were added and pellet was resuspended and allowed to incubate on ice for 30 minutes. The cells were centrifuged as above for 10 minutes, and the supernatant was removed. The pellet was resuspended in 4 ml of sterile RF-II solution (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, and 15% glycerol at pH 6.8) and was incubated on ice for 15 minutes. Aliqouts of 100 µl were dispensed into pre-chilled 1.5 ml Eppendorf tubes and stored at -70 °C until used.

For transformation, one Eppendorf tube containing frozen cells was thawed on ice, and ligation mixture prepared as described above was added. The mix was incubated on ice for 5 minutes then transferred to a water bath set at 42 °C for 1 minute. The tube was then incubated on ice for 3 minutes. A 100 µl of LB broth was added, and 15 µl were spread on an LB plate supplemented with kanamycin (LB-KM) at a concentration of 200µl/ml. Plates were incubated at 37 °C O/N and observed for any colony growth. Colonies that grew were analyzed using whole-cell PCR or miniprep to confirm the transformation of the plasmid.

4.2.8. Whole-cell PCR:

This technique was used to confirm the presence of *gfp* gene sequence in the colonies growing on LB-KM plates. The side of the colony of interest was picked using a sterile toothpick. The bacteria carried on a toothpick were transferred to 100 µl of sterile RNase/DNase-free water (SIGMA, Oakville, ON) in 1.5 ml Eppendorf tube. The water was boiled for 5 min in Thermolyne Dri bath (Dubuque, Iowa), followed by incubation on ice for 3 minutes. Two µl of boiled bacteria were transferred to a PCR mix prepared as described above. Primers used in the reaction are described in Table 1. The PCR reaction was carried out as follows: hot denaturation at 96 °C for 1 minute, then 25 cycles of denaturation for 30 sec at 95 °C, annealing for 30 sec at 60 °C, and extension for one minute and fifity seconds at 68 °C. A final extension step lasted 5 minutes at 68 °C

4.2.9. Screening for spontaneous Streptomycin-resistant *C. jejuni* mutants:

To obtain a streptomycin-resistant isolate, a freshly grown *C. jejuni* was harvested in 5 ml MHB. The harvested bacteria were centrifuged at 2500 X *g* and resuspended to a

concentration of 10^8-10^9 CFU/ml *C. jejuni*, plated on MHA-SM plates and incubated at 42 °C for 24-48 hours. Colonies growing were restreaked twice to confirm streptomycin resistance of the isolate.

4.2.10. Construction of *gfp* reporter system:

A fragment containing a *Campylobacter* plasmid origin of replication, ColE1 origin of replication, *Campylobacter* plasmid mobility element (*mob*), and *Campylobacter* kanamycin resistance gene was obtained from plasmid AF292555 (Fig1). To obtain a *gfp* gene that is constitutively expressed, a primer containing *Campylobacter* consensus promoter sequence described by Wosten et al (Wosten et al., 1998) (Table 1), ribosomal binding site sequence (Miller et al., 2000), and 18 bp necessary to amplify *gfp* from plasmid pQBI-25(GFP) from Quantum Biotechnologies (Montreal, QC). Reaction conditions used were the same as described above with the exception of that the annealing temperature was 42 °C. The PCR products amplified with the primers were analyzed using agarose gel electrophoresis to confirm band size and purity. To purify the PCR product, 3µl of amplified PCR products were separated on 1% agarose gel and purified using UltraClean 15 kit (MO Bio Labs, Montreal, QC) silica binding particle method according to the manufacturer instructions.

To construct the final plasmid that was transformed into *E. coli*, purified plasmid AF292555 and purified PCR product were digested with enzymes EcoRI and AatII as described above. The reaction mixture was separated on 1% agarose and purified with UltraClean 15 cleaning kit. The resulting two DNA fragments were ligated and transformed into *E. coli* DH5α as described above. The transformation mixture was plated on LB-KM plates and incubated at 37 °C for 24 hours. Colonies were screened by whole-cell PCR. Colonies that contained the correct size PCR band were selected for sequencing for confirmation. Primers used for sequencing are listed in Table 4.1 and maps of plasmids AF292555 and pQBI-25 are illustrated in Fig 4.1. A frozen stock was made for future studies as described above. The isolate used for the remaining experiments was called EC1690.

4.2.11. Transformation of *C. jejuni* NCTC11168V1 and V26 with plasmid pHG101:

This protocol has been modified from Miller *et al.* (Miller et al., 2000). Three strains were involved in the process of transformation: *C. jejuni* (streptomycin-resistant), DH5α (helper, containing no plasmids), and EC1690 (containing plasmid pHG101). *C. jejuni* was freshly grown from frozen as described above, restreaked on MHA supplemented with streptomycin and allowed to grow for 9 hours. *E. coli* DH5α and EC1690 were grown in a 5 ml broth overnight. Strains were mixed in a 1:1:10 ratio (donor:helper:recipient) in a sterile 1.5 ml Eppendorf tube and spotted in 10µl mating spots on blood agar. Mating spots were harvested after 24 hours. *E. coli* cells were removed by centrifugation for 40 seconds at 3000 X *g*. Supernatant was plated on MHA plates amended with kanamycin and streptomycin. Plates were incubated for 24-48 hours. Fluorescent *C. jejuni* colonies were detected by UV light. Colonies were restreaked on MHA supplemented with streptomycin and kanamycin. Frozen stock was prepared as described above. The plasmid was extracted by miniprep extraction and complete transformation of plasmid was confirmed by digestion with enzymes EcoRI and AatII, and by sequencing using primers listed in Table 4.1. The isolate selected for future experiments was named *C. jejuni* NCTC11168V1G.

4.2.12. Electro-transformation of *C. jejuni* with pHG101:

One plate of *C. jejuni* was grown overnight from frozen stock. Growing bacteria were restreaked onto 3 blood agar plates with a sterile cotton swab to obtain confluent growth. Plates were incubated overnight and bacteria were harvested with 800 µl electroporation buffer (15% glycerol /9% sucrose) and centrifuged for 1.5 min at 9000 X *g*. Supernatant was decanted and cells were resuspended in 800 µl of the same buffer and centrifuged again. Supernatant was decanted and pellet was resuspended in 125 µl of the same buffer and kept on ice. For every 40 µl of cells 1 µg of plasmid DNA was added and mixed. The mix was transferred into a pre-chilled electroporation cuvette and cells were pulsed with 1.25 kV with a constant range of 4-6 mS in Gene Pulser xcell (BioRad) electroporater. Immediately 500 µl of SOC media was added to the cuvette and mixed well. Cells were plated on MHA-KM and incubated at 37 °C. Transformation of the bacteria was confirmed by whole-cell PCR, enzyme digestion, and sequencing.

4.2.13. Stability of transformed pHG101 in *C. jejuni in vitro*:

Primer	Sequence (5'→ 3')
GFP-Fwd	AATTGAATTCGGCTTATTCCCTAACTAACTAAAGATTAACTTTATA AGGAGGAAAAACATGGCTAGCAAAGGAGAAGAACTCTTCACTGG
GFP-Rev	ACGTGACGTCAGTTGTACAGTTCATCCATGCCATGTGT
1 FWD	AAGCTTGCATGCCTGCAGGTCG
1 REV	CAAGGAAGATGGCAACATTCTG
2 FWD	ACAAATAGGGGTTCCGCGCACA
2 REV	GAATGTTGCCATCTTCCTTGAA

Table 4.1. Primers used for amplification, colony PCR, and sequencing of plasmid pHG101. GFP-FWD and GFP-Rev are the sequences of primers used for amplification of *gfp* gene from pQBI-25(GFP). The forward primer contained a ribosome binding site sequence and the starting sequence of *gfp* gene and overhanging site for AatII restriction enzyme. The reverse primer contained the necessary sequence for amplification at low annealing temperature and overhang sequence for digestion enzyme EcoRI. The rest of the primers in the table were used for sequencing of pHG101. Primers were designed to span a terminal part of the gene and the vector.
FWD: forward, Rev: reverse, GFP: green fluorescent protein.

To determine the stability of plasmid pHG101 in the absence of selective antibiotic pressure, freshly grown NCTC11168V1G and NCTC11168V26G were restreaked on Karmali selective agar plates and Karmali agar plates supplemented with kanamycin. Agar plates were incubated for 24 hours before bacteria were harvested in PBS and centrifuged at 9000 X *g* for 5 minutes. The pellet was resuspended to an absorbance of 0.1 at $O.D_{600}$, and 25 µl were subcultured on corresponding plates and incubated for 24 hours. The ratio of fluorescent *C. jejuni* growing on antibiotic free Karmali agar plates to kanamycin-supplemented Karmali plates was determined. The same process was repeated for 10 consecutive subcultures. A ratio of one would indicate that the plasmid is stable.

4.2.14. Stability of transformed pHG101 in NCTC11168V1G and NCTC11168V26G *in vivo*:

The stability of plasmid pHG101 is critical in detecting *C. jejuni in vivo* using fluorescence microscopy since the bacterium that loses the plasmid will not be detected using fluorescence microscopy. *In vivo* stability of plasmid pHG101 was tested by challenging 21-day old broiler chickens orally and testing for fluorescent bacteria in chickens. Two groups of 21-day old broiler chickens, each consisting of 12 birds, received a dose of 1×10^9 CFU/0.5 ml of *C. jejuni*. Group one received NCTC11168V1G, and group two received NCTC11168V26G. Cloacal swabs were collected on daily bases for the duration of the experiment. On day 7, chickens were euthanized and cecal contents were collected aseptically. Samples were serially diluted as described in General Materials and Methods chapter, and duplicate 25 µl were plated from each dilution on Karmali and Karmali agar supplemented with kanamycin. Plates were incubated for 48 hours at 42 °C under microaerophilic conditions. The ratio of *C. jejuni* growing in the absence of antibiotic to the ratio of *C. jejuni* growing in the presence of antibiotic was determined by dividing colony counts of respective dilutions. A ratio of 1 indicated that the plasmid is stable *in vivo*.

4.2.15. Colonization of *C. jejuni* NCTC11168V1G and NCTC11168V26G in broiler chickens:

To study colonization and systemic spread of *C. jejuni* in broiler chickens, two groups of 14 chickens were housed in VIDO animal care. Birds were *C. jejuni*-free when tested upon arrival for presence of *C. jejuni* using cloacal swabs. On day 21, each bird in group one received 1 X 10^9 CFU of *C. jejuni* NCTC11168V1G suspended in 0.5 ml 0.85% NaCl orally, while birds in group two received 1 X 10^9 CFU/0.5 ml *C. jejuni* NCTC11168V26G. After 24 hours 2 birds from each group were euthanized by cervical dislocation, and tissue samples were collected aseptically from crop, gizzard, small intestine, large intestine, cecum, liver, and spleen. Each sample was placed in a separate sterile Petri dish and immediately processed. Tissues were cut using a surgical carbon steel blade (VWR, Mississauga, ON) to produce small pieces of 1-3 mM X 3-5 mM thickness and were placed in an individual Cryomold (VWR) that had a thin film of Sakura Finetek Tissue-Tek Optimal Cutting Temperature media (OCT) (VWR) covering the bottom, and were covered completely with OCT and placed immediately either in liquid nitrogen for 90 second or -80 °C freezer for 20 minutes. A cloacal swab was dipped into the cecal contents and streaked on Karmali agar plate to confirm the presence of *C. jejuni* by culture. The same sampling procedure was repeated every 24 hours for the remaining birds. All samples were stored at -20 °C until processed.

Tissue sections were cut in a cryostat (Microm HM505E, Thermo Scientific, Nepean, ON) at 5-10 μm thickness depending on tissue type. The optimal cutting temperature varied depending on tissue type, generally liver, spleen, crop, and gizzard were cut at -12 °C and the remainder of the tissues were cut at -20 °C. Tissue sections were transferred to room-temperature SuperFrost Excell adhesion microscopic slides (VWR). Each slide contained 2-3 sections. From each tissue sample 9 sections in total were made. Slides were air-dried for 15 minutes, washed by dipping in PBS (+4 °C) for 3 times, air-dried for additional 10 minutes, and fixed in acetone at 4 °C for 1 hr. Tissues were either covered directly by cover slip and sealed with nail polish, or mounted using Fluoromount (Pleaston, CA) or Cytoseal 60 low viscosity mounting medium (Richard-Allan Scientific, Kalamazoo, MI) that does not interfere with fluorescence. Slides were stored in a closed box at -20 °C until inspected with fluorescent microscope.

Slides were studied using fluorescent microscope (Carl Zeiss Inc) using light transmission and fluorescein isothiocyanate (FITC) filters. Each tissue section was

visualized using 4X, 20X, 40X, and 100 X magnifications. If any section contained fluorescent *C. jejuni*, the sample was considered positive. If *C. jejuni* was not found in any of the 9 sections, the sample was considered negative (Tables 2 and 3).

Tissue samples collected from four 14-day old *C. jejuni*-free broiler chickens were used as negative controls. For positive controls, cecal contents collected from broiler chickens positive for *C. jejuni* NCTC11168V1G by culture were resuspended in normal saline 2 X weight, embedded in OCT media, frozen, and treated as tissue samples.

4.2.16. Confocal microscopy:

Samples for confocal microscopy (Leica Microsystems) were prepared as per fluorescence microscopy, only Fluoromount mounting media was used for slide preparation to avoid any distortion in image sharpness (Mathiesen, 1973).

4.2.17. Electron Microscopy:

Tissue samples collected on day 4 from a bird challenged with *C. jejuni* NCTC11168V1G and another challenged with *C. jejuni* NCTC11168V26G were processed for electron microscope imaging in addition to fluorescence microscopy. Selected tissues from the cecum, spleen, and liver were processed as described in General Materials and Methods chapter and visualized using electron microscopy.

4.2.18. Immunohistochemistry (IHC):

To confirm the presence of *C. jejuni* in positive tissues, and to verify that negative tissues did not contain non-fluorescent *C. jejuni*, IHC was performed on several tissue samples from birds sampled on different days that were either positive or negative for presence of *C. jejuni* by culture. Biotinylated secondary antibody (Vectastain Elite ABC kit, VectorLabs, QC) was used to detect the attachment of an anti- *C. jejuni* NCTC11168V1 polyclonal antibody (primary antibody) generated in rabbits (a generous gift from Dr. Brenda Allan, VIDO) to *C. jejuni* in tissues. Tissues to be tested were sectioned at 10 μm thickness, mounted on microscopic slides, and allowed to air dry for 15 minutes then immediately fixed in acetone at 4 °C for 1 hour. Sections were rinsed for 5 minutes in 0.1 M PBS, and treated with 3% hydrogen peroxide for 3 minutes to quench any endogenous peroxidase activity. Slides were washed with a gentle stream of PBS

from a wash bottle, and incubated in PBS that contained 2% normal goat serum to eliminate any background staining. Sections were incubated with rabbit anti-*C. jejuni* NCTC11168V1 polyclonal antibody that was diluted 1:100 for 4 hours at 4 °C. Samples were washed with PBS and incubated for 10 minutes with biotinylated secondary antibody followed by a wash with buffer. Sections were incubated with VectaStain ABC reagent for 5 minutes and washed with PBS. Peroxidase substrate 3, 3'-diaminobenzidine (DAB) was prepared according to the manufacturer instructions (VectorLabs, QC) and incubated with sections for 4 minutes to allow for color development. *C. jejuni* appeared brownish in color if present in the section.

4.3. Results:

4.3.1. Construction of *gfp* reporter system (pHG101):

Construction of a plasmid containing *gfp* gene was a critical step for *C. jejuni* visualization *in vivo*. The *gfp* gene was amplified using primers listed in Table 4.1, digested with AaTII and EcoRI and ligated to the plasmid backbone derived from plasmid AF292555 that was digested with the same restriction enzymes. The constructed plasmid contained a strong constitutively expressed promoter (Pc), *Campylobacter repB* gene required for plasmid replication, *mob* genes required for plasmid mobility, and *Campylobacter* KM resistance gene (*aphA*) (Fig 4.1). After ligation, plasmid pHG101 was transformed into *E. coli* DH5α using electroporation. Transformation was confirmed by whole-cell PCR (Fig 4.2), restriction enzyme digestion (Fig 4.2), and sequencing.

Streptomycin-resistant *C. jejuni* NCTC11168V1 and *C. jejuni* NCTC11168V26 were obtained by screening for spontaneous resistant mutants using minimally passaged colonies. Once streptomycin-resistant isolates were obtained, the resistance was confirmed by subculturing two times on MHA supplemented with streptomycin.

The plasmid was transferred into streptomycin-resistant *C. jejuni* 11168V1 by a tri-parental mating using 1:1:10 donor:helper:recipient ratio. Colonies growing on MHA supplemented with streptomycin and kanamycin after 48 hours of incubation were tested for fluorescence directly under a UV lamp, and the presence of the desired plasmid was confirmed using whole-cell PCR, restriction enzyme digestion, and sequencing. Colonies growing on MHA supplemented with streptomycin-kanamycin were green-creamy in color under room light and fluorescent under UV illumination. Some of the *C. jejuni* colonies became fluorescent only after subculture for one time on MHA supplemented with streptomycin and kanamycin. Most of the colonies were found to contain the complete plasmid pHG101 as confirmed by sequencing.

Plasmid pHG101 was purified from *C. jejuni* NCTC11168V1G and electro-transformed into streptomycin-resistant *C. jejuni* NCTC11168V26. Colonies were confirmed to contain pHG101 using whole-cell PCR, restriction enzyme digestion, and sequencing (Fig 4.2).

C. jejuni NCTC11168V1G and *C. jejuni* NCTC11168V26G showed various shapes and forms when viewed using fluorescent and electron microscopy (Fig 4.3). Both

strains showed the typical *C. jejuni* spiral shape, and the viable but not-culturable form. Both strains were fluorescent regardless of their form. *C. jejuni* fluorescence faded gradually when the cells were exposed to UV illumination for more than 10 minutes. This process is known as photobleaching (Cormack et al., 1996).

4.3.2. Stability of pHG101 *in vitro*:

To determine the stability of plasmid pHG101 in the absence of antibiotic selective pressure, the ratio of fluorescent *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC1168V26G colonies growing on Karmali agar to fluorescent colonies growing on Karmali agar supplemented with kanamycin was calculated. Both strains had a ratio of ~1 after 10 subcultures (Fig 4.4), indicating the stability of pHG101in the absence of kanamycin, and the suitability of the plasmid for the *in vivo* experiment.

4.3.3. Stability of pHG101 *in vivo*:

One of the main objectives of this study was to develop a reliable system for tracking *C. jejuni in vivo*. The stability of plasmid pHG101in *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G was tested in broiler chickens. *C. jejuni* colonies cultured from cloacal swabs and cecal contents on Karmali agar plates were green in color when examined directly under room light and fluorescent under UV illumination. The level of *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G detected in the ceca after euthanization were similar to levels reported previously in the literature for the parent strains *C. jejuni* 11168V1 and V26 (Fig 4.5 and 4.6) (Carrillo et al., 2004). The ratio of colonies growing on Karmali agar to colonies growing on Karmali agar supplemented with kanamycin was approximately one (Fig 4.5), indicating that the plasmid is stable in both strains *in vivo*.

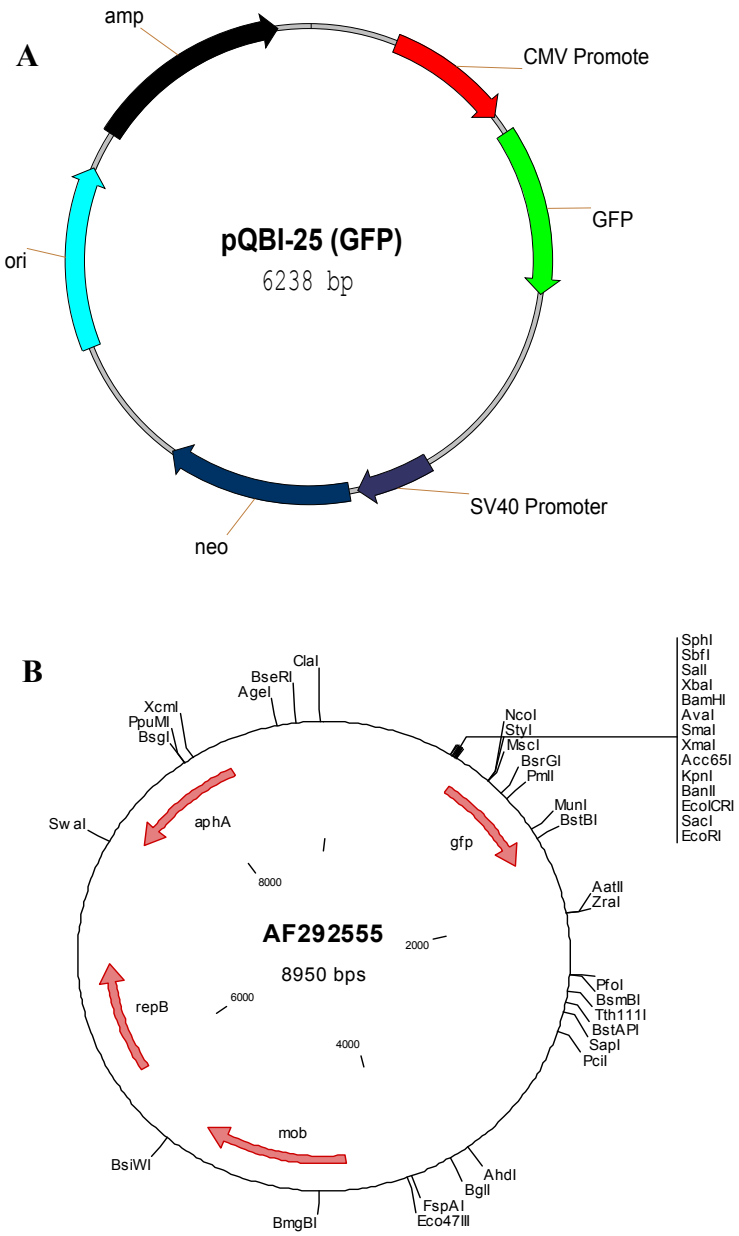

Fig 4.1. Plasmids used in construction of pHG101 (A) Map of pQBI-25 GFP plasmid, the source for *gfp* gene used in the final construct. A forward primer containing *Campylobacter* consensus sequence and 18 bp of *gfp* sequence was used to amplify the *gfp* gene. The reverse primer only amplified *gfp* gene. No other elements from this plasmid were amplified. (B) Structural map of plasmid AF292555 (8.9 kb). Plasmid contained *Campylobacter* mobility element (*mob*), *repB* gene required for plasmid replication, constitutively expressed promoter attached to *gfp* gene, and kanamycin resistance gene (*aphA*).

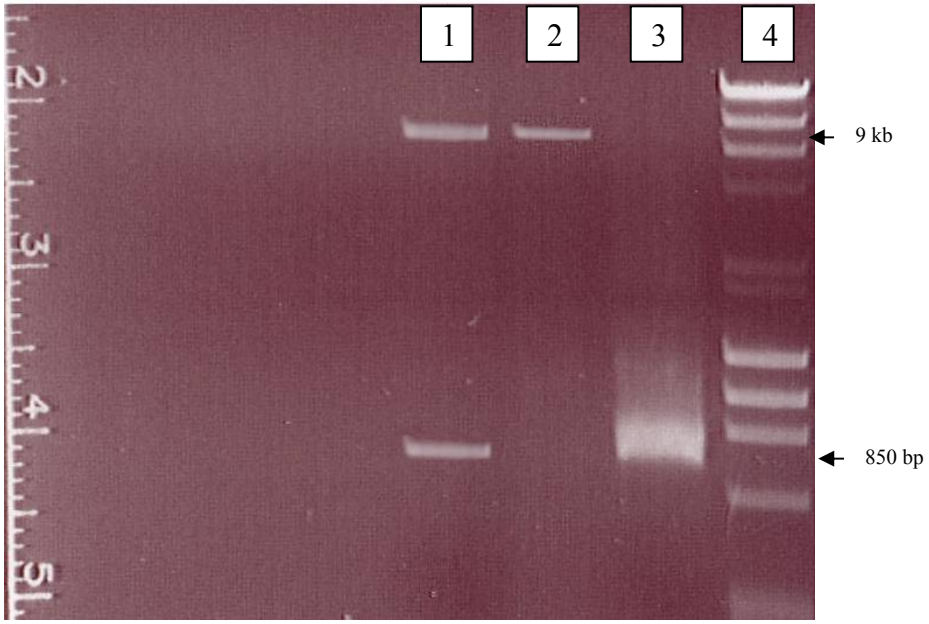

Fig 4.2. Confirmation of pHG101 size using restriction enzyme digestion. The final plasmid pHG101 was obtained by ligation of two DNA segments, the backbone from plasmid AF292555 was digested using EcoRI and AatII (lane 2). The *gfp* gene was amplified using primers listed in Table 4.1, extracted from agarose gel, and digested with the same restriction enzymes (lane 3). Growing colonies were screened for pHG101 and confirmation of the correct plasmid size was obtained by restriction analysis (lane 1). DrigestIII DNA marker was used to determine band size (lane 4)

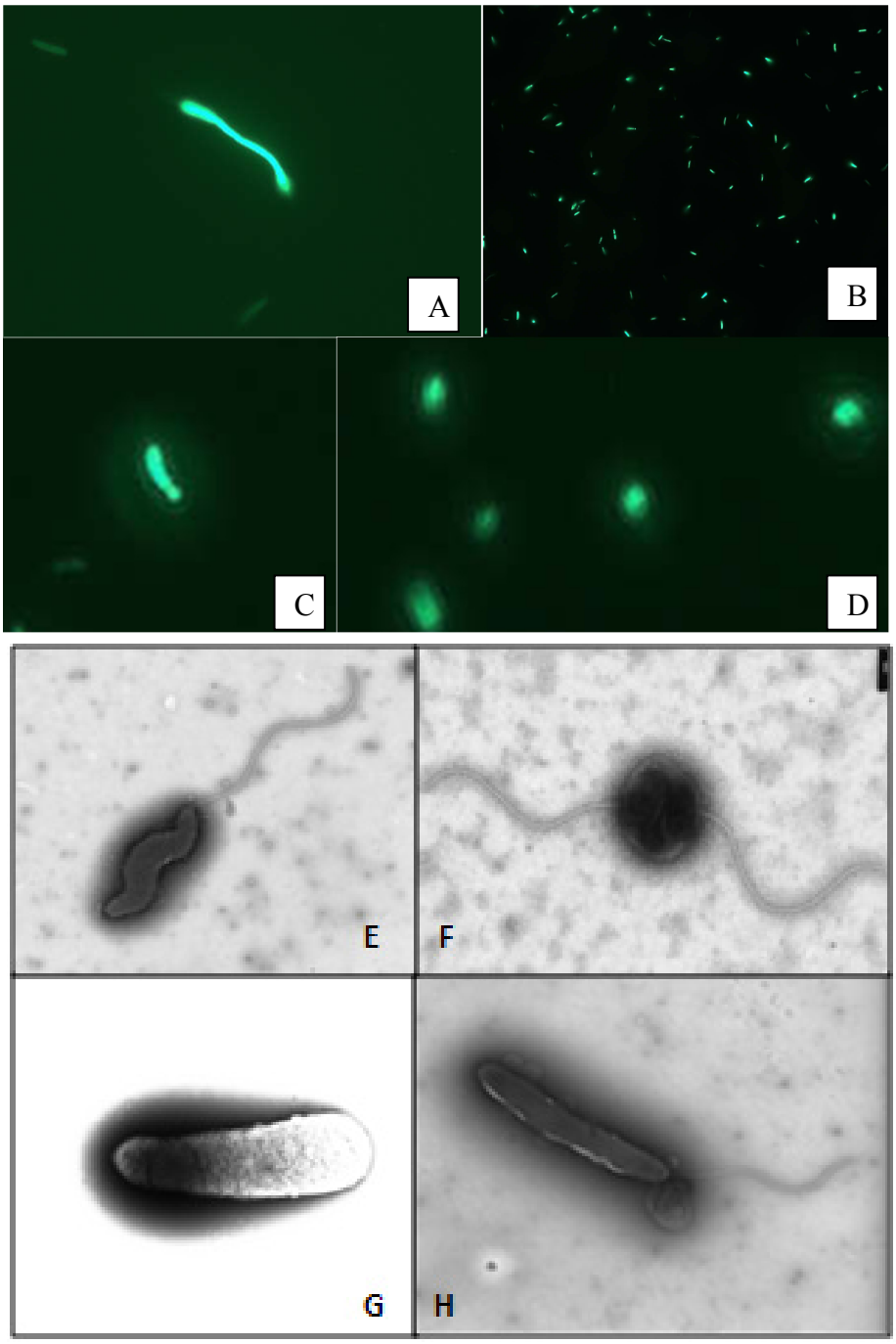

Fig 4.3. Various shapes and forms of *C. jejuni* visualized by microscopy. All samples were prepared from a culture grown overnight except (D) which was prepared form 48 hours old culture. (A) *C. jejuni* NCTC11168V1G 1,000x, (B) Wet-mount preparation of NCTC11168V1G under fluorescent microscope 630x, (C) non-typical NCTC11168V1G in the same sample 630x, (D) NCTC11168V1G in an aging bacterial culture 1000x, (E) typical NCTC11168V1G as seen under TEM 18,000x, (F) and (G) non-typical appearance of NCTC11168V1 from the same sample of (E), (G) is similar in appearance to (C) 18,000x, (H) NCTC11168V26G expressing a unipolar flagellum.

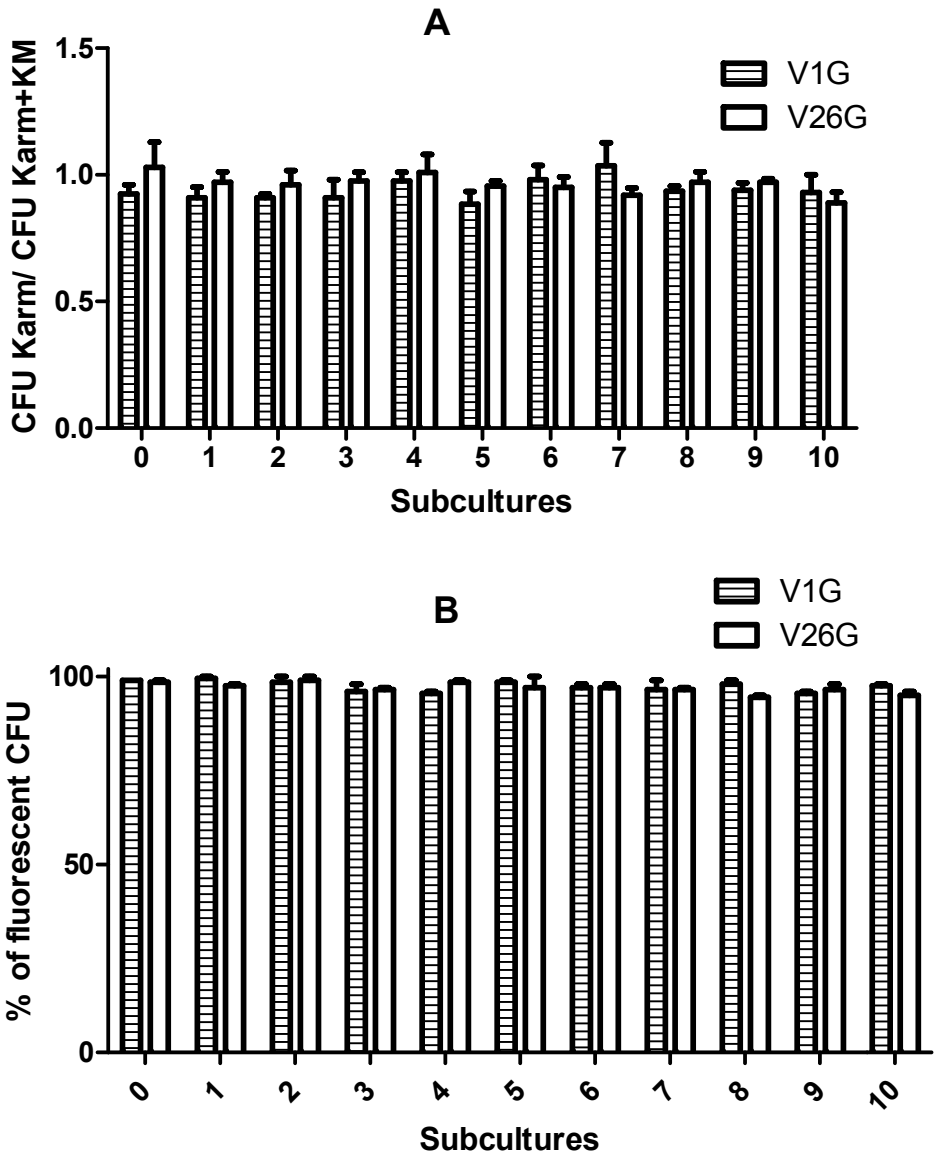

Fig 4.4. Stability of plasmid pHG101 in *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G *in vitro*. (A) Ratio of *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G growing on Karmali agar to the same strains growing on Karmali agar supplemented with kanamycin after subculture. Subculture 0 is the ratio of cells growing on Karmali agar. Bacterial growth was not affected by the presence or absence of antibiotic as growth ratio was ~1 after 10 subcultures. (B) Plasmid stability was determined by dividing the ratio of fluorescent *C. jejuni* growing on antibiotic free Karmali agar plates to Karmali agar plates supplemented with kanamycin. Each set of subcultures was repeated twice. Both strains maintained a fluorescence ratio above 94%. The same process was repeated for 10 consecutive subcultures. V1G: *C. jejuni* NCTC11168V1G, V26G: *C. jejuni* NCTC11168V26G.

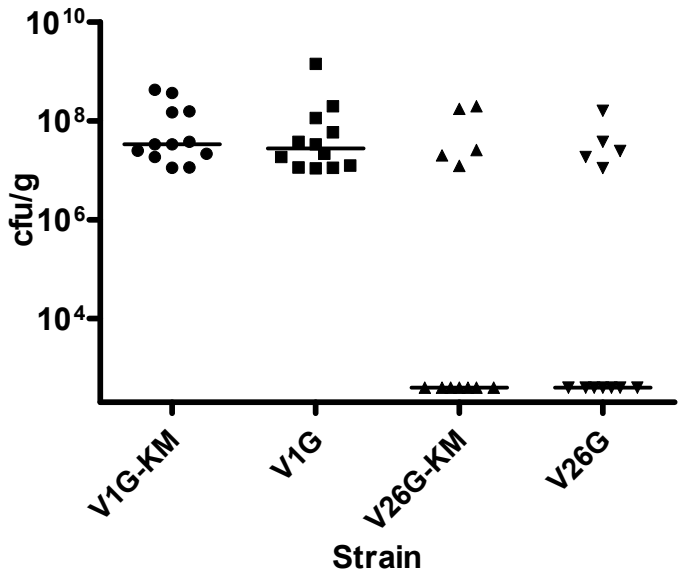

Fig 4.5. Stability of plasmid pHG101 *in vivo*. To determine stability of pHG101 two groups of 21-day old broiler chickens, each consisting of 12 birds, received a dose of 1 X 10^9 CFU/0.5ml in 0.85% NaCl of *C. jejuni* NCTC11168V1G or NCTC11168V26G orally. On day 7 post-challenge, chickens were euthanized and cecal contents were collected aseptically from ceca. Samples were serially diluted and duplicates of 25 µl were plated from each dilution on Karmali and Karmali-KM. Plates were incubated for 48 hours at 42 °C under microaerophilic conditions. The ratio of *C. jejuni* growing in the presence of antibiotic to the ratio of *C. jejuni* growing in the absence of antibiotic was determined by dividing colony counts of respective dilutions. A ratio of 1 indicated that the plasmid is stable *in vivo*. All the ceca of birds challenged with *C. jejuni* NCTC11168V1G were colonized, while only 5 birds were colonized with *C. jejuni* NCTC11168 V26G. Birds that had no *C. jejuni* detected by culture were assigned a value of 400.
V1G: *C. jejuni* NCTC11168V1G growing on Karmali agar plates, V26G: *C. jejuni* NCTC11168V26G growing on Karmali agar plates, V1G-KM: growth on Karmali agar plates supplemented with kanamycin, V26G-KM: growth on Karmali agar plates supplemented with kanamycin.

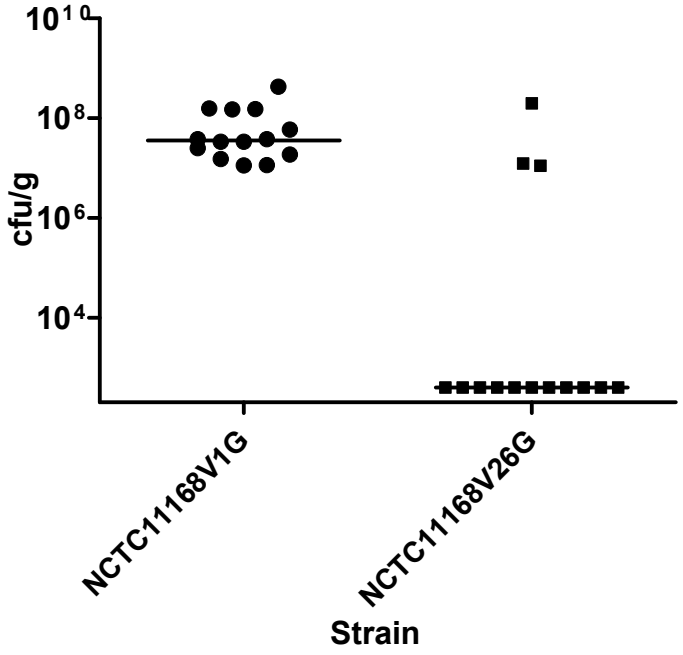

Fig 4.6. Colonization of broiler chickens by *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G. Two groups of 14 broiler chickens were challenged with 1 X 10^9 CFU suspended in 0.5 ml 0.85% NaCl of either *C. jejuni* NCTC11168V1G or *C. jejuni* NCTC11168V26G at day 21 of age. Every 24 hours following the infection two birds from each group were euthanized and tissue samples were collected aseptically including the ceca. Cecal contents were processed as described in the text and samples were plated on Karmali selective agar plates. All the ceca of birds challenged with *C. jejuni* NCTC11168V1G were colonized, while only 3 birds were colonized with *C. jejuni* NCTC11168V26G. Birds not shedding *C. jejuni* were assigned the lowest detection level possible (400).

4.3.4. Colonization of *C. jejuni* NCTC11168V1G and NCTC11168V26G in broiler chickens:

To detect the systemic dissemination of *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G in broiler chickens tissues samples were collected from crop, gizzard, small and large intestine, ceca, spleen, and liver every 24 hours after the challenge. Samples were preserved by freezing in OCT embedding media and studied using fluorescent microscope, confocal microscope, and electron microscope. All the ceca of chickens challenged with *C. jejuni* NCTC11168V1G were colonized for the duration of the experiment as detected by culture of cloacal swabs and tissue sections (Table 4.2), while only the ceca of 3 chickens were colonized by *C. jejuni* NCTC11168V26G (Fig 4.6 and Table 4.3). Fluorescent *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G were visible in the ceca of chickens sampled 24 hours after challenge (Table 4.2 and 4.3), and resided in close proximity with the intestinal surface. No bacteria were detected in layers beneath the mucosal surface. The large intestine of the same 3 birds that had *C. jejuni* NCTC11168V26G visible in the ceca contained the fluorescent bacteria, which appeared as early as 24 hours post-challenge. *C. jejuni* NCTC11168V26G was not detected in any crop, gizzard, spleen, liver, or small intestine of the challenged chickens for the duration of the experiment (Table 4.2 and Fig 4.7).

C. jejuni NCTC11168V1G was detected in the small intestine, liver, and spleen only 3 days post-challenge (Table 4.2), but only in 29%, 35.7%, 42.9% of the total samples respectively. Large intestines were colonized in 64.3% of the samples and were colonized as early as 24 hours post-challenge. Bacteria were found either in the crypts or the lumen of the small and large intestine, but not in any layers beneath the mucosal surface. In the liver and spleen, bacteria appeared to be mainly intracellular, which was confirmed using IHC (Fig 4.8).

	Crop	Gizzard	Small intestine	Large intestine	Cecum	Liver	Spleen	Swab
Day 1								
Bird 1	-	-	-	+	+	-	-	+
Bird 2	-	-	-	-	+	-	-	+
Day 2								
Bird 3	-	-	-	-	+	-	-	+
Bird 4	-	-	-	+	+	-	-	+
Day 3								
Bird 5	-	-	+	-	+	+	+	+
Bird 6	-	-	+	+	+	-	+	+
Day 4								
Bird 7	-	-	+	+	+	+	+	+
Bird 8	-	-	-	-	+	+	-	+
Day 5								
Bird 9	-	-	-	+	+	+	+	+
Bird 10	-	-	-	+	+	-	+	+
Day 6								
Bird 11	-	-	-	-	+	-	-	+
Bird 12	-	-	+	+	+	+	+	+
Day 7								
Bird 13	-	-	-	+	+	-	-	+
Bird 14	-	-	-	+	+	-	-	+

Table 4.2. Colonization of *C. jejuni* NCTC11168V1G in various tissues of broiler chickens over the period of 7 days. A total of 14 birds were challenged with 1 X 10^9 CFU orally. Tissue samples were collected from gizzard, crop, small and large intestine, ceca, liver, and spleen from two birds every 24 hours following the challenge. Bacteria were detected using fluorescent microscope. Tissues were embedded in OCT frozen section media and sectioned at 3-5 µm thickness. (-) No fluorescent bacteria detected in any of the 9 sections made from the tissue sample, (+) bacteria detected in any of the 9 sections made from the tissue sample. No bacteria were detected in Crop or gizzard for 7 days or in small intestine on days 1, 2, 5, and 7. Ceca and large intestine were colonized from day one, while spleen and liver were colonized as of day 3.

	Crop	*Gizzard*	*Large intestine*	*Small intestine*	*Cecum*	*Liver*	*Spleen*	*Swab*
Day 1								
Bird 1	-	-	+	-	+	-	-	+
Bird 2	-	-	-	-	-	-	-	-
Day 2								
Bird 3	-	-	+	-	+	-	-	+
Bird 4	-	-	-	-	-	-	-	-
Day 3								
Bird 5	-	-	-	-	-	-	-	-
Bird 6	-	-	+	-	+	-	-	+
Day 4								
Bird 7	-	-	-	-	-	-	-	-
Bird 8	-	-	-	-	-	-	-	-
Day 5								
Bird 9	-	-	-	-	-	-	-	-
Bird 10	-	-	-	-	-	-	-	-
Day 6								
Bird 11	-	-	-	-	-	-	-	-
Bird 12	-	-	-	-	-	-	-	-
Day 7								
Bird 13	-	-	-	-	-	-	-	-
Bird 14	-	-	-	-	-	-	-	-

Table 4.3. Colonization of *C. jejuni* NCTC11168V26G in tissues of broiler chickens over the period of 7 days. A total of 14 birds were challenged with 1 X 10^9 CFU orally. Tissue samples were collected from Gizzard, crop, small and large intestine, ceca, liver, and spleen from two birds every 24 hours following the challenge. *C. jejuni* was detected using fluorescent microscope. Tissues were embedded in OCT frozen section media and sectioned at 3-5 µm thickness. (-) No fluorescent bacteria detected in any of the 9 sections made from the tissue sample, (+) bacteria detected in any of the 9 sections made from the tissue sample. No bacteria were detected in Crop, gizzard, small intestine, spleen. No fluorescent bacteria were detected in ceca sampled on day 4 onward.

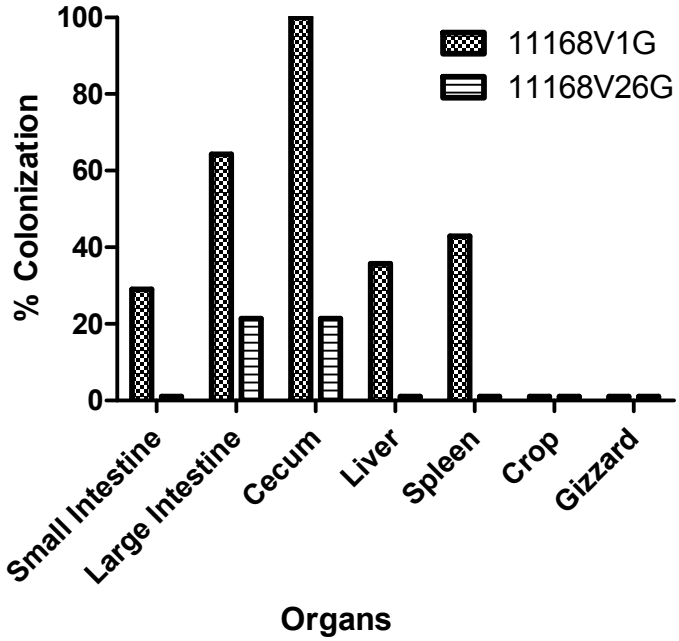

Fig 4.7. Systemic spread of *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G in broiler chickens. Two groups each conssiting of 14 birds were challenged with 1 X 10^9 CFU orally with either of the strains. Tissue samples were collected from Gizzard, crop, small and large intestine, ceca, liver, and spleen from two birds every 24 hours following the challenge. Bacteria were detected using fluorescent microscope. *C. jejuni* was absent from crop and gizzard of birds challenged with both strains, and from the spleen, liver, and small intestine of *C. jejuni* NCTC11168V26G-challenged birds. All the ceca of *C. jejuni* NCTC11168V1G-challenged birds were colonized, while only 64.3%, 42.9%, and 35.7% of large intestine, spleen, and liver were colonized respectively.

11168V1G: *C. jejuni* NCTC11168V1G, 11168V26G: *C. jejuni* NCTC11168V26G

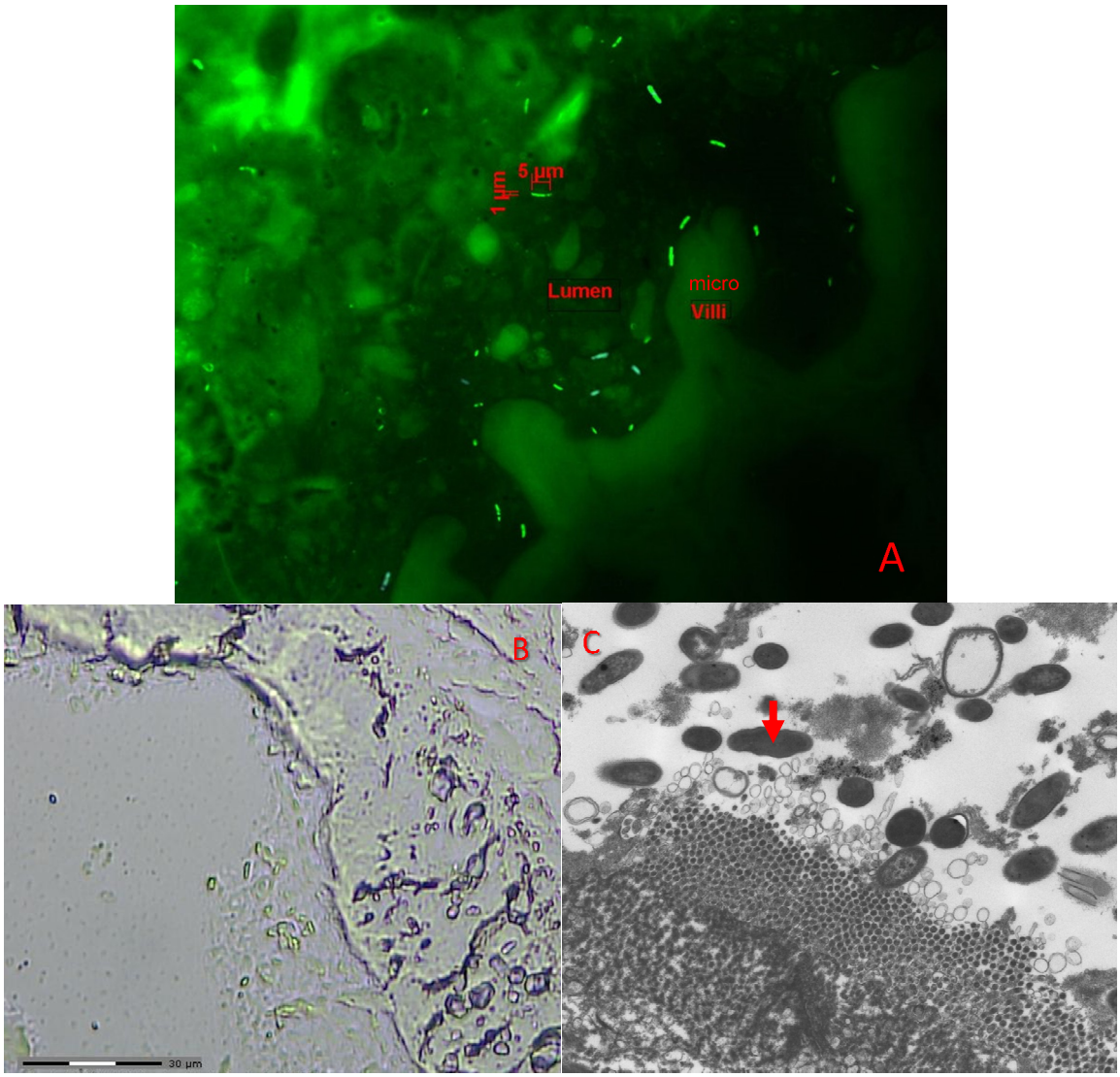

Fig 4.8. Presence of fluorescent *C. jejuni* in the ceca of broiler chickens. (A) Fluorescent *C. jejuni* NCTC11168V1G in the cecum of a broiler chicken 3 days post-challenge detected using fluorescent microscope. Bacteria were associated with mucus layer and floating in the lumen 1000x. (B) Another section from the same tissue sample used in (A) visualized using light transmission, the bacteria appears to be swimming freely in the lumen and not attaching to the surface 1000x. (C) *C. jejuni* NCTC11168V26G (arrow) in the cecum of a broiler chicken 3 days post-challenge as detected by transmission electron microscope 18,000x. Bacteria were associated mainly with the mucus.

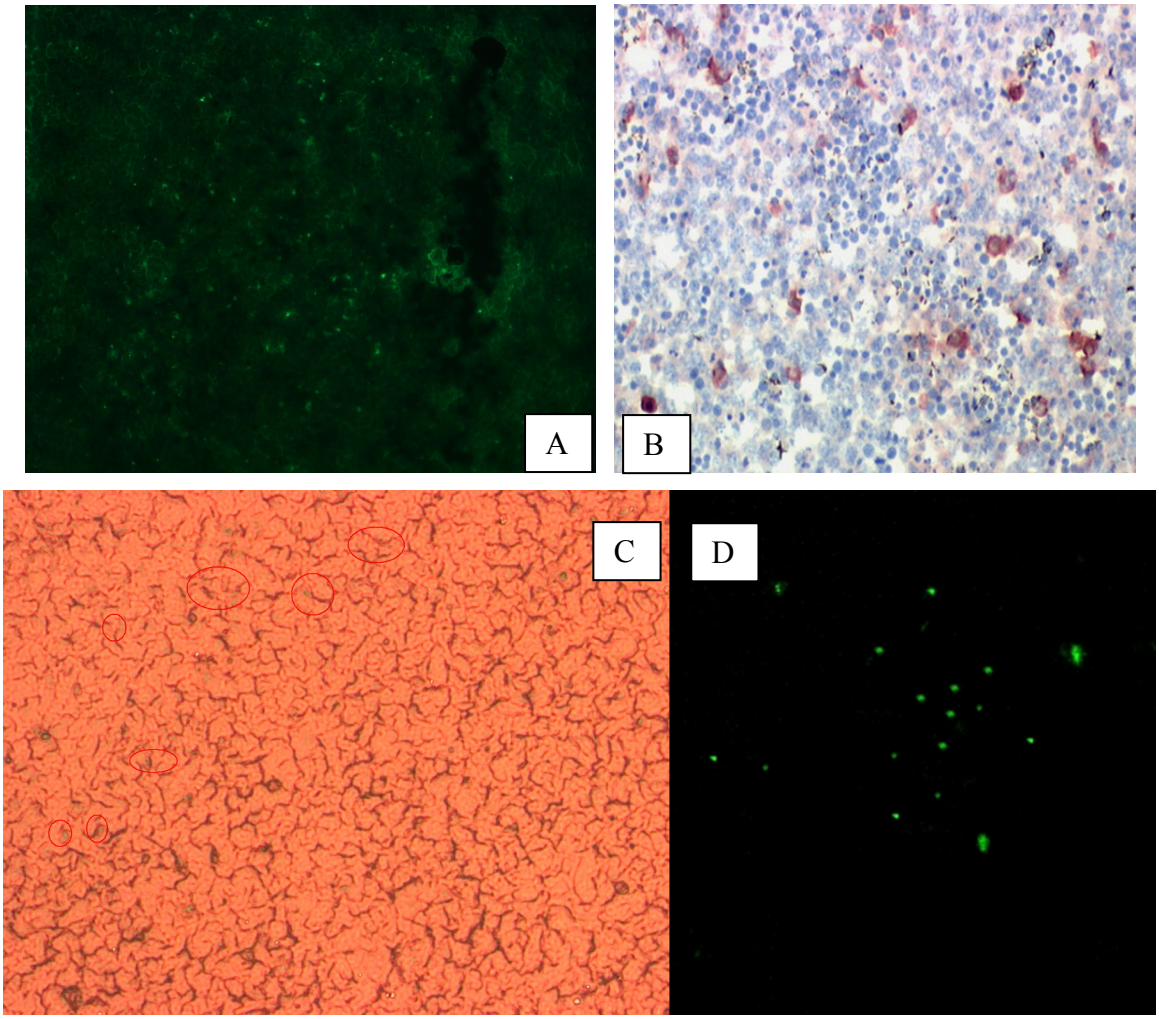

Fig 4.9. Fluorescent *C. jejuni* NCTC11168V1G in the spleen of broiler chickens. (A) *C. jejuni* in the spleen of a broiler chicken 5 days post-challenge. Bacteria appeared to be mainly intra-cellular, with some spleenocytes or macrophages containing more than bacterium 630X. (B) Immunohistochemical detection of *C. jejuni* using polyclonal antibody generated in rabbits 1000X (C) Light transmission microscope of a another section of tissue sample in (A) with some of the bacteria visible 1000X. (D) Laser scanning confocal microscope of another section from the same tissue sample used in (A). Background has been completely eliminated and the fluorescent spots present are *C. jejuni* NCTC11168V1G.

4.4. Discussion:

Despite the important role broiler chickens play in transmitting disease to humans, little is known about *C. jejuni* colonization and systemic spread. Early studies have focused mainly on the gastrointestinal tract and detected no presence in the liver or a limited presence in the spleen (Beery et al., 1988). Other studies that have used standard bacterial culture detected the systemic spread of *C. jejuni* beyond the gastrointestinal tract (Cox et al., 2006; Van Deun et al., 2008). Some of the findings in these studies were controversial, as *C. jejuni* was detected in the thymus, ceca, spleen, liver, intestine, and bursa of fabricius only 1 hour post oral challenge with 10^5-10^6 CFU (Cox et al., 2006). This is highly unlikely, as the bacteria have to disseminate systemically and reach lymphoid organs in numbers high enough to be detected using traditional bacterial culture within 1 hour.

Theunique autofluorescence characteristic green fluorescent protein using only ultra violet light without any requirement for an exogenous substrate makes it an ideal tool for detection of *C. jejuni in vitro* and *in vivo*. The presence of a strong promoter (P_c) that is insensitive to regulatory signals and constitutively expressed when fused to the *gfp* gene resulted in very brightly fluorescent *C. jejuni* cells both *in vitro* (Fig 4.4) and *in vivo* (Fig 4.8). The Pc sequence has been predicted by Wosten and colleagues (Wosten et al., 1998) and has been used successfully by Miller et al to detect *C. jejuni* on several plant and skin surfaces and in Caco-2 cells (Miller et al., 2000). The plasmid pHG101 was very stable both *in vitro* after several passages, and *in vivo* 7 days post-challenge. This level of stability made plasmid pHG101 ideal for tracking the bacteria *in vivo* over the course of several days. The absence of kanamycin did not affect stability (Fig 4.5), and this allowed for an experimental design that does not require the treatment of birds with antibiotics that might alter the intestinal microflora. Miller et al. reported that a plasmid that encoded a *gfp* gene was very stable *in vitro* in several *C. jejuni* strains, but no data were presented regarding stability in animals except that *C. jejuni* isolated from the ceca of challenged birds was fluorescent (Miller et al., 2000). Green fluorescent protein has been used successfully to detect intestinal colonization of *Lactobacillus delbrueckii* ssp. *lactis* D17 (Yu et al., 2007). The plasmid carrying the *gfp* gene were stable *in vitro* after 10 subcultures and GFP was fully expressed, while the fluorescent

bacteria was present in high numbers (up to 1 X 10^7 CFU/g of cecal contents) *in vivo* after 72 hours of the challenge (Yu et al., 2007). Yu and colleagues were able to detect fluorescent *Lactobacillus* in tissue sections of gastrointestinal tract, but unable to detect any in spleen or liver using fluorescent microscope (Yu et al., 2007). *C. jejuni* NCTC11168V1G and *C. jejuni* NCTC11168V26G fluorescence was not affected by tissue processing procedures or depletion of nutrients from the growth medium. Using the same protocol intended for tissues collected for histopathology, including freezing, fixation with acetone, and mounting; the two strains remained fluorescent at the end of the process (Fig 4.3, 4.8, and 4.7). Taken together, the stability of the plasmid and the fluorescence were adequate for using this system to track *C. jejuni in vivo*.

In this study, *C. jejuni* NCTC11168V1G was detected in the ceca and large intestine 24 hours post-challenge, but was not detected in the liver or spleen samples collected at 24 or 48 hours post-challenge (Table 4.1). *C. jejuni* NCTC11168V26G was not detected in the liver, spleen, or small intestine of any of the birds sampled (Table 4.2). This might be a direct result of the reduced ability to colonize the ceca and large intestine, as *C. jejuni* NCTC1168V26G was detected only in 21.4% of ceca and large intestine sampled (Fig 4.7), while *C. jejuni* NCTC1168V1G colonized 100% of the ceca of challenged birds. Birds colonized with *C. jejuni* NCTC11168V26G had a 1.5 X 10^7 CFU/g of cecal contents recovered, while birds colonized with *C. jejuni* NCTC11168V1G had a slightly higher mean of 8 X 10^7 CFU/g of cecal contents. These finding are linked to previous reports in literature that wild-type *C. jejuni* NCTC11168V26 required a high dose (10^8 CFU) to colonize day-old chicks, and had a poor bird-to-bird transmission rate that did not exceed 5% (Carrillo et al., 2004), which demonstrates an overall reduced virulence compared to *C. jejuni* NCTC11168V1 and *C. jejuni* NCTC11168V1G.

In this study *C. jejuni* preferably colonized the lumen and mucus layer of ceca and large intestine. Most the fluorescent bacteria detected in the intestinal tract of challenged birds were either in the lumen or closely associated with the mucus layer, with no direct observation of fluorescent bacteria in the submucusal layers. Some bacteria were closely associated with the mucosal surface (Fig 4.8), which is an important step in establishing infection and crossing intestinal barrier to establish systemic infection. It is difficult to

rule-out that *C. jejuni* crosses the intestinal barrier since the bacteria have been detected in spleen and liver in this study and other reports in the literature (Barot et al., 1983; Fernandez and Pison, 1996; Forbes et al., 2009; Whyte et al., 2006). This systemic spreading is likely to be initiated in the ceca or large intestine where *C. jejuni* colonizes in larger numbers. Other researchers have also found that *C. jejuni* colonization of the intestine is mainly associated with mucosal layer and could be rarely detected in the deeper mucosal layers (Beery et al., 1988; Smith et al., 2008; Van Deun et al., 2008).

C. jejuni NCTC11168V1G spreads systemically beyond the gastrointestinal tract as it was detected in both spleen and liver of 42.9% and 35.7% of the samples examined respectively. All of the birds that had *C. jejuni* in the liver or spleen had their ceca colonized but not necessarily their large intestine (Tables 4.2 and 4.3), which emphasizes the role the ceca are playing as a foothold in establishing *C. jejuni* infection. Almost all of the birds that have *C. jejuni* positive livers also had the bacteria in their spleen, with the exception of one bird. The detection of systemic spreading of *C. jejuni* using the system developed in this study is generally in agreement with studies that used bacterial culture to detect the presence of *C. jejuni* in liver and spleen. Meade and colleagues were not able to detect any *C. jejuni* in spleen and liver samples collected at 6 hours post-infection with 1 X 10^{10} CFU using bacterial cultures (Meade et al., 2009). Only one bird had detectable *C. jejuni* in liver and spleen 20 hours post infection (Meade et al., 2009). After 48 hours, 62.5% of birds challenged had *C. jejuni* in their liver, and only 25% in their spleen (Meade et al., 2009). The systemic spread of *C. jejuni* is probably facilitated by cells that can carry it to a secondary lymphoid organ. The spleen in poultry has limited hematopoietic functions and consists mainly of lymphoid aggregations (macrophages and heterophils) (Jeurissen, 1991). It is considered a secondary lymphoid organ to which the local lymphatic system drains. The cecal tonsils consist of lymphoid aggregations that play a role in antigen sampling and antimicrobial peptide secretion (Akbari et al., 2008; Haghighi et al., 2008), and are located at the proximal part of the cecum. The continuous presence of *C. jejuni* in this area increases the chances of interaction with macrophages present in the cecal tonsils, which might be transferring the bacteria to the spleen.

Another possibility for how *C. jejuni* spread arises from a recent observation by Smith and colleagues who observed an influx of heterophils in the ceca of birds infected

with *C. jejuni* without any signs of pathology (Smith et al., 2008). *C. jejuni* might be able to use any of the cells attracted to the site of infection as a vehicle to spread systemically. These cells will home to a secondary lymphoid organ, which is probably the spleen, given the high percentage of birds that contained *C. jejuni* in their spleens.

This study has demonstrated that GFP is appropriate for studying *C. jejuni* localization in various tissues if the gene is carried on a stable plasmid. Tissue processing and microscopy require more labor and special equipment compared to traditional culture, but fluorescent tags are particularly useful in pathogenesis and co-infection studies (wild-type vs. mutant), as different fluorescence tags can be used to track each bacteria. Another disadvantage for GFP use is that tissue sections exposed to UV light will photobleach, and eventually lose their fluorescence, limiting the ability to archive the sections. Our study has demonstrated that the GFP-encoding plasmid prepared did not interfere with colonization or invasion and systemic spreading, which confirms the finding by Clark and colleagues who demonstrated that the expression of GFP itself does not affect the invasiveness of *Salmonella* but it is the architecture of the reporter system itself (Clark et al., 2009). This study also showed that *C. jejuni* NCTC111618V1 and *C. jejuni* NCTC111618V26 have a different colonization pattern. It also demonstrated that the colonization of the bird's ceca is directly correlated to colonization of other organs. The process of systemic spread required at least 48 hours of initial colonization in the ceca before *C. jejuni* was detected in the spleen.

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Chapter V: Colonization of Chickens and BALB/c mice by Wild-type and Paralyzed *Campylobacter jejuni* Mutants

5.1. Introduction:

Gastro-intestinal infection with *Campylobacter jejuni* remains the leading cause of diarrhea in humans (Ailes et al., 2008; Gillespie et al., 2008; Kubota et al., 2008; Thomas et al., 2008b; Yang et al., 2008). The number of *C. jejuni* cases per community in Canada ranges from 23-49 cases (Thomas et al., 2008b). The exact cost of campylobacteriosis is not known in Canada, but the economical burden from acute gastroenteritis illness caused by *C. jejuni*, *E. coli* O157, and *Salmonella* is $115 CAD per capita (Thomas et al., 2008b). In the United States, up to 4 million cases of campylobacteriosis are estimated to occur every year leading to an estimated 400 deaths caused by the bacteria or its immune-mediated sequel; Guillain-Barré Syndrome (CDC, 2009). *C. jejuni* is a problem among younger population in developing countries. The incidence of campylobacteriosis is estimated to be as high as 40,000/100,000 cases in children under the age of 5 according to World Health Organization estimates (Rao et al., 2001), in comparison to 300/100,000 for the same age group in developed countries (Janssen et al., 2008).

Handling poultry and poultry meat products is the main cause of campylobacteriosis, and despite the importance of this source, the pathogenesis of *C. jejuni* in broiler chickens, the major carrier, is poorly explored. Broiler chickens are asymptomatically infected with *C. jejuni* although they carry up to 10^{10} colony forming units per gram of cecal contents (Dhillon et al., 2006).

Despite the limited understanding of *C. jejuni* pathogenesis in broiler chickens, several factors involved in colonization of the cecum and invasion of cell lines have been characterized. *Campylobacter* invasion antigens (Cia), play a role in invasion and internalization of *C. jejuni* into human intestinal cells (Rivera-Amill and Konkel, 1999), and deletion of the *ciaB* gene, and thus the only characterized Cia protein, reduces the ability of the mutant to colonize broiler chickens upon oral infection (Ziprin et al., 2002a).

C. jejuni flagella is probably the most studied component of this bacterium. The fast darting motility facilitated by this structure (Guerry, 2007), and the suggested role of

Campylobacter invasion antigens, flagella secreted proteins, and FlaC, that are secreted through the flagellum in cecal colonization (Konkel et al., 2004; Poly et al., 2007), have led many researchers to consider the *C. jejuni* flagellum the main virulence factor for this bacterium. Researchers have studied the effect of various mutations on the expression of the flagellum and the ability to colonize chickens and mice. Mutations in the *flaA* gene which encodes flagellin A, the main flagellar subunit, produced a truncated flagellum that is non-motile and has a reduced ability to invade human intestinal cells *in vitro* (Wassenaar et al., 1991). A *C. jejuni flaA* mutant did not secrete *Campylobacter* invasion antigens, while a *flaB* mutant , the minor flagellin subunit, secrete Cia (Konkel et al., 2004). A *flaA* mutant colonized chickens efficiently (Wassenaar et al., 1993), while a mutation in *flaB* enhanced the ability to colonize chickens' cecum (Wassenaar et al., 1994).

Mice are used often in studying many aspects of human diseases including immune response and pathogenesis. They are also used to study *C. jejuni* mutations that might affect colonization (Bacon et al., 2000). Under natural conditions, *C. jejuni* is not normally isolated from the intestinal tract of laboratory mice (Diker et al., 1992; Jesudason et al., 1989; Newell et al., 1985), but using various experimental settings colonization could be induced. Establishing a reproducible challenge model and understanding the pathogenesis of *C. jejuni* in mice will allow the utilization of genetic and immunological tools available. Indeed, mice have already been used to study the role of MyD88 (Watson et al., 2007) and NF-κB (Fox et al., 2004) in the immune response induced by *C. jejuni*.

The role *Campylobacter* invasion antigens play in mucosal and systemic spread of *C. jejuni* in broiler chickens and mice is unknown. In addition, to date, no published study has investigated the impact of flagellar paralysis on pathogenesis of *C. jejuni* in broiler chickens and mice. In this study, the role of motility in pathogenesis of *C. jejuni* is studied using paralyzed flagella mutants that express a full-length flagellum, but are motility impaired (Yao et al., 1994). This paralysis is a result of an insertion mutation in the *pflA* gene, the function of which is still unknown in *C. jejuni*, but it is common to *C. jejuni* strains sequenced to date (Gaynor et al., 2004; Parkhill et al., 2000).

In this study, mucosal and systemic spread of *C. jejuni* is studied in broiler chickens and BALB/c mice. *C. jejuni* transformed with green fluorescent protein (GFP) was tracked using fluorescence microscopy. The *Campylobacter* invasion antigens secretion profile of these bacteria is also determined, and the impact these proteins might have on colonization is determined.

5.2. Materials and Methods:

5.2.1. Bacterial strains:

Four *C. jejuni* strains were used in this study. The wild-type strain *C. jejuni* 81-176 is described in detail in General Materials and Methods chapter. Strains *C. jejuni* K2-37 and K2-55 were a generous gift from Dr. Patricia Guerry (Naval Medical Research Center, Silver Spring, Maryland), and they are isogenic mutants of wild-type strain *C. jejuni* 81-176. The mutation was generated by an insertion mutation of kanamycin-resistance cassette into *pflA* gene (Yao et al., 1994). The mutants expressed full-length flagella that were completely paralyzed. The ability of K2-37 and K2-55 to adhere to INT407 cells was not significantly affected, but their ability to invade cells was reduced (Yao et al., 1994). Strain NCTC11168V1G was used only in BALB/c mice and is described in detail in General Materials and Methods chapter.

5.2.2. Screening for spontaneous streptomycin-resistant *C. jejuni*:

To obtain a streptomycin-resistant isolate, the same procedure described in Chapter IV was followed. Briefly, freshly grown *C. jejuni* culture was harvested in 5 ml MHB. The harvested bacteria were centrifuged at 2500 X *g* and resuspended to a concentration of 10^8-10^9 CFU/ml. *C. jejuni* was plated on MHA plates supplemented with 100 μg/ml streptomycin and incubated at 42 °C for 24-48 hours. Colonies growing were restreaked twice to confirm streptomycin resistance.

5.2.3. Construction of Green Fluorescent Protein (GFP) plasmid and bacterial transformation:

Plasmid pHG101 was constructed as described in chapter IV. The plasmid contained a GFP reporter system, a *Campylobacter* kanamycin resistance gene, a mobility gene (*mob*), and *Campylobacter* origin of replication (*repB*), *E. coli* ColE1

origin of replication, consensus primer (Pc), and translation initiation region (TIR) (Miller et al., 2000). Plasmid pHG101 was purified from *C. jejuni* NCTC 11168V1G and transformed by electroporation into the three strains used in this study as described in chapter IV. The presence of the plasmid in the strains was confirmed by whole-cell PCR, enzyme digestion, and sequencing.

5.2.4. Motility test of *C. jejuni* 81-176, *C. jejuni* K2-37, and *C. jejuni* K2-55:

To establish the motility pattern of *C. jejuni* 81-176, *C. jejuni* K2-37, and *C. jejuni* K2-55, bacteria freshly grown on MHA supplemented with 5% blood at 42 °C for 24 hours were harvested using 0.85% NaCl and diluted to an absorbance of 0.1 at 600 nm wavelength ($O.D_{600}$). Using a sterile inoculating needle (VWR), approximately 2 µl of the suspension were stabbed into the side of an 0.4% MHA and incubated at 37 °C under microaerophilic conditions for 48 hours. The radius of the ring of growth formed by the mutant was compared to that of the wild-type to determine relative motility. Wild-type was grown on each plate to rule out plate-to-plate variation. The experiment was repeated 3 separate times.

5.2.5. Transformation of plasmid pHG101:

The plasmid was transferred into wild-type *C. jejuni* 81-176, K2-37, and K2-55 using electroporation. The experiment was conducted as described in chapter IV using electroporation buffer (15% glycerol /9% sucrose). For every 40 µl of cells, 1 µg of plasmid DNA was added and mixed. The mix was transferred into pre-chilled electroporation cuvettes and cells were pulsed with 1.25 kV and constant range of 4-6 mS in a Gene Pulser xcell (BioRad) electroporater. The resulting transformants were named *C. jejuni* 81-176G, K2-37G, and K2-55G.

5.2.6. Electron microscope examination of flagella:

After transformation with pHG101, the three strains used in this study were negatively stained and examined using transmission electron microscopy to confirm flagellar expression. Samples were prepared as described in General Materials and Methods chapter.

5.2.7. *C. jejuni* protein secretion assay:

The secretion assay described by Konkel and colleagues (Konkel et al., 1999c; Rivera-Amill and Konkel, 1999) was used to determine if *Campylobacter* invasion antigens were secreted by the strains used in this study. *C. jejuni* strains were grown overnight on MHA supplemented with 0.1% (w/v) sodium deoxycholate at 37 °C under microaerophilic conditions. *C. jejuni* growing on each plate was harvested into 5 ml Eagle minimum essential medium (EMEM, ICN Biomedicals, Solon, OH). The bacterial suspension was centrifuged at 3,000 X *g*, and the supernatant was decanted. The pellet was resuspended in 5ml EMEM and the procedure repeated. The resulting pellet was resuspended in EMEM supplemented with 0.5% fetal bovine serum to an absorbance of 0.3 at 590 nm wavelength ($O.D_{590}$). Fetal bovine serum used was filtered with Amicon Ultra centrifugal filter (MW 30K; Millipore) to avoid gel distortions. To label secreted proteins, [^{35}S] methionine (Amersham Biosiences, Baie d'Urfé, QC) was added to the culture medium at a final concentration of 25 µCi/ml. The mixture was transferred to a 25 ml tissue culture flask (Corning) and incubated at 37 °C for 3 hours. After incubation, flask contents were centrifuged at 6,000 X *g* for 15 minutes and the supernatant filtered through 0.2 µm sterile filter (Nalgene). Equal volumes of the samples were loaded in the wells of a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous buffer system described by Laemmli (Laemmli, 1970). Gels were treated with Amplify (Amersham Life Sciences) according to the manufacturer's instructions, and autoradiography was performed with Kodak BioMax MR film (SIGMA). Strain *C. jejuni* NCTC11168V1 was used as a positive control, and *C. jejuni* NCTC11168V1*flgK* mutant was used as a negative control (Fernando et al., 2007). The secretion assays were repeated twice.

5.2.8. Stability of plasmid pHG101 in *C. jejuni* 81-176G and K2-55G *in vitro*:

To determine the stability of plasmid pHG101 in the absence of selective antibiotic pressure, freshly grown *C. jejuni* 81-176G or K2-55G were restreaked on Karmali agar plates supplemented with 200 µg/ml kanamycin. Agar plates were incubated for 24 hours, bacteria were harvested in PBS, centrifuged at 9000 X *g* for 5 minutes, resuspended to an absorbance of 0.1 at 600 nm wavelength ($O.D_{600}$) , and 25 µl were subcultured on Karmali agar plates and Karmali agar plates supplemented with

kanamycin and incubated for 24 hours at 42 °C. The ratio of *C. jejuni* growing on antibiotic free Karmali agar plates to Karmali agar plates supplemented with kanamycin was determined. The same process was repeated for 10 consecutive subcultures using the bacteria growing on Karmali agar plates. Each set of subcultures was repeated twice. A ratio of one indicates that the plasmid is stable. The number of fluorescent colonies was determined by counting colonies illuminated by a UV lamp.

5.2.9. Dose titration of *C. jejuni* 81-176G and K2-55G in day-old chicks:

The ability of strains *C. jejuni* 81-176G and K2-55G to colonize day-old chicks was determined by challenging 90 birds divided into 6 groups each consisting of 15 chickens with the doses listed in Table 5.1. The challenge dose was suspended in 0.5 ml 0.85% NaCl and delivered orally. Cecal contents were collected 7 days post challenge and colonization level represented by colony forming units/g (CFU/g) of feces were determined using the procedure described in General Materials and Methods chapter.

5.2.10. Sites of colonization of *C. jejuni* 81-176G and K2-55G in broiler chickens:

To study mucosal and systemic spread of *C. jejuni* 81-176G wild-type and paralyzed flagella mutant *C. jejuni* K2-55G in broiler chickens, two groups of 16 chickens were housed at VIDO animal care. Birds were *C. jejuni*-free when tested upon arrival for presence of *C. jejuni* using cloacal swabs. To confirm that the birds remained *Campylobacter*-free until challenged, cloacal swabs were collected on days 1, 7, 14 and 21 of the experiment and plated on Karmali selective agar. On day 21, each bird in group one received 1 X 10^9 CFU *C. jejuni* 81-176G suspended in 0.5ml 0.85% NaCl orally, while birds in group two received 1 X 10^9 CFU/0.5 ml *C. jejuni* K2-55G by the same route. After 24 hours, 2 birds from each group were euthanized by cervical dislocation, and tissue samples were collected aseptically from small intestine, large intestine, cecum, liver, spleen, crop, and gizzard. Each sample was placed in a separate sterile Petri dish and immediately processed. Tissues were cut using a surgical carbon steel blade (VWR, Mississauga, ON) to produce small pieces of 1-3 mm X 3-5 mm thickness and were placed in individual Cryomold (VWR) that had a thin film of Sakura Finetek Tissue-Tek Optimal Cutting Temperature media (OCT) (VWR) covering the bottom, and were covered completely with OCT and placed immediately either in liquid nitrogen for 90

Group	Challenge dose (CFU)	Strain
1	1.5×10^4	K2-55G
2	2×10^3	81-176G
3	2×10^6	K2-55G
4	1.8×10^6	81-176G
5	2×10^8	K2-55G
6	1.5×10^8	81-176G

Table 5.1. Dose titration of *C. jejuni* 81-176G and K2-55G in day-old chicks. The ability of strains *C. jejuni* 81-176G and *C. jejuni* K2-55G to colonize day-old chicks was determined by challenging 90 birds divided into 6 groups each consisting of 15 chickens. Challenge dose was suspended in 0.5 ml saline and delivered orally. Cecal contents were collected 7 days post challenge.

second or -80 °C freezer for 20 minutes. A cloacal swab was dipped into the cecal contents and streaked on Karmali agar plate to confirm the presence of *C. jejuni* by culture. The same sampling procedure was repeated every 24 hours for the remaining birds. All samples were stored at -20 °C until processed.

Tissues were processed as described in Chapter IV to obtain sections. From each tissue sample 9 sections in total were made. Slides were air-dried for 15 minutes, washed by dipping in PBS (+4 °C temperature) for 3 times, air-dried for additional 10 minutes, and fixed in acetone at 4 °C for 1 hr. Tissues were either covered directly by cover slip and sealed with nail polish, or mount using Fluoromount (Pleaston, CA) or Cytoseal 60 low viscosity mounting medium (Richard-Allan Scientific, Kalamazoo, MI). Slides were stored in a closed box at -20 °C until inspected with fluorescent microscope.

Slides were studied using fluorescent microscopy (Carl Zeiss Inc) with light transmission and fluorescein isothiocyanate (FITC) filters. Each tissue section was visualized using 4X, 20X, 40X, and 100 X magnifications. If any section contained fluorescent *C. jejuni*, the sample was considered positive. If *C. jejuni* was not found in any of the 9 sections, the sample was considered negative.

Tissue samples collected from four 14-day old *C. jejuni*-free broiler chickens were used as negative controls. For positive controls, cecal contents collected from broiler chickens positive for *C. jejuni* 11168V1G by culture were resuspended in normal saline 2 X weight, embedded in OCT media, frozen, and treated as tissue samples.

5.2.11. Sites of colonization of *C. jejuni* NCTC11168V1G in BALB/c mice:

To establish a reliable challenge model in BALB/c mice, a pilot study was conducted using 5 mice housed at the VIDO animal care. Mice were treated with streptomycin in drinking water (3g/L) for 3 days (Wadolkowski et al., 1990). On day 4, each mouse orally received 1 X 10^{10} CFU of *C. jejuni* NCTC11168V1G suspended in 0.5 ml 0.85% NaCl. Fecal samples were collected on days 1, 5, 6, 7, 9, 11, and 14 from individual mice using a sterile Eppendorf. Fecal material was spread on Karmali selective agar to determine the level of *C. jejuni* colonization.

To study mucosal and systemic colonization of *C. jejuni* NCTC11168V1G in the mouse, a group of 10 BALB/c mice were challenged with 1 X 10^9 CFU using the same protocol in the pilot study. Starting 24 hours after the challenge, two mice were euthanized daily and tissue samples were collected from liver, spleen, small intestine,

large intestine, and cecum. Fecal samples were collected from cecum to confirm shedding of *C. jejuni* from the sampled animal. Tissue samples were treated as described in chicken studies, and the same controls were used.

5.2.12. Sites of colonization of *C. jejuni* 81-176G and K2-55G in BALB/c mice:

Mucosal and systemic colonization of *C. jejuni* 81-176G and K2-55G in BALB/c mice was studied using two groups of 15 mice. Each mouse received 1 X 10^9 CFU/0.5 ml 0.85% NaCl orally after 3 days of streptomycin treatment as described for the pilot study. On days 1, 3, and 6 post challenge 5 animals from each group were euthanized and tissue samples collected from liver, spleen, small and large intestine, and cecum. Tissues were treated following the same protocol described for chicken's tissues. All samples were stored at -20 °C until processed.

5.3. Results:

5.3.1. Plasmid pHG101 transformation and stability *in vitro*:

The plasmid pHG101 containing GFP reporter system was transformed using electroporation, into a streptomycin-resistant isolate of *C. jejuni* 81-176 and K2-55. The transformed isolates were named *C. jejuni* 81-176G and K2-55G. The presence of the plasmid was confirmed using whole-cell PCR, enzyme restriction digestion, and sequencing (Fig 5.1).

5.3.2. Stability of plasmid pHG101 in *C. jejuni* 81-176G and K2-55G *in vitro*:

To determine the stability of plasmid pHG101 in transformed strains, the ratio of *C. jejuni* 81-176G or K2-55G growing on antibiotic-free Karmali agar plates to Karmali agar plates supplemented with kanamycin was approximately 1 after 10 subcultures, indicating a very stable plasmid in these strains (Fig 5.2-A). The percentage of fluorescent *C. jejuni* 81-176G and K2-55G cells was almost always above 95% of CFU (Fig 5.2-B)

5.3.3. Motility tests:

Motility test were performed to determine if strains *C. jejuni* K2-37 and K2-55 remained paralyzed after screening for streptomycin resistance, transformation with pHG101, and challenging chickens. The motility test confirmed that both strains *C. jejuni* K2-37 and K2-55 had an impaired motility. It also confirmed that the bacteria remained paralyzed in samples recovered from cecal contents of broiler chickens and mice (Fig 5.4).

5.3.4. Electron Microscopy:

In order to confirm the expression of full-length flagella in strains *C. jejuni* 81-176G, K2-37G, and K2-55G, samples of these strains were visualized using electron microscopy. Strains *C. jejuni* 81-176G, K2-37G, and K2-55G expressed full-length intact flagella upon examination with transmission electron microscope (Fig 5.3). No visible differences were observed among the three strains.

The presence of *C. jejuni* K2-55G in the ceca was also confirmed by electron microscopy (Fig 5.3).

5.3.5. Proteins secreted by *C. jejuni*:

A protein secretion assay using radioactively-labeled methionine detected *Campylobacter* invasion antigens secretion by strain *C. jejuni* 81-176, while strains K2-37, and K2-55 had no detectable secreted proteins (Fig 5.4). Strain *C. jejuni* NCTC11168V1 was used as a positive control, and *C. jejuni* NCTC11168V1*flgK* mutant was used as a negative control (Fernando et al., 2007).

5.3.6. Dose titration of *C. jejuni* 81-176G and K2-55G in day-old chicks:

The ability of strains *C. jejuni* 81-176G and K2-55G to colonize day-old chicks was determined by orally challenging 90 birds divided into 6 groups each consisting of 15 chickens (Table 5.1). All birds that received 1 X 10^8 CFU/0.5 ml challenge dose in Groups 5 and 6 of either *C. jejuni* 81-176G or K2-55G respectively were colonized when sampled (Fig 5.5). All birds that were challenged with 1X 10^5 CFU/0.5 ml of *C. jejuni* 81-176G were colonized, while 14 birds challenged with *C. jejuni* K2-55G were colonized when sampled on day 7 (Fig 5.5). Group two challenged with 1X 10^3 CFU/0.5 ml *C. jejuni* 81-176G had 6 birds that were colonized, while Group one that received an actual dose of 1.5 X10^4 *C. jejuni* K2-55G had 8 colonized birds (Fig 5.5). This suggests a limited effect of motility on colonization of day-old broiler chickens over the period of 7 days.

5.3.7. Sites of colonization of *C. jejuni* 81-176G and K2-55G in broiler chickens:

Mucosal and systemic presence of *C. jejuni* 81-176G and K2-55G in broiler chickens was determined by examining tissue sections, collected from broiler chickens, using fluorescence microscopy. *C. jejuni* K2-55G was not detected in any tissue sections of crop, gizzard, liver or spleen examined (Tables 5.2 and 5.3). *C. jejuni* 81-176G was not detected in any crop or gizzard tissue sections. Both strains were detected in cecal tissue sections, as *C. jejuni* 81-176G was present in 100% of samples, while *C. jejuni* K2-55G was present in 81% of the sampled birds. *C. jejuni* 81-176G was present in 62% of large intestine samples, while *C. jejuni* K2-55G was present in only 43% of the samples. Only 25% of the small intestine of birds challenged with K2-55G had detectable bacteria, while 43% challenged with *C. jejuni* 81-176G had detectable bacteria (Fig 5.6).

5.3.8. Sites of colonization of *C. jejuni* NCTC11168V1G in BALB/c mice:

To determine the sites of colonization of *C. jejuni* NCTC11168V in BALB/c mice, a group of 10 mice were challenged with 1 X 10^9 CFU orally. Starting 24 hours after the challenge, two mice were euthanized daily and tissue samples were collected. Although all the ceca of the mice challenged had detectable *C. jejuni*, no *C. jejuni* was detected in the liver or spleen of any of the samples, while only two mice had detectable *C. jejuni* in their small intestine, and 6 mice had the bacteria in their large intestine (Table 5.4 and Fig 5.7).

5.3.9. Sites of colonization of *C. jejuni* 81-176G and K2-55G in BALB/c mice:

To determine mucosal and systemic spread of *C. jejuni* 81-176G and K2-55G in BALB/c mice, two groups consisting of 15 mice each were challenged with either strain. Neither *C. jejuni* 81-176G and K2-55G were detected in any of the spleen or liver tissue sections of BALB/c. *C. jejuni* was present in 66.6% of the ceca , 33% of large intestine, and 20% of small intestine of the mice challenged. *C. jejuni* K2-55G was present in only 53.3% of ceca sampled, 20% of large intestine tissue sections, and in none of the small intestine examined (Tables 5.5 and 5.6 Fig 5.8).

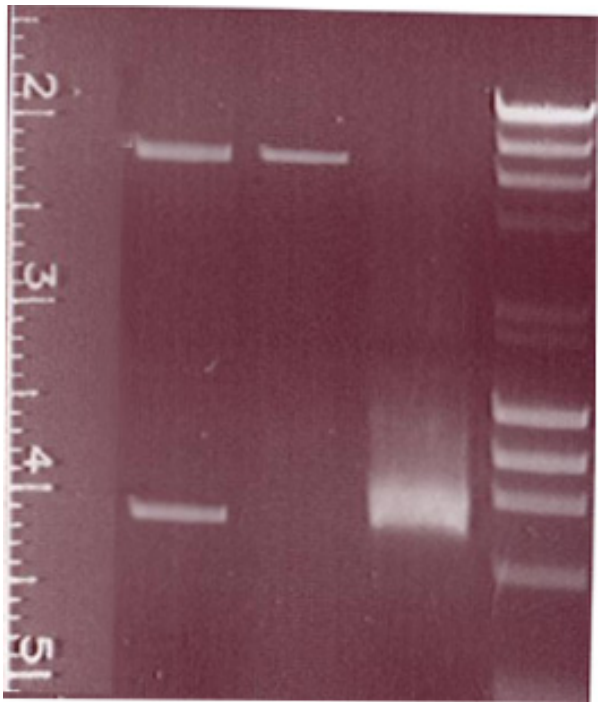

Fig 5.1.Confirmation of the presence of pHG101 in *C. jejuni* 81-176G. The plasmid size was confirmed using restriction enzyme digestion. The final plasmid pHG101 was obtained by ligation of two DNA segments, the backbone from plasmid AF292555 was digested using EcoRI and AatII (lane 2). The *gfp* gene was amplified using primers listed in Table 4.1, extracted from agarose gel, and digested with the same restriction enzymes (lane 3). Growing colonies were screened for pHG101 and confirmation of the correct plasmid size was obtained by restriction analysis (lane 1). DrigestIII DNA marker was used to determine band size (lane 4)

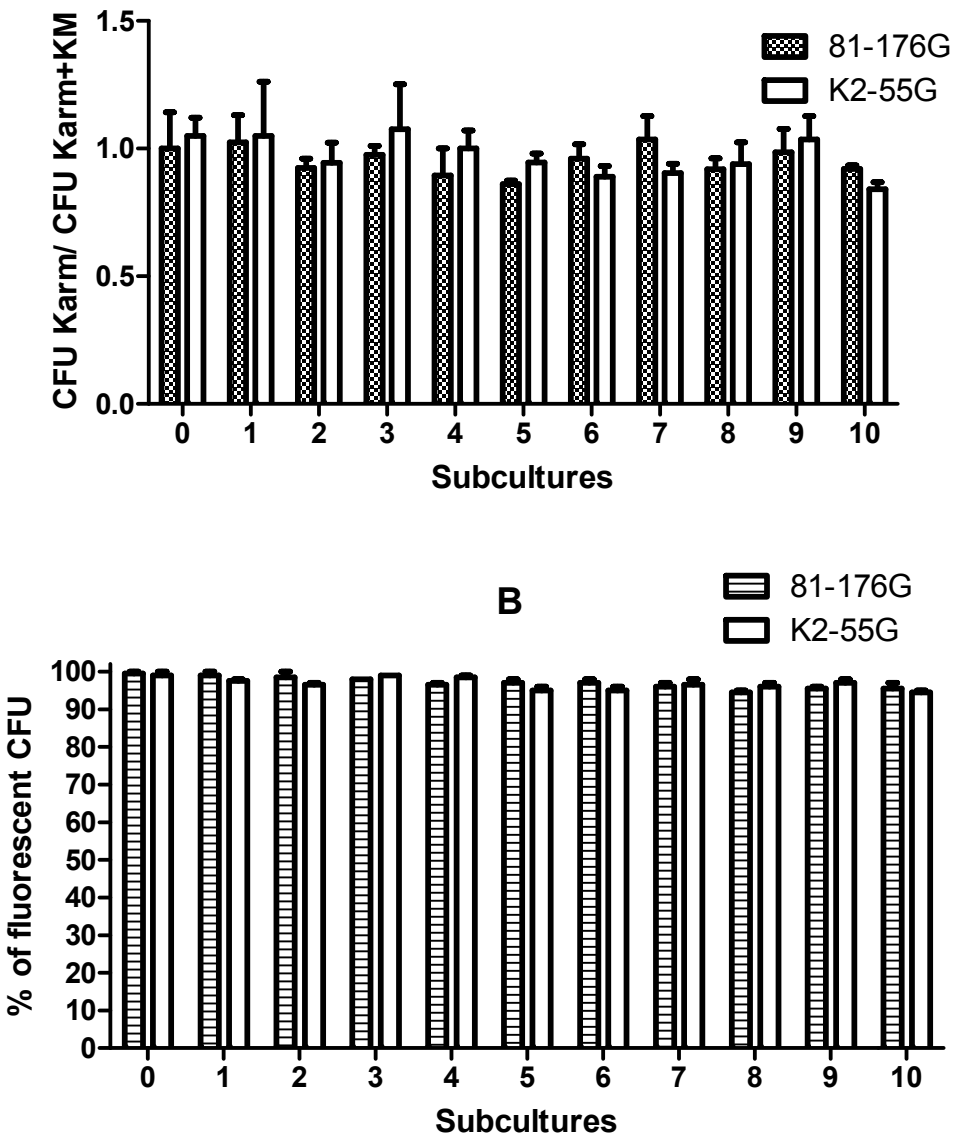

Fig 5.2. Stability of plasmid pHG101 in *C. jejuni* 81-176G and K2-55G *in vitro*. (A) Ratio of *C. jejuni* 81-176G or K2-55G growing on Karmali agar to the same strains growing on Karmali agar supplemented with kanamycin after subculture. Subculture 0 is the ratio of cells growing on Karmali agar Bacterial growth was not affected by the presence or absence of antibiotic as growth ratio was ~1 after 10 subcultures. (B) Plasmid stability was determined by dividing the ratio of fluorescent *C. jejuni* growing on antibiotic free Karmali agar plates to Karmali agar plates supplemented with kanamycin. Each set of subcultures was repeated twice. Both strains maintained a fluorescence ratio above 94%. The same process was repeated for 10 consecutive subcultures.

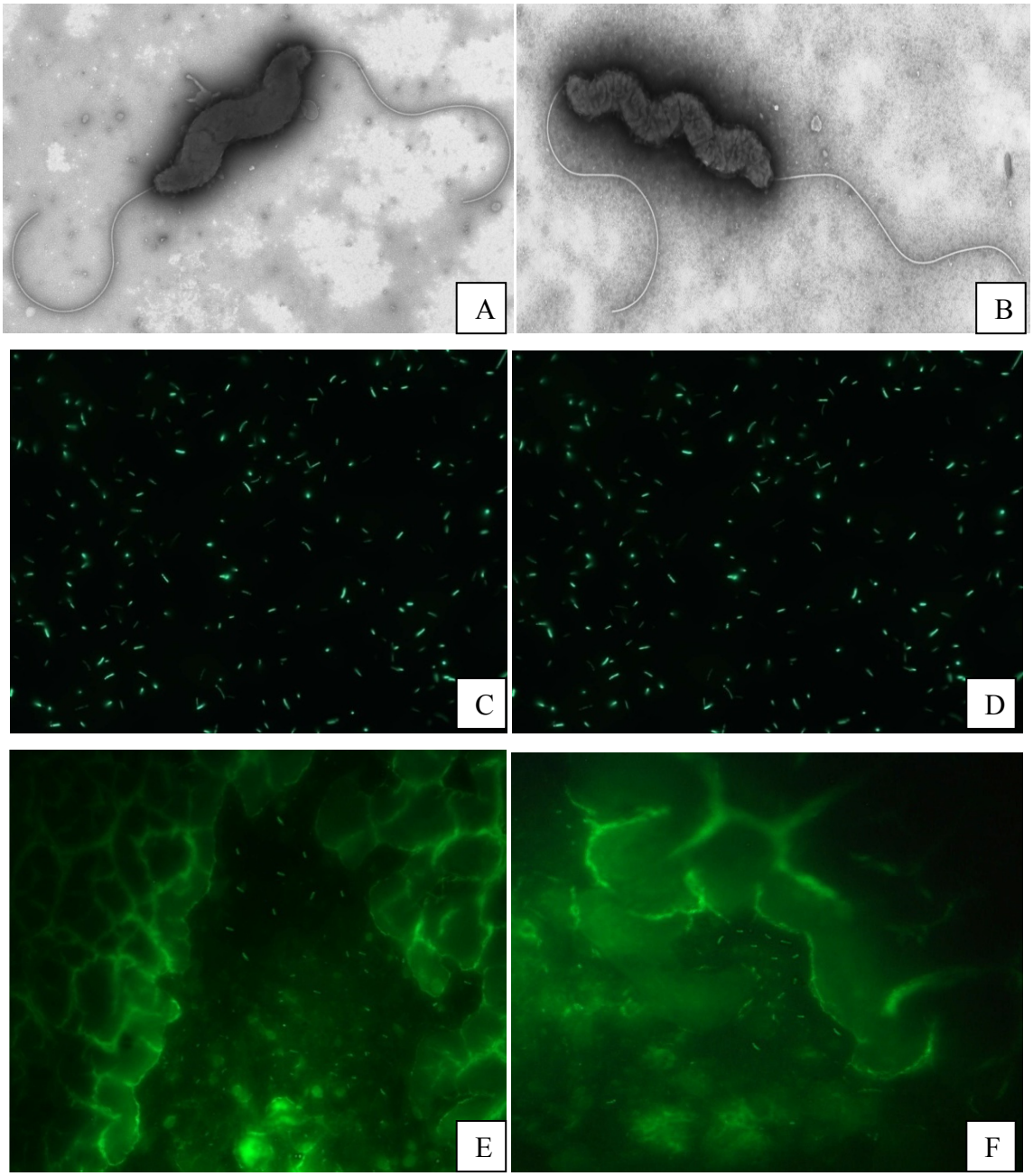

Fig 5.3. Electron and fluorescence microscopy images of *C. jejuni* 81-176G and K2-55G. (A): Scanning EM of *C. jejuni* 81-176G with bi-polar flagellum. (B): Scanning EM of K2-55G expressing fully length but paralyzed bi-polar flagellum (14,000x). (C) and (D) are fluorescence microscopy images of *C. jejuni* 81-176G and K2-55G (respectively) suspended in water (1000x). (E) Fluorescence microscopy image of a cross section of the cecum of a BALB/c mouse 3 days post-challenge with *C. jejuni* 81-176G. Bacteria are closely associated with the mucosal surface or freely in the lumen but are not found in the submucosal layers. (F) Fluorescent microscope image of a cross section of the cecum of a BALB/c mouse 2 days post-challenge with *C. jejuni* K2-55G.

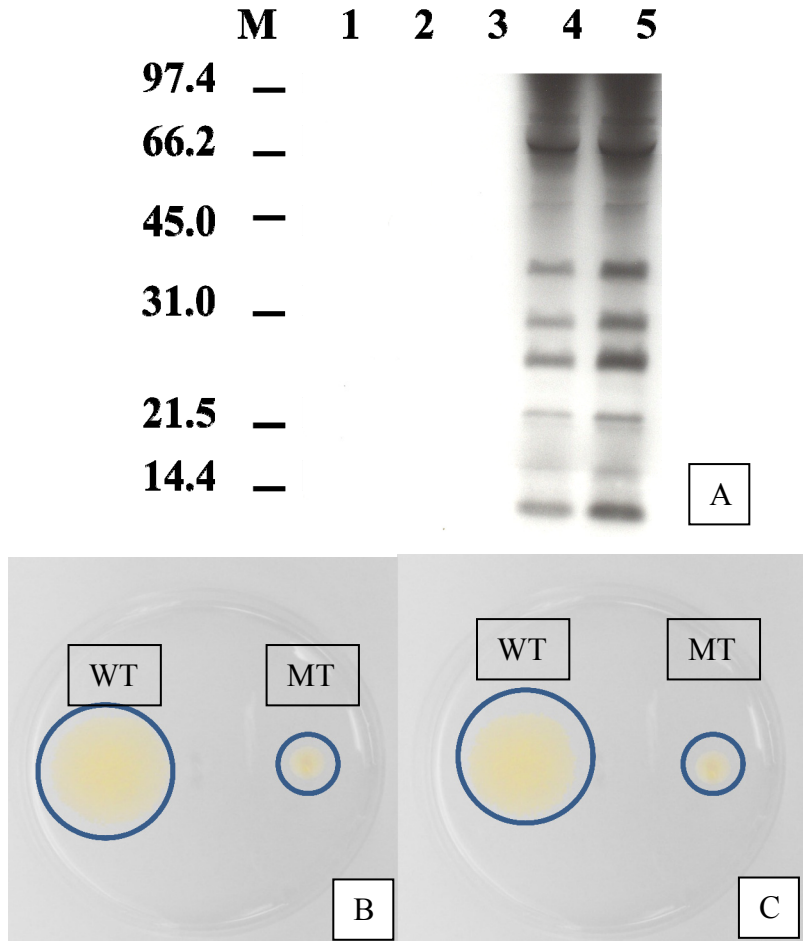

Fig 5.4. Characterization of *C. jejuni* K2-37 and K2-55 motility and secreted patterns. (A) Proteins secreted through *C. jejuni* flagella (Cia) were labeled using [^{35}S] methionine as described in materials and methods section and resolved using 12.5% SDS-PAGE. Lane 1: negative control (*flgK* mutant), lane 2: K2-37, Lane 3: K2-55, lane 4: *C. jejuni* 81-176, and lane 5: *C. jejuni* NCTC1168V1. Strains K2-37 and K2-55 had no detectable secretions in the supernatant of the culture when co-incubated with FBS. Wild-type 81-176 had a secretion profile comparable to the positive control (Fernando et al., 2007). (B) Motility pattern of K2-37 and (C) K2-55 compared to the wild-type 81-176. Freshly grown bacteria were diluted to an absorbance of 0.1at 600 nm wavelength ($O.D_{600}$). Using a sterile inoculating needle (VWR), approximately 2 µl of the suspension were stabbed into 0.4% MHA and incubated at 37 °C under microaerophilic conditions for 48 hours. The radius of the ring formed by mutant swarm was compared to that of the wild-type to determine motility. Wild-type was grown on each plate to rule out plate-to-plate variation. The experiment was repeated 3 separate times. Both strains showed an impaired motility compared to the wild-type.

WT: wild-type, MT: mutant.

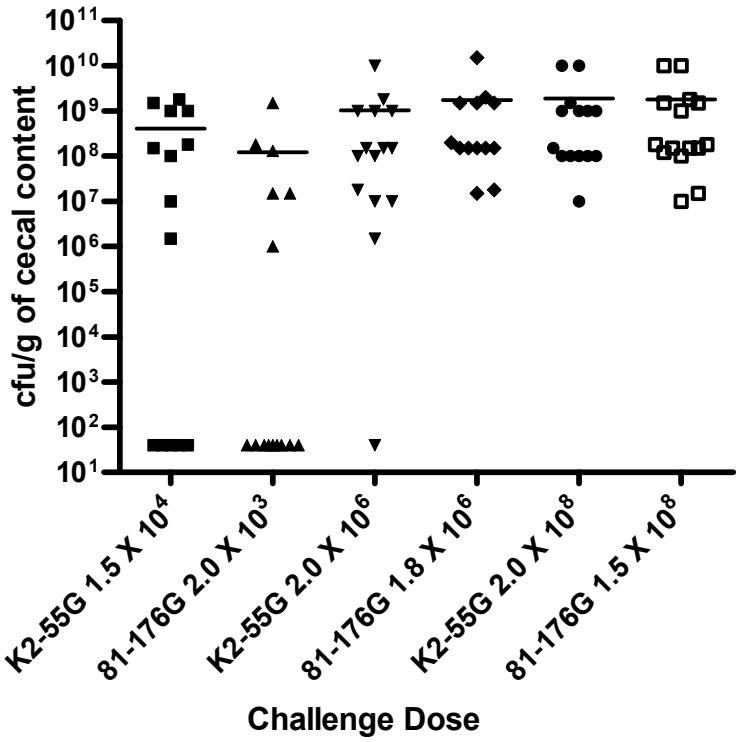

Fig 5.5. Dose titration of *C. jejuni* 81-176G and K2-55G in day-old chicks. To determine the ability of both strains to colonize day-old broiler, six groups each consisting of 15 birds were challenged as following: Group 1: 1.5 X 10^4 K2-55G, Group 2: 2 X 10^3 81-176G, Group 3: 2 X 10^6 K2-55G, Group 4: 1.8 X 10^6 81-176, Group 5: 2 X 10^8 K2-55G, and Group 6: 1.5 X 10^8 CFU/ 0.5 ml. Challenge dose was suspended in 0.85% NaCl and was administered orally. Birds were euthanized and cecal contents were collected on day 7. All birds in groups 4, 5, and 6 had detectable *C. jejuni* in their ceca. Birds with no detectable *C. jejuni* in cecal contents were assigned the minimum detection level value of 400. Only one bird in Group 3 had no detectable *C. jejuni*, while 9 birds in Group 2 and 5 birds in Group 1 had no detectable *C. jejuni* in their cecal contents.

	Crop	Gizzard	Large intestine	Small intestine	Cecum	Liver	Spleen	Cloacal swab
Day 1								
Chicken 1	-	-	-	-	+	-	-	+
Chicken 2	-	-	-	-	+	-	-	+
Day 2								
Chicken 3	-	-	+	+	+	-	-	+
Chicken 4	-	-	+	-	+	-	-	+
Day 3								
Chicken 5	-	-	+	-	+	+	-	+
Chicken 6	-	-	+	-	+	-	-	+
Day 4								
Chicken 7	-	-	+	+	+	+	+	+
Chicken 8	-	-	-	+	+	+	-	+
Day 5								
Chicken 9	-	-	-	-	+	-	+	+
Chicken 10	-	-	-	+	+	+	+	+
Day 6								
Chicken 11	-	-	+	+	+	-	-	+
Chicken 12	-	-	+	-	+	-	-	+
Day 7								
Chicken 13	-	-	+	-	+	-	-	+
Chicken 14	-	-	-	-	+	+	+	+
Day 8								
Chicken 15	-	-	+	+	+	+	+	+
Chicken 16	-	-	+	+	+	-	-	+

Table 5.2: Sites of colonization of *C. jejuni* 81-176G in broiler chickens. A group of 16 birds were challenged with 1 X 10^9 CFU *C. jejuni* 81-176G suspended in 0.5ml 0.85% NaCl orally on day 0. Every 24 hours after the challenge two birds were euthanized and tissue samples were collected from crop, gizzard, small and large intestine, ceca, liver, and spleen. Samples were frozen and processed for examination by fluorescence microscopy. All birds had detectable *C. jejuni* in their ceca, and none in the crop or gizzard, while 10 birds had *C. jejuni* in their large intestine, seven in small intestine, six in the liver, and 5 in the spleen.

	Crop	Gizzard	Large intestine	Small intestine	Cecum	Liver	Spleen	Cloacal swab
Day 1								
Chicken 1	-	-	+	-	+	-	-	+
Chicken 2	-	-	-	-	+	-	-	+
Day 2								
Chicken 3	-	-	-	-	+	-	-	+
Chicken 4	-	-	+	+	+	-	-	+
Day 3								
Chicken 5	-	-	-	-	+	-	-	+
Chicken 6	-	-	-	-	-	-	-	-
Day 4								
Chicken 7	-	-	+	+	+	-	-	+
Chicken 8	-	-	-	-	+	-	-	+
Day 5								
Chicken 9	-	-	-	-	+	-	-	+
Chicken 10	-	-	+	-	+	-	-	+
Day 6								
Chicken 11	-	-	-	-	-	-	-	-
Chicken 12	-	-	+	+	+	-	-	+
Day 7								
Chicken 13	-	-	+	-	+	-	-	+
Chicken 14	-	-	-	-	+	-	-	+
Day 8								
Chicken 15	-	-	-	+	+	-	-	+
Chicken 16	-	-	+	+	+	-	-	+

Table 5.3. Sites of colonization of *C. jejuni* K2-55G in broiler chickens. A group of 16 birds were challenged with 1 X 10^9 CFU *C. jejuni* K2-55G suspended in 0.5ml 0.85% NaCl orally on day 0. Every 24 hours after the challenge two birds were euthanized and tissue samples were collected from crop, gizzard, small and large intestine, ceca, liver, and spleen. Samples were frozen and processed as described in materials and methods section. *C. jejuni* was detected in the ceca of 13 birds, seven large intestines, five small intestines, and none of the crops, gizzards, spleens, or livers sampled.

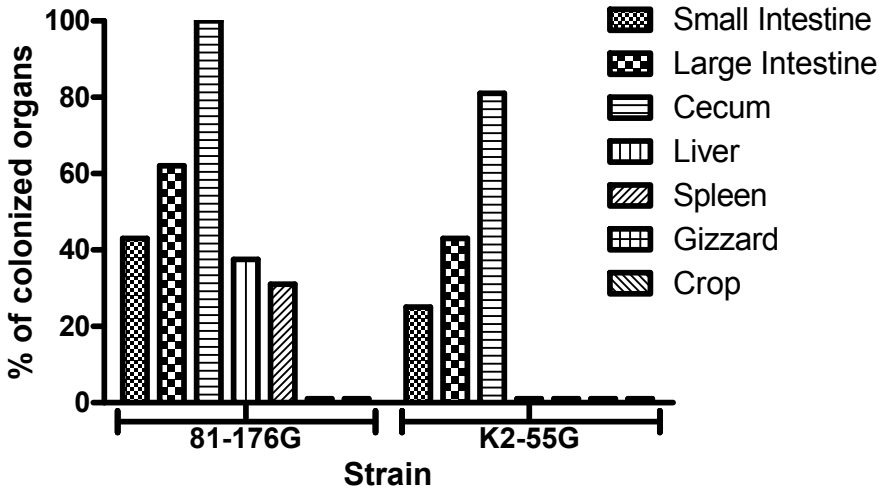

Fig 5.6. Comparison of sites of colonization of *C. jejuni* 81-176G and K2-55G in broiler chickens. Two groups of 16 birds each were challenged with 1 X 10^9 CFU of either *C. jejuni* 81-176G or K2-55G suspended in 0.5ml 0.85% NaCl orally. Every 24 hours after the challenge two birds from each group were euthanized and tissue samples were collected from crop, gizzard, small and large intestine, ceca, liver, and spleen. Samples were frozen and processed for examination by fluorescence microscopy. Neither of the two strains were detected in the crop or gizzard, while *C. jejuni* K2-55 G was not detected in the liver or spleen. *C. jejuni* 81-176 was detected in 37.5% of liver and 31% of spleen samples examined. All the ceca of chickens challenged with *C. jejuni* 81-176G were colonized, while only 81% of the ceca of chickens challenged with *C. jejuni* K2-55G were colonized. Large intestine of 62% and 43% of the birds, and small intestine of 43% and 25% of birds were colonized with *C. jejuni* 81-176G and K2-55G respectively.

	Large intestine	Small intestine	Cecum	Liver	Spleen	Swab
Day 1						
Mouse 1	-	-	+	-	-	+
Mouse 2	-	-	+	-	-	+
Day 2						
Mouse 3	+	+	+	-	-	+
Mouse 4	+	-	+	-	-	+
Day 3						
Mouse 5	+	-	+	-	-	+
Mouse 6	+	-	+	-	-	+
Day 4						
Mouse 7	+	+	+	-	-	+
Mouse 8	-	-	+	-	-	+
Day 5						
Mouse 9	-	-	+	-	-	+
Mouse 10	+	-	+	-	-	+

Table 5.4. Sites of colonization of *C. jejuni* NCTC11168V1G in BALB/c mice. Mice were treated with streptomycin in drinking water (3g/L) for 3 days. On day 0, each mouse orally received 1 X 10^9 CFU of *C. jejuni* NCTC11168V1G in 0.5 ml 0.85% NaCl. Starting 24 hours after the challenge, two mice were euthanized daily and tissue samples were collected from liver, spleen, small intestine, large intestine, and cecum. Fecal samples were collected from cecum to confirm shedding of *C. jejuni* the sampled mice. Tissue samples were treated as described in chickens studies, and the same controls were used. Although all the ceca of the mice challenged had detectable *C. jejuni*, no *C. jejuni* was detected in the liver or spleen of any of the samples, while only two mice had detectable *C. jejuni* in their small intestine, and 6 mice had the bacteria in their large intestine.

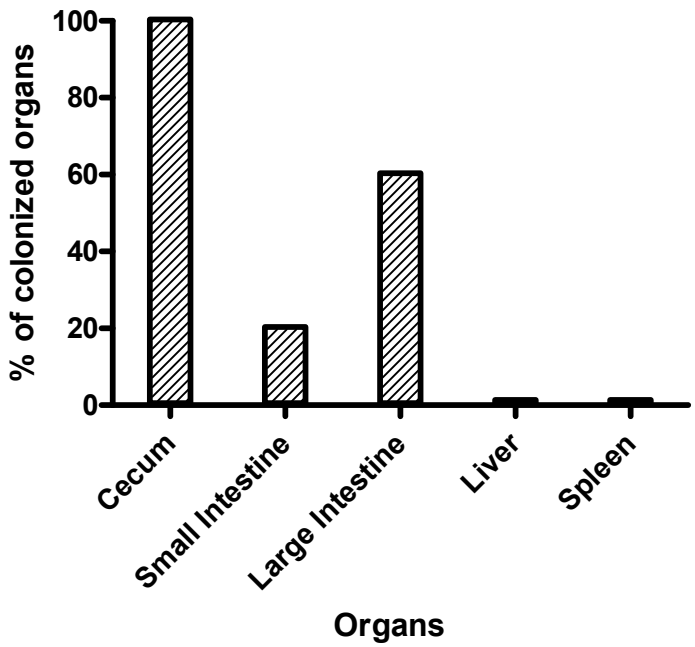

Fig 5.7. Colonization of *C. jejuni* NCTC11168V1G in BALB/c mice. Mice were treated with streptomycin in drinking water (3g/L) for 3 days. On day 0, each mouse orally received 1 X 10^9 CFU of *C. jejuni* NCTC11168V1G in 0.5 ml 0.85% NaCl. Starting 24 hours after the challenge, two mice were euthanized daily and tissue samples were collected from liver, spleen, small intestine, large intestine, and cecum. Fluorescent *C. jejuni* NCTC11168V1G was detected in 100% of the ceca, but in none of the livers or spleens examined. *C. jejuni* was detected in 60% of the large intestine and only 20% of the small intestine examined.

	Small intestine	Large intestine	Cecum	Liver	Spleen	Swab
		Day 1				
Mouse 1	-	-	+	-	-	+
Mouse 2	-	+	+	-	-	+
Mouse 3	-	-	-	-	-	-
Mouse 4	-	-	+	-	-	+
Mouse 5	-	+	+	-	-	+
		Day 3				
Mouse 6	-	-	-	-	-	-
Mouse 7	-	-	+	-	-	+
Mouse 8	+	+	+	-	-	+
Mouse 9	-	-	-	-	-	-
Mouse 10	-	-	-	-	-	-
		Day 6				
Mouse 11	+	+	+	-	-	+
Mouse 12	-	-	+	-	-	-
Mouse 13	-	-	+	-	-	+
Mouse 14	-	-	-	-	-	-
Mouse 15	-	+	+	-	-	+

Table 5.5. Sites of colonization of *C. jejuni* 81-176G in BALB/c mice. Mice were treated with streptomycin in drinking water (3g/L) for 3 days. On day 0, each mouse orally received 1 X 10^9 CFU of *C. jejuni* 81-176G in 0.5 ml 0.85% NaCl. On days 1, 3, and 6 after the challenge, 5 mice were euthanized and tissue samples were collected from liver, spleen, small intestine, large intestine, and cecum. Fecal samples were collected from cecum to confirm the presence of *C. jejuni* in the in feces of sampled mice. Tissue samples were treated as described in chickens studies, and the same controls were used. No *C. jejuni* was detected in the liver or spleen of any of the samples, while only two mice had detectable *C. jejuni* in their small intestine, and 5 mice had the bacteria in their large intestine.

	Small intestine	Large intestine	Cecum	Liver	Spleen	Swab
			Day 1			
Mouse 1	-	-	-	-	-	-
Mouse 2	-	+	+	-	-	+
Mouse 3	-	-	+	-	-	+
Mouse 4	-	-	-	-	-	-
Mouse 5	-	-	+	-	-	+
			Day 3			
Mouse 6	-	-	+	-	-	+
Mouse 7	-	-	-	-	-	-
Mouse 8	-	-	-	-	-	-
Mouse 9	-	+	+	-	-	+
Mouse 10	-	-	-	-	-	-
			Day 6			
Mouse 11	-	+	+	-	-	+
Mouse 12	-	-	+	-	-	+
Mouse 13	-	-	-	-	-	-
Mouse 14	-	-	+	-	-	+
Mouse 15	-	-	-	-	-	-

Table 5.6. Sites of colonization of *C. jejuni* K2-55G in BALB/c mice. Mice were treated with streptomycin in drinking water (3g/L) for 3 days. On day 0, each mouse orally received 1 X 10^9 CFU of *C. jejuni* K2-55G in 0.5 ml 0.85% NaCl. On days 1, 3, and 6 after the challenge, five mice were euthanized and tissue samples were collected from liver, spleen, small intestine, large intestine, and cecum. Fecal samples were collected from cecum to confirm the presence of *C. jejuni* in the in feces of sampled animal. Tissue samples were treated as described in chickens studies, and same controls were used. No bacteria were detected in the spleen, liver, or small intestine of any of the mice sampled. The bacteria were found in 3 of large intestine samples, and 8 ceca.

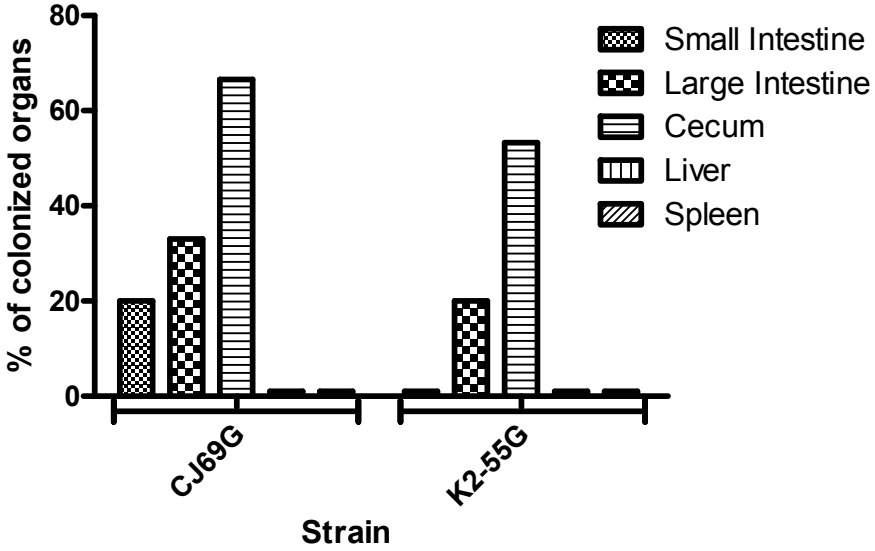

Fig 5.8. Comparison of sites of colonization of *C. jejuni* 81-176G and K2-55G in BALB/c. Two groups of 15 mice each were treated with streptomycin in drinking water (3g/L) for 3 days. On day 0, each mouse orally received 1 X 10^9 CFU of *C. jejuni* 81-176G in 0.5 ml 0.85% NaCl. On days 1, 3, and 6 after the challenge, five mice were euthanized and tissue samples were collected from liver, spleen, small intestine, large intestine, and cecum. Both strains were not detected in any of liver or spleen samples. *C. jejuni* 81-176G was detected in only 20% of small intestinal samples, while *C. jejuni* K2-55G was not detected in any of the small intestine samples. Strain *C. jejuni* 81-176G was detected in 66.6% of sampled ceca while *C. jejuni* K2-55G was detected in only 53.3% of sampled ceca.

5.4. Discussion:

Campylobacter jejuni pathogenesis in poultry is still poorly understood when compared to the pathogenesis of other enteric bacteria. Although there has been an increase in published studies investigating *C. jejuni*, the main focus has been the epidemiology and mechanisms of survival in the environment, and only a limited number of studies investigated pathogenesis. From this limited knowledge, flagella have been identified as a major contributor to *C. jejuni*'s pathogenesis (Guerry, 2007). Flagella are not only a motility organelle, but also serve as a secretion system that exports several sets of proteins (Konkel et al., 1999c; Poly et al., 2007). The lack of appropriate tools for studying the pathogenesis of this unique organism has contributed to low number of these studies. In this study we used a green fluorescent protein reporter system to detect the mucosal and systemic presence of *C. jejuni* 81-176 and its paralyzed flagella mutant at various time points in broiler chickens and BALB/c mice. Two earlier studies have utilized *C. jejuni* that harbored a GFP reporter system; a study by Miller and colleagues that mainly investigated the stability of a plasmid carrying a GFP reporter system. Miller and colleagues, although they did not investigate *C. jejuni*'s pathogenesis, have reported the use of a constitutively expressed *Campylobacter* promoter, which enabled the detection of the fluorescent bacteria without any external substrates or induction and under a variety of environmental conditions (Miller et al., 2000).The study by Konkel and colleagues investigated the cells associated with intraperitoneal infection of mice with *C. jejuni* expressing GFP (Mixter et al., 2003). In our study, the promoter identified by Wosten and colleagues was used (Wosten et al., 1998), along with other elements from the same plasmid used by Miller et al. (Miller et al., 2000). The plasmid pHG101 was very stable *in vitro* after 10 subcultures with and without selective antibiotic pressure in both strains *C. jejuni* 81-176 and K2-55. The plasmid was also very stable *in vivo* based on the results obtained from the various animal trials conducted in broiler chickens and BALB/c mice. The study by Konkel and colleagues drew its conclusions though intraperitoneal administration of the bacteria and collecting peritoneal fluids a few hours later (Konkel et al., 2004), which is a highly artificial challenge method, as the bacteria are introduced to an environment in which they do not exist in under natural infection conditions. The model used in this study uses an oral challenge route that mimics the

natural infection and allows the monitoring of *C. jejuni* dissemination into the various organs of the bird or mouse providing possibly more relevant information.

C. jejuni K2-55 and K2-37 expressed a full-length uni- or bipolar flagella that appeared to be intact, but were paralyzed in both strains, resulting in a very limited motility. Both strains did not secret any *Campylobacter* invasion antigens that were detectable in secretion assays, which allowed us to investigate the importance of these proteins in pathogenesis of *C. jejuni*. The most noticeable difference was the detection of the wild-type *C. jejuni* 81-176 in liver and spleen, while *C. jejuni* K2-55 was not detectable in the internal organs of the birds challenged. *Campylobacter* invasion antigens have a role in internalization (Rivera-Amill and Konkel, 1999) and invasion of cell lines (Rivera-Amill et al., 2001), and a deletion mutation of *ciaB* gene reduces the organism's ability to colonize the ceca of broiler chickens (Ziprin et al., 1999). In this study, a challenge dose of 1 X 10^6 of *C. jejuni* K2-55 was able to colonize the ceca of challenged birds at levels that had no statistically significant difference from the level of colonization of the wild-type parent strain (Fig 5.5). The difference in the outcome of these experiments could be due to the strain-to-strain variation and experimental design. The *C. jejuni* K2-55 strain used in this study has a different genetic background than the strain used by Ziprin et al. (Ziprin et al., 1999). Another important factor is that the strains used in this study had an insertion mutation in *pflA* gene, while the strain used by Ziprin and colleagues had a deletion mutation of the *ciaB* gene. Although both strains had no detectable *Campylobacter* invasion antigens in the culture supernatant, *ciaB* could have functions other than producing the secreted protein that might have affected colonization. Also, the deletion mutation might have had another effect that did not affect bacterial growth *in vitro* but was detrimental *in vivo*. In this study, the lack of *Campylobacter* invasion antigens proteins and motility contributed to the reduced ability of *C. jejuni* K2-55G in the cecal tissues when compared to the wild-type *C. jejuni* 81-176G which was present in all of the ceca examined (Tables 5.2 and 5.3). These finding are in agreement with results reported in the literature that loss of motility did not affect colonization level (Wassenaar et al., 1993). It is the loss of flagellar structure that impairs intestinal colonization (Fernando et al., 2007; Nachamkin et al., 1993). A difference in the colonization ability of *C. jejuni* K2-55 of day-old chicks and 21-days old chickens

was observed. The mutant was able to colonize all day-old chickens after one week of infection at a dose of $2X10^8$ CFU, while only 81% of 21-days old birds were colonized in total. The changes in immune system, gut physiology and microflora might have influenced the ability. The difference in experimental design might also had an effect, as birds were euthanized daily from the 21-days old chickens challenged with *C. jejuni* K2-55, reducing the density of the birds, therefore reducing the chances of bird-to-bird transmission.

C. jejuni colonization of the ceca did not necessarily extend to the small and large intestine for either *C. jejuni* 81-176 or K2-55. Although 100% of the ceca of birds challenged with *C. jejuni* 81-176 had detectable *C. jejuni*, only 62% of the large intestines and 43% of the small intestines had detectable *C. jejuni*. *C. jejuni* K2-55 had the same pattern of colonization and was detectable in 81% of the ceca, but only in 43% of the large intestine and 25% of the small intestine. These finding are in agreement with the results obtained using strains *C. jejuni* NCTC11168V1 and V26 in Chapter IV, and confirms that the ceca is the main colonization site for *C. jejuni*. These finding are also in agreement with the literature where numbers of *C. jejuni* were found significantly higher in the ceca than in other parts of the intestinal tract (Subler et al., 2006; Wallace et al., 1997). The chicken's ceca are blind–ended sacs extending from the proximal end of the colon at its junction with small intestine. They are the perfect environment for *C. jejuni* to thrive as they are abundant in volatile fatty acids and other nutrients, and they have a very limited intestinal motility compared to other parts of the chicken's intestine which has a bi-directional movement and only empty once every 24-48 hours (Clench, 1995).

In this study, fluorescent *C. jejuni* was only detected in the lumen and at the surface of the intestinal epithelium. Although many bacteria were present, no inflammatory reaction was observed when tissue sections were examined using light and transmission microscopes, confirming similar findings in the literature (Beery et al., 1988; Meinersmann et al., 1991; Van Deun et al., 2008).

C. jejuni 81-176 and K2-55 colonized the intestinal tract of streptomycin treated BALB/c mice, but were not detectable in the spleen or liver of the mice. The absence of motility and *Campylobacter* invasion antigens affected the ability of *C. jejuni* K2-55 to colonize the mice, as only 53.3% of the ceca were colonized compared to 66.6%.

Researchers have used a variety of BALB/c mouse models making it difficult to draw a direct comparison. *C. jejuni* spread systemically to spleen and liver in germ-free and immuno-deficient mice (Chang and Miller, 2006; Jesudason et al., 1989), but not in this study.

In conclusion, the presence of flagella was sufficient for *C. jejuni* to colonize the intestine of broiler chickens and BALB/c, while the absence of motility and *Campylobacter* invasion antigens had an impact only on the systemic spread to the liver and spleen of broiler chickens.

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Chapter VI: Vaccination of Broiler Chickens Against *Campylobacter jejuni*

6.1. Introduction:

Campylobacter jejuni is the most common cause of human bacterial gastroenteritis in North America. Ingestion of few hundred organisms is enough to cause the onset of the clinical signs (Robinson, 1981) which includes abdominal cramps, fever, and diarrhea (Drake et al., 1981).

Consumption or handling of poultry meat that is contaminated with *C. jejuni* has been identified as the main source of infections in humans (Grant et al., 1980; Petersen et al., 2001). *C. jejuni* can be introduced into broiler houses via various sources, including flies and other insects (Rosef and Kapperud, 1983), contaminated water (Kazwala et al., 1990; Pearson et al., 1993), rodents (Annan-Prah and Janc, 1988), and farm workers (Annan-Prah and Janc, 1988; Kazwala et al., 1990). Once *C. jejuni* has been introduced into the chicken farm it spreads throughout the flock (Shanker et al., 1988, 1990), and by the end of the production cycle almost 100% of the chickens shed the bacteria (Shanker et al., 1988). Various studies have shown that fresh poultry meat products are frequently contaminated with *C. jejuni*, and depending on the study and the method of sampling, up to 98% of poultry products tested were positive (Madden et al., 1998; Pearson et al., 2000).

Several methods have been used in an attempt to control *C. jejuni* levels in the poultry products. These methods include on-farm strategies such as water chlorination and bacteriophages in feed, and post-harvest strategies such as chemical treatment of poultry products. Given the difficulty of controlling the sources of *C. jejuni* on the farm using biosecurity measures only, and public concerns over adding chemicals to poultry products, vaccination remains the most feasible alternative approach (de Zoete et al., 2007; Wagenaar et al., 2006). The exact type of immune response that is protective in broiler chickens is not known. Several studies have correlated increased levels in the serum of IgG and IgA directed against several *C. jejuni* antigens with a decrease in shedding of *C. jejuni* (Cawthraw et al., 1994; Myszewski and Stern, 1990). Antibodies purified from the bile of chickens colonized by *C. jejuni* and given to naïve birds have increased the infective dose by 50% (Stern et al., 1990). Generally, antibodies are generated against *C. jejuni* flagellin within 2-3 weeks, but it can take up to 8 weeks to

generate an immune response to other *C. jejuni* proteins such as outer membrane proteins (OMP) (Cawthraw et al., 1994; Rice et al., 1997). The successful vaccine should also be cross protective as the same chicken flock is usually colonized by more than one strain of *C. jejuni* (Denis et al., 2008).

In this study, the ability of vaccination with a *C. jejuni* paralyzed flagella mutant to reduce the carriage of wild-type in broiler chickens was evaluated in a variety of challenge models and in combination with antibiotic treatment.

6.2. Materials and Methods:

6.2.1. Bacterial strains and growth conditions:

Three *C. jejuni* strains were used for vaccination studies. These include strain *C. jejuni* 81-176, strain *C. jejuni* K2-55, which were a generous gift from Dr. P. Guerry (Naval Medical Research Institute, Bethesda, Md.) and strain *C. jejuni* NCTC11168 variant 1 genetic background (Carrillo et al., 2004). Strain *C. jejuni* K2-55 shares the same genetic background of strain *C. jejuni* 81-176 but with an insertion mutation in *pflA* gene (Yao et al., 1994). These strains are described in detail in chapter III.

C. jejuni strains were cultured on Mueller-Hinton agar (MHA), (Difco), or Mueller-Hinton agar supplemented with 5% sheep blood (PML Microbiological, Richmond, BC) under microaerophilic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) at 42°C.

The spontaneous streptomycin-resistant (SMR) isolates of *C. jejuni* were obtained by plating 10^9-10^{10} CFU/ ml on MHA plates amended with 100 µg streptomycin (MHA-SM). The colonies that grew were restreaked on MHA-SM plates to confirm their resistance.

6.2.2. Detection of *C. jejuni* in the ceca of infected chickens:

Immediately after the birds were euthanized, the ceca were aseptically removed and placed in individual, sterile Petri dishes. To determine the number of colony forming units per gram of cecal contents, approximately 0.2-0.4 grams of cecal content were aseptically collected from each cecum, and weighed. Contents were diluted 1:10 using ice-cold 0.85% NaCl, and were vortexed for 10 seconds each. From each sample, dilutions of 10^{-1} to 10^{-6} were made, and 25 µl from each dilution were plated on one-third

of dry Karmali agar (Oxoid). Plates were incubated under microaerophilic conditions at 42 °C for 48 hours. Chickens that had no detectable colonies were assigned the value of 400 CFU/g (level of detection).

6.2.3. Collection of serum from blood samples:

Blood from each bird was collected from the external jugular vein into blank Beckton-Dickinson (BD) Microtainer micro-vacutainer tubes (BD Diagnostics, Oakville, ON). Tubes were incubated overnight at room temperature, and centrifuged at 8000 x *g* in a table top centrifuge at room temperature. Serum was collected, stored in cryovials and stored at -20 °C.

6.2.4. Collection of cecal contents for determination of IgA level:

Ceca collected for detection of *C. jejuni* were also used to determine IgA level in cecal contents. From each bird, 0.2-0.4 g of feces were diluted 4 times in PBS and vortexed for 30 seconds. Samples were centrifuged in a tabletop centrifuge at 12,000 X g for 10 minutes, and the supernatant was collected and stored at -20 °C.

6.2.5. Treatment of birds with streptomycin in drinking water:

When appropriate, birds were treated with streptomycin in drinking water (Myhal et al., 1983; Wadolkowski et al., 1990). For each treated group, 3g/L streptomycin (SIGMA-ALDRICH, Oakville, ON) was added to the drinking water after the birds were deprived for two hours of drinking water. Any water left was disposed after two hours of adding streptomycin. Birds were treated on days 25, 26, and 27 of the experiment.

6.2.6. Enzyme Linked Immuno-Sorbent Assay (ELISA):

Whole-cell *C. jejuni* NCTC1168V1 or K2-55 were used to coat ELISA plates. Bacteria were grown overnight from frozen stock. A sterile swab was used to restreak growing bacteria on MHA agar supplemented with 5% sheep blood. The plates were grown for 18 hours at 42 °C to obtain confluent growth. Cells were harvested into 5 ml 0.85% NaCl and resuspended in Tris-buffered saline (TBS) to an absorbance of 0.25 at 600 nm wavelength ($O.D_{600}$). Immunolon HB2 plates (Dynatech) were coated using 100µl of antigen and incubated for 24 hours at 4 °C. Plates were washed 8 times with distilled water. To block the plates, 100 µl of TBS containing 0.05% Tween-20 (Fisher

Scientific) and 0.5% gelatin (TBS-T) (Fisher Scientific) were added to each well and plates were incubated over night at 4 °C. Plates were washed 8 times with distilled water and 100 µl of TBS-T were added to all wells except those in the first row. To each well of the first row, 200 µl of serum or feces containing primary antibody diluted 1:20 in TBS-T were added and doubling dilutions were made. Plates were incubated for 1 hour at room temperature, and then washed 8 times with distilled water. Alkaline phosphatase-conjugated goat anti-chicken immunoglobulin (IgG 200 ng/ml or IgA 50 ng/ml; Bethyl Laboratories Inc, Montgomery, TX, USA) were used to detect immunoglobulin (Ig) captured by the coating antigen. Anti-IgG antibody was used at a 1:7500 dilution in TBS-T, while anti-IgA was used at a 1:5000 dilution. To each well, 100 µl of secondary antibody was added. Plates were incubated for 1 hour at room temperature, and then washed 8 times using distilled water. Alkaline phosphatase activity was detected using the substrate *p*-nitrophenyl phosphate (PNPP; Sigma, St. Louis, MO, USA), which was prepared immediately prior to use at 3 mg/ml in DE buffer (1M diethanolamine, 0.5 mM magnesium chloride, pH 9.5). To each well, 100 µl of PNPP solution was added. The absorbance was determined at 405 nm wavelength with a reference of 490 nm wavelength in a Bio Rad microplate reader (model # 1350) after 1 hour for serum IgG and after an overnight incubation for intestinal IgA. Serum IgG titers were calculated by the intersection of least-square regression of absorbance at 405 nm wavelength versus the logarithm of dilution, while the absorbance at 405 nm wavelength was used to represent IgA activity in cecal contents.

6.2.7. Animal Experiments:

All procedures involving animals were carried out using protocols that were approved by the University of Saskatchewan Committee on Animal Use and Supply and followed the guidelines established by the Canadian Council for Animal Care.

6.2.8. Statistical analysis:

One-Way Analysis of Variance (ANOVA), assuming non-parametric data distribution, was used for analysis of level of colonization (CFU/g) as well as IgG and IgA antibody levels obtained from ELISA readings.

6.3. Vaccination of Broiler Chickens:

C. jejuni strain K2-55 was used as the vaccine strain. Bacteria were grown, from a pure frozen stock, on MHA supplemented with 5% sheep blood for 24 hours under microaerophilic conditions in a CO_2 incubator at 42 °C. A sterile swab was used to restreak growing bacteria on MHA agar supplemented with 5% sheep blood and incubated for approximately 18 hours to obtain confluent growth. Cells were harvested into 5 ml 0.85%NaCl/plate and diluted in normal saline so that each vaccine dose contained 1 x 10^5 CFU/0.5 ml. The vaccine was delivered orally using a ball taped needle. From each batch of prepared vaccine 0.5 ml were used to confirm CFU/ml. The viable cell count was determined as previously described.

6.3.1. Evaluation of *C. jejuni* K2-55 ability to protect chickens from *C. jejuni* 81-176 (homologous) infection:

Four groups each consisting of 30 day-old chickens each were housed at the VIDO Animal Care Facility. Groups were housed separately for the duration of the experiment as illustrated in Fig 6.1. On the day of arrival, 10 cloacal swabs were collected from each group to confirm that the birds were *C. jejuni* free. On day 7, groups 1, 2, and 3 were vaccinated as described above (Fig 6.1 and Table 6.1). Cloacal swabs for bacterial culture and blood samples for determination of serum antibodies were collected from 8 birds. From each group, 4 birds were euthanized and the ceca were collected and used to determine the level of *C. jejuni* colonization (CFU/gram counts) as described in chapter II. Cecal contents were also used to determine the level of IgA activity. On day 21, groups 1, 2, and 3 received another vaccination as a booster, and the same sampling procedure was repeated (Fig 6.1 and Table 6.1). On days 25, 26, and 27, Groups 2 and 4 were treated with streptomycin at a dose of 3g/L of drinking water, and on day 28, groups 1, 2, and 4 received a challenge dose of 1 x 10^5 of streptomycin-resistant *C. jejuni* 81-176. The challenge strain was grown as described for the vaccine strain (*C. jejuni* K2-55) and the same sampling procedure was repeated. On day 35, four birds from each group were sampled as described for day 7. Cecal contents from groups 1, 2, and 4 were plated on Karmali agar that was supplemented with kanamycin, while contents from group 2 were plated on unsupplemented Karmali agar. On day 40, all remaining birds were euthanized after collecting blood samples, and cecal contents were plated as described above to obtain CFU/g for each bird (Fig 6.1 and Table 6.1).

6.3.2. Evaluation of the ability of vaccination with *C. jejuni* K2-55 ability to protect chickens from *C. jejuni* NCTC1168V1 (heterologous) infection:

The same experimental design for protection against *C. jejuni* 81-176 was followed as described above (Fig 6.1), with the exception of the challenge strain. Additionally, each sample from the cecal contents from groups 2 and 3 were plated on media with and without streptomycin.

6.3.3. Evaluation of K2-55 ability to protect chickens from *C. jejuni* 81-176 (homologous) infection using a seeder challenge model:

A seeder model is intended to mimic natural mode of infection and is intended mainly to study horizontal transmission of the bacteria (Lowry et al., 1999). In a seeder model, a group of chickens are challenged with a precise dose of *C. jejuni* orally, and introduced to a group of uninfected/vaccinated chickens (Fig 6.2). Three groups of 20 chickens each were housed separately. All birds were numbered. Groups 1 and 3 received the first vaccination on day 7 and a booster dose on day 21. Group 2 did not receive any vaccination and served as unvaccinated control. These 3 groups were treated with streptomycin on days 25, 26, and 27. On day 28 all birds from group 3 were euthanized and birds from group 1 and 2 were housed together (Fig 6.2). Blood samples and cecal contents were collected from group 3. A fourth group of 10 *Campylobacter*-free chickens received $1x10^9$ CFU/0.5 ml of *C. jejuni* 81-176 challenge orally on day 28 and housed with groups 1 and 2. Blood samples were collected from 6 birds from each group on day 35. On day 42 all birds were euthanized. Blood samples were collected from all remaining birds and serum was collected as described above (Fig 6.2). Cecal contents were collected and plated as described above after euthanization.

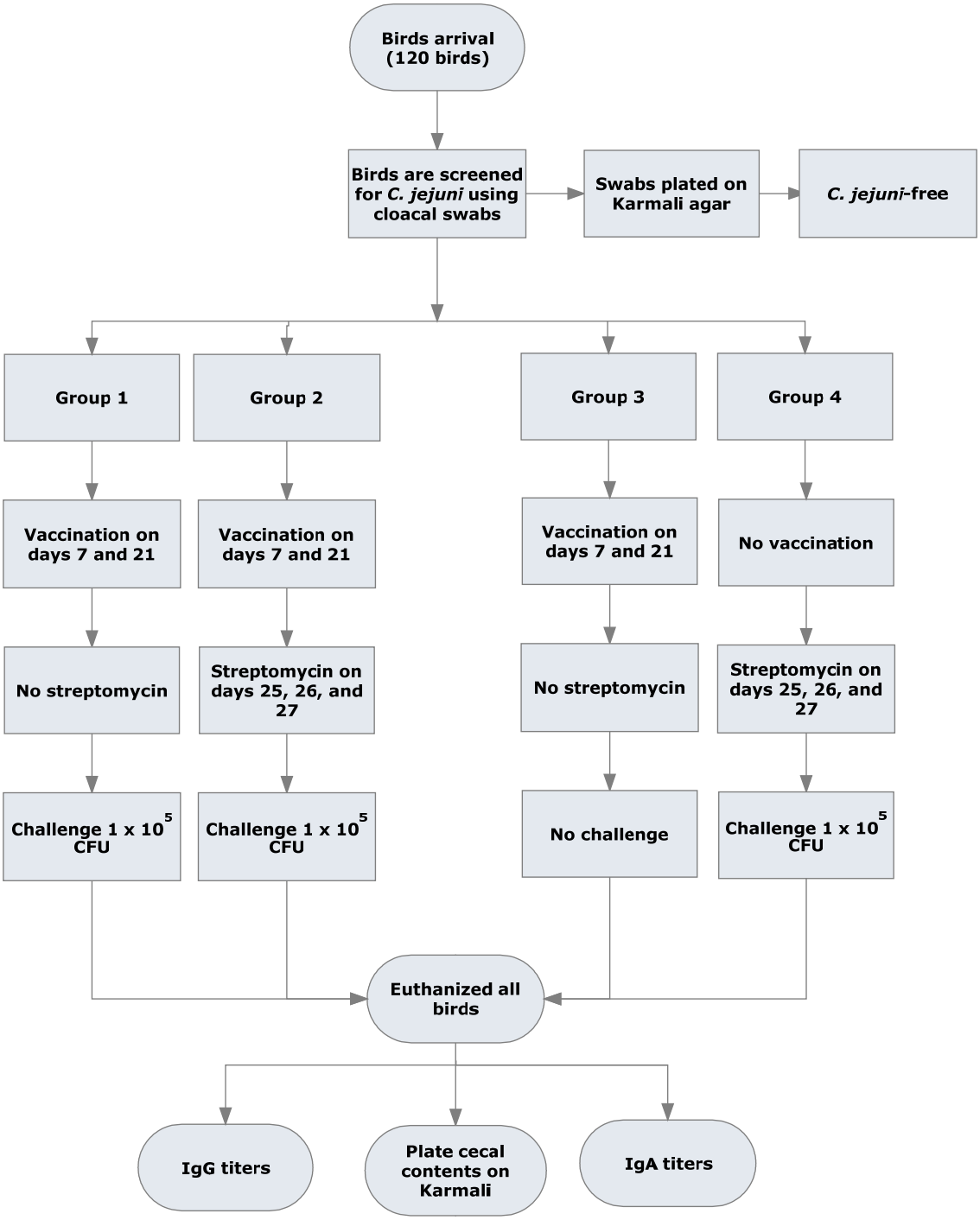

Fig 6.1. Overview of experimental design to evaluate the ability of *C. jejuni* K2-55 to protect broiler chickens against colonization with the wild-type. Four groups of 30 day-old chickens each were housed separately. Birds were vaccinated using 1 X 10^5 CFU/0.5 ml of *C. jejuni* K2-55 orally on day 7 of age and a booster of the same strain on day 21 of age, and challenged with 1 X 10^5 CFU/0.5 ml on day 28 were applicable. For a complete description please refer to section 6.3.1.

Day	Procedure
Day 1	• Chickens arrived and screened for *C. jejuni*
Day 7	• Vaccination of groups 1, 2, and 3. Group 4 remained *C. jejuni* free. • Cecal contents, blood, and cloacal swabs were collected
Day 21	• Vaccination of groups 1, 2, and 3. Group 4 remained *C. jejuni* free. • Cecal contents, blood, and cloacal swabs were collected
Day 28	• Challenge of groups 1, 2, and 4 • Group 3 was not challenged • Cecal contents, blood, and cloacal swabs were collected
Day 35	• Cecal contents, blood, and cloacal swabs were collected
Day 40	• All remaining birds were euthanized. • Cecal contents and blood were collected

Table 6.1. Experimental design used to evaluate the ability of vaccination with *C. jejuni* K2-55 to protect chickens from *C. jejuni* 81-176 (homologous) infection. Each group contained 30 birds in total. Chickens were vaccinated orally using a dose of 1 x 10^5 CFU/0.5 ml normal saline and challenged orally with 1 x 10^5 CFU/0.5 ml *C. jejuni* 81-176. Cloacal swabs were used for screening chickens for *C. jejuni* shedding. The same experimental design was followed to evaluate the protection provided by C. jejuni K2-55 against a heterologous challenge with *C. jejuni* NCTC11168V1. Full description of the experiments is provided in sections 6.3.1 and 6.3.1.

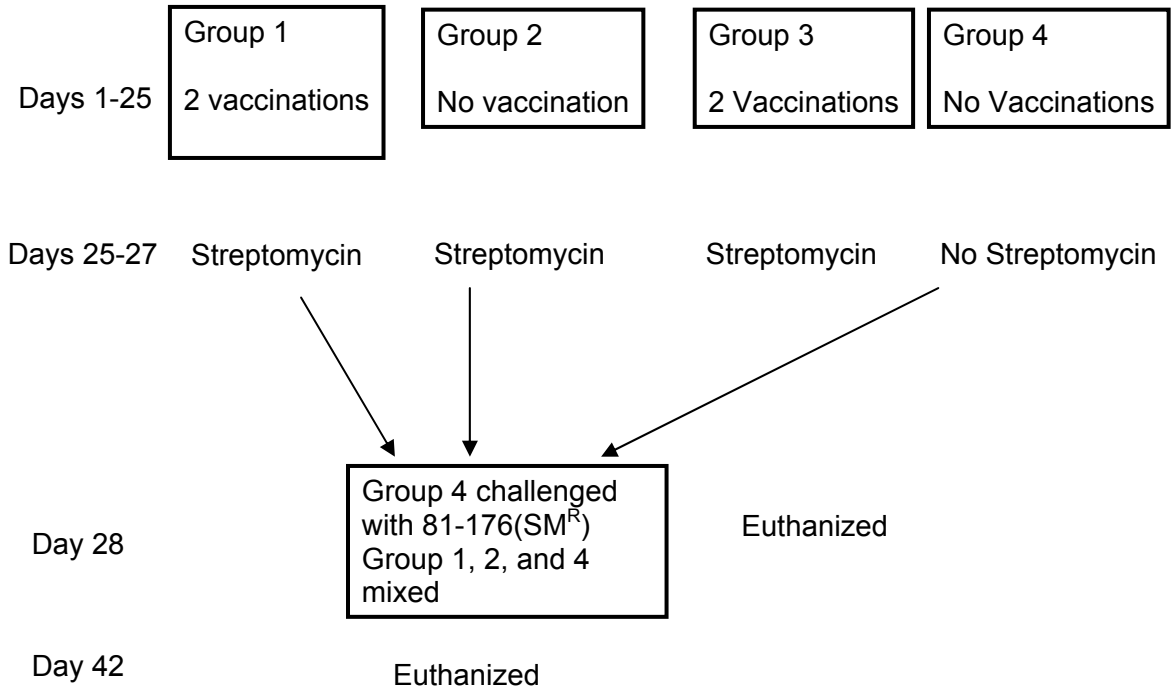

Fig 6.2. Experimental design for evaluation of *C. jejuni* K2-55 (paralyzed flagella mutant) ability to protect broiler chickens using a seeder model. Birds in groups 1 and 3 received two vaccinations orally on day 7 and 21. Each vaccine dose consisted of 1 X 10^5 CFU/0.5 ml of normal saline. Groups 2 and 4 received no vaccination. On days 25, 26, and 27 birds in groups 1, 2, and 3 received streptomycin in drinking water to eliminate the vaccine strain *C. jejuni* K2-55. On day 28 of the experiment all birds from group 3 were euthanized, and birds from group 4 received 1 X 10^9 CFU/0.5 ml *C. jejuni* 81-176. Birds from groups 1, 2, and 4 were housed in the same room thereafter. On day 42 all birds were euthanized and cecal contents were collected. Full description of the experiment is provided in section 6.3.3.

6.4. Results:

6.4.1. Vaccination of broiler chickens with *C. jejuni* K2-55:

Motility and proteins secreted via the flagella export apparatus are important in *C. jejuni* pathogenesis of broiler chickens. The vaccine strain *C. jejuni* K2-55 used in this study has paralyzed flagella and does not secret *Campylobacter* invasion antigens (see sections 5.3.3 and 5.3.5). The first step in evaluating the vaccine strain was to study the level at which it colonizes the cecum throughout a broiler chicken's life. Results from group 3 in the experiment described in section 6.3.1were used to establish the pattern of *C. jejuni* K2-55 colonization for the duration of the experiment. Chickens that received $1x10^5$ CFU/0.5 ml of the *C. jejuni* K2-55 vaccine strain orally on day 7 of age and a booster vaccination of the same strain on day 21 of age remained colonized for the duration of the experiment as shown in Fig 6.3. No statistically significant change in the median of *C. jejuni* recovered from the cecal contents on days 21, 28, 35, and 40. *C. jejuni* K2-55 CFU counts (Fig 6.3) were 2 logs lower on day 40 than on day 35 ($2 \text{ X } 10^6$ versus $1.3 \text{ X } 10^8$ respectively).

6.4.2. Evaluation of *C. jejuni* K2-55 ability to protect chickens from *C. jejuni* 81-176 (homologous) infection:

To evaluate the ability of the paralyzed flagella mutant *C. jejuni* K2-55 to protect broiler chickens from subsequent *C. jejuni* infection, three groups of chickens were vaccinated with *C. jejuni* K2-55 strain and challenged with *C. jejuni* 81-176 strain. Each bird in groups 1 and 2 received $1 \text{ X } 10^5$ CFU/0.5 ml of *C. jejuni* K2-55 orally on day 7 of age and a booster of the same strain on day 21 of age. Birds in group 2 were treated with streptomycin in drinking water on days 25, 26, and 27 of the experiment to clear the vaccination strain. Birds in group 4 received no vaccination, and all groups were orally challenged with $1 \text{ X } 10^5$ of streptomycin-resistant *C. jejuni* 81-176 isolate. Chickens in groups 1 and 2 were colonized by vaccination strain *C. jejuni* K2-55 as shown in Fig 6.4. The mean of colonization in both groups was very close as $1.8 \text{ X } 10^7$ and $1.4 \text{ X } 10^7$ CFU were detected on day 21. No *C. jejuni* was detectable in the ceca of group 4 chickens on day 21, indicating that the chickens remained *C. jejuni*-free as intended and no environmental contamination occurred.

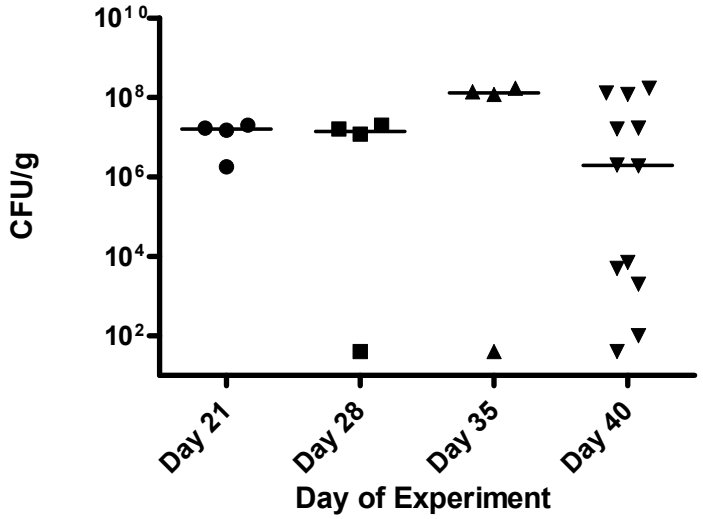

Fig 6.3. Colonization of broiler chickens by *C. jejuni* K2-55 (vaccine strain). Each chicken received a vaccination of 1 X 10^5 CFU/0.5 ml of *C. jejuni* K2-55 orally on day 7 of age and a booster of the same strain on day 21 of age. To serve as a vaccinated control, this group received no challenge with the wild-type strain. Cecal contents were collected on days 7, 21, 28, and 35 from 4 birds and on day 40 from all remaining birds and plated on Karmali plates to determine the level of colonization. Full details of the experiment are found in section 6.3.1 and Fig 6.1.

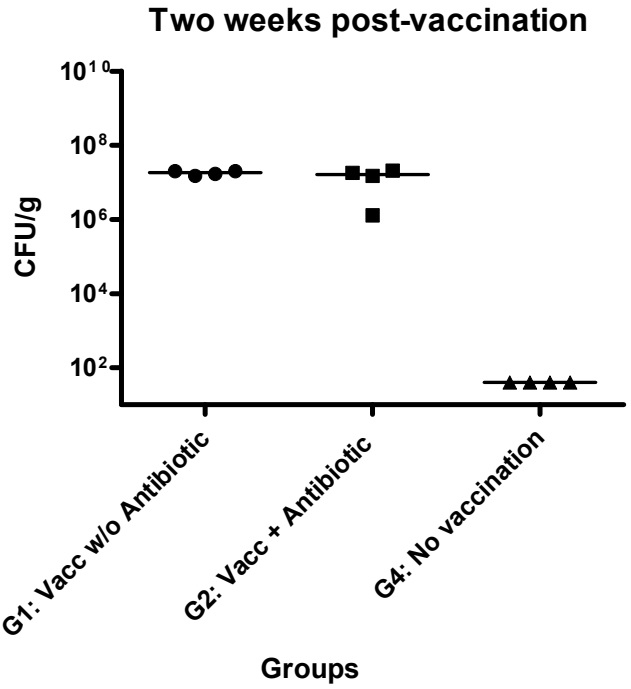

Fig 6.4. Colonization of broiler chickens by *C. jejuni* K2-55 on day 21 of the experiment. Each chicken received 1 X 10^5 CFU/0.5 ml orally on day 7 of age. Cecal contents from 4 birds were collected from each group on day 21 and plated on Karmali agar plates to determine *C. jejuni* colonization level. Full details of the experiment are found in section 6.3.1 and Fig 6.1.
G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G4: No Vaccination: Group 4: No vaccination.

No *C. jejuni* K2-55 was detected in the cecal contents of chickens in group 2 (vaccinated twice and was not challenged at this point) after three days of treatment with streptomycin in drinking water when samples were collected on day 28 (Fig 6.5-A). Chickens in group 4 (challenge control, did not receive any vaccination) remained *C. jejuni*-free as no colony forming units were detectable in the cecal contents of birds sampled or cloacal swabs collected (Fig 6.5-A). Chickens in group 1 (vaccinated twice, was not treated with streptomycin) remained colonized with vaccine strain *C. jejuni* K2-55, and the median for the 4 chickens sampled was 1.3 X 10^7 CFU/g of cecal contents (Fig 6.5-A).

The cecal contents of the 4 birds sampled from each group on Day 35 (one week post-challenge) were plated on streptomycin-supplemented Karmali agar plates to determine the level of colonization of streptomycin-resistant *C. jejuni* 81-176. The birds in group 1 (vaccinated twice and did not receive any streptomycin) had a median of 1 X 10^7 CFU/g of cecal contents of *C. jejuni* 81-176 (Fig 6.5-B). Birds in group 2 (vaccinated twice and received streptomycin treatment) had a median of 0.5 X 10^2 CFU/g of cecal contents as shown in Fig 6.5-B. The median of *C. jejuni* detected in cecal contents of birds in group 4 (was 2 X 10^8, which is comparable to group 1 (Fig 6.5-B).

All remaining birds were euthanized on day 40, and cecal contents were plated from each bird on Karmali agar plates supplemented with streptomycin to detect *C. jejuni* 81-176. Groups 1(vaccinated twice and was not treated with streptomycin) and 4 (challenge control, did not receive any vaccination) had comparable medians of CFU/g of cecal contents, as group 1 had 1 X 10^8 and group 4 had 2 X 10^8 (Fig 6.5-C). Birds in group 2 (vaccinated twice and treated with streptomycin) had significantly lower ($P < 0.05$) median of CFU/g of cecal contents compared to groups 1 and 4 on day 40, as only 1.3 X 10^2 CFU/ were detected as shown in Fig 6.5-C.

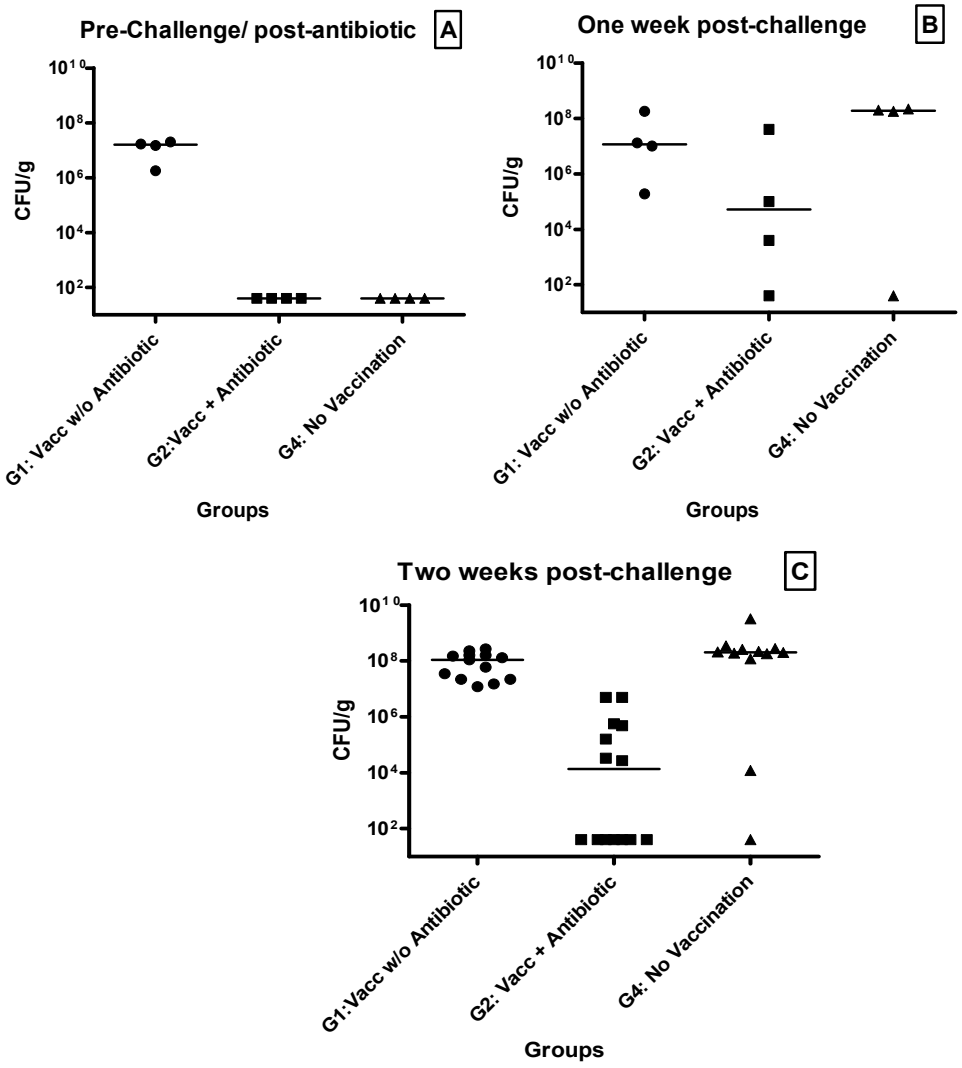

Fig 6.5. Colonization of broiler chickens by *C. jejuni* K2-55 (vaccination strain) on day 28 (A), and 81-176 on day 35 (B) and 40 (C) of the experiment. Group 1 (G1: Vacc w/o Antibiotic) contained birds that received vaccination but were not treated with streptomycin. Group 2 (G2: Vacc + Antibiotic) contained birds that were vaccinated and treated with streptomycin. Group 4 (G4: No Vaccination) contained birds that were not vaccinated but were challenged with the wild-type strain later in the experiment (challenged-unvaccinated control). Each bird in group 1 and 2 received 1 X 10^5 CFU/0.5 ml of vaccine strain K2-55 orally on day 7 of age and a booster on day 21 of age. Birds in groups 2 and 4 were treated with streptomycin in drinking water on days 25, 26, and 27. Cecal contents were collected from groups 1, 2, and 4 on day 28 of the experiment (prior to challenge and post streptomycin treatment), day 35 (one week post-challenge), and day 40 (12 days post-challenge) and plated on Karmali agar plates (A) and Karamali agar supplemented with streptomycin (B and C). Chickens sampled from Group 2 had no detectable *C. jejuni* in cecal contents sampled (A). Group 4 remained *C. jejuni* free until day 28 (A). G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.1 and Fig 6.1.

6.4.3. ELISA:

Serum was collected from blood samples and ELISA was performed as described in the materials and methods section to determine the activity level of IgG antibodies. IgA antibody activity in cecal contents was determined using a similar ELISA protocol but plates were incubated overnight with the enzyme substrate (DNPP) to allow for the proper color intensity to develop.

The pre-vaccination IgG titers in the sera of the birds sampled on day 7 were comparable (Fig 6.6-A) as the medians were not significantly different when tested using one-way ANOVA. When IgG activity in the sera of the birds was tested on day 21, no significant difference in titers of groups 1, 2, and 4 was found when they were compared to each other. However, the titers of birds in group 3 (birds that were vaccinated but not challenged with the wild-type over the duration of the experiment), were significantly higher ($P < 0.05$) than those of group 4 (Fig 6.6-A). IgG titers in group 3 were not significantly higher than group 1 or 2. The same trend continued on day 28 (Fig 6.7-A), as IgG titers in group 3 remained higher than those of groups 2 and 4 ($P < 0.05$), but not significantly higher than the titers in group 1. On day 35 (Fig 6.7-B), birds in group 3 had significantly higher ($P < 0.5$) IgG titers than the birds sampled in all other groups. No significant difference was found among the other groups although the titers of group 4 (did not receive vaccination, challenged on day 28) were lower.

IgA activity level in the cecal contents of chickens did not change over the course of the experiment and no significant difference among the 4 groups was observed (Fig 6.9-6.12). The cut-off point for a sample optical density (O.D.) to be considered positive was double the O.D. of the negative control.

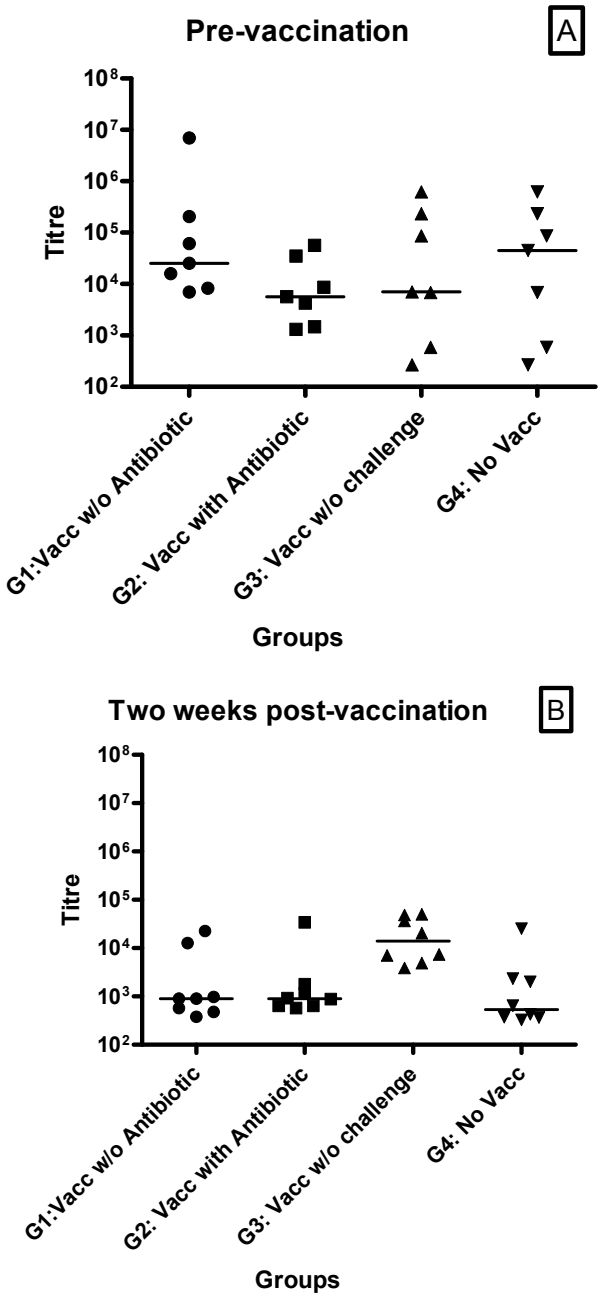

Fig 6.6. IgG antibody activity in the sera of: 7 day old chickens (A) (pre-vaccination) and 21 day old chickens (B) (two weeks post-vaccination and prior to vaccine boost). From each group 8 blood samples were collected and processed to generate serum. Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination. None of the groups received streptomycin treatment up to this point. Chickens were found to be *C. jejuni* free using cloacal swabs (A). G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc w/o challenge: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.1 and Fig 6.1.

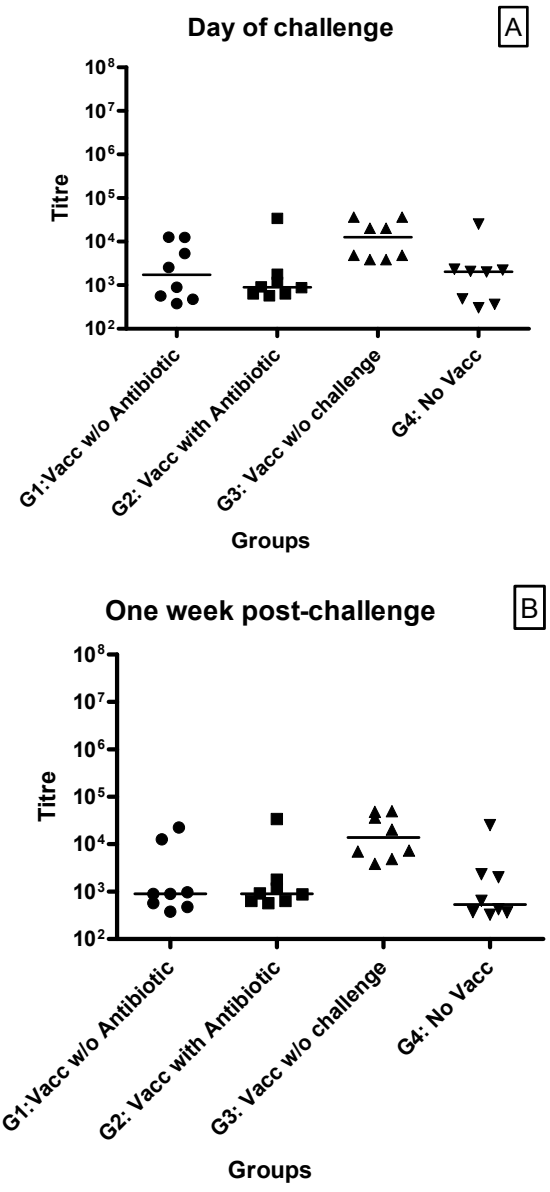

Fig 6.7. IgG antibody activity in the sera of 28 days-old chickens. (A) (one week after a vaccine boost and day of challenge with *C. jejuni* 81-176) and 35 day old chickens (B) (one week post challenge with *C. jejuni* 81-176). From each group 8 blood samples were collected and processed to generate serum. Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination. Analysis using one-way ANOVA showed significant difference ($P < 0.05$) in IgG antibody activity in the sera of group 3 compared to group 4 (A). On day 35, group 3 had significantly higher ($P < 0.05$) IgG titers than all other groups (B). Titers among other groups were not significantly different. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc w/o challenge: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.1 and Fig 6.1.

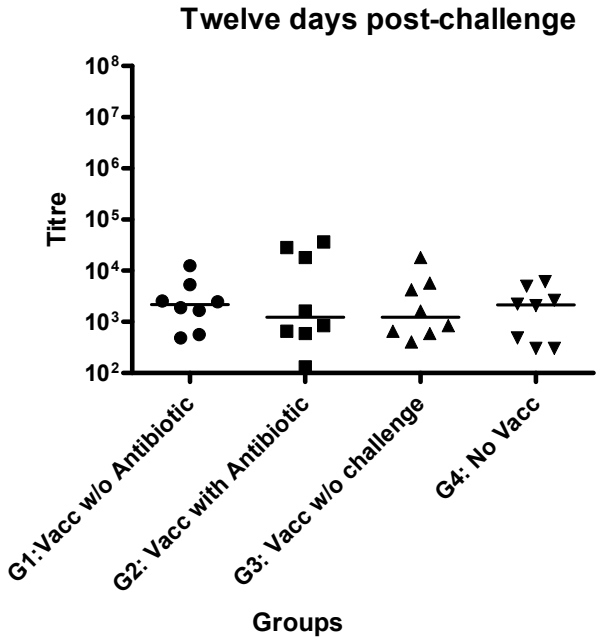

Fig 6.8. IgG antibody activity in the sera of chickens 12 days post-challenge. From all the birds remaining in each group blood samples were collected and processed to generate serum. Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination. Analysis using one-way ANOVA showed no significant difference among the groups IgG titers. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc w/o challenge: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.1 and Fig 6.1.

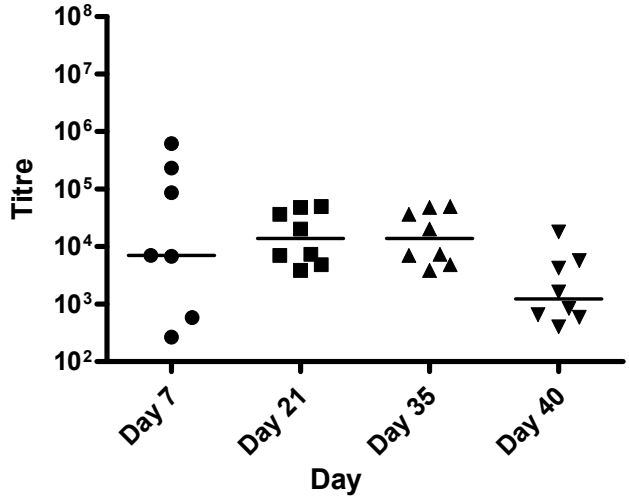

Fig 6.9. IgG antibody activity in the sera from chickens vaccinated with *C. jejuni* K2-55 for the duration of the experiment (Group 3, which received two vaccinations and no challenge or antibiotic treatment). Blood samples were collected from 8 chickens on each time point. Analysis using one-way ANOVA showed no significant difference among the time points. Full details of the experiment are found in section 6.3.1 and Fig 6.1. Day 7: day of first vaccination, Day 21: day of second vaccination, Day 35: one week post-challenge, Day 40: twelve days post-challenge.

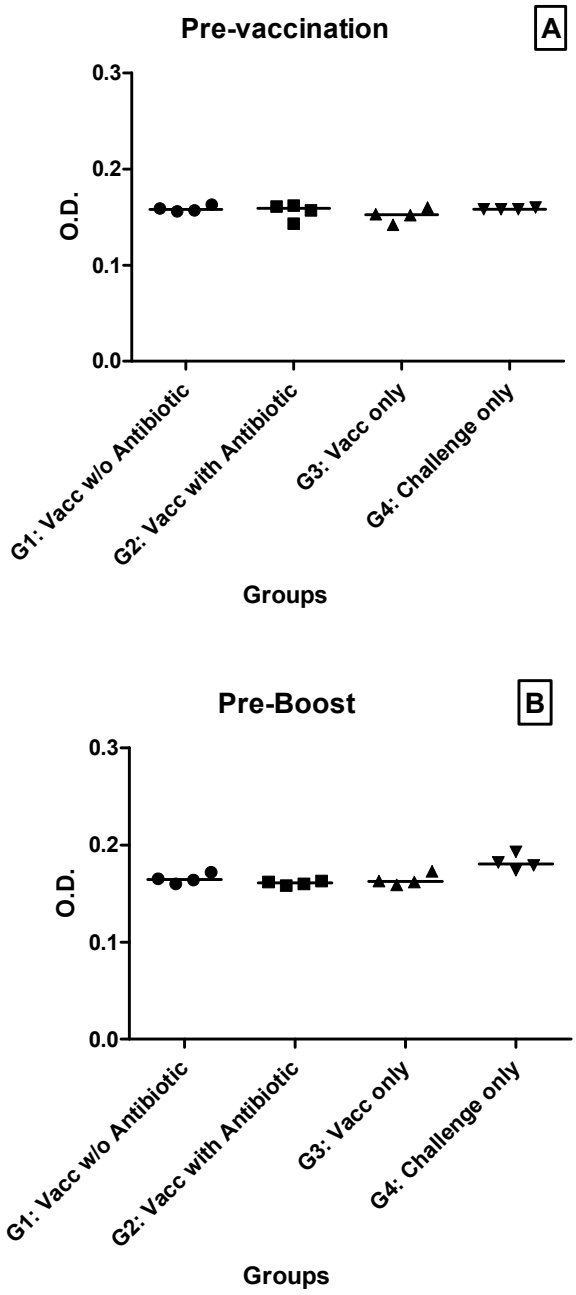

Fig 6.10. IgA antibody activity level in cecal content of 7 days-old chickens (pre-vaccination) (A) and 21 days-old chickens (pre-boost and 14 days post-vaccination) (B). Ceca were collected from 4 chickens from each group and processed as described in text. Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain *C. jejuni* K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination. Analysis using one-way ANOVA showed no significant difference among groups. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc only: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.1 and Fig 6.1.

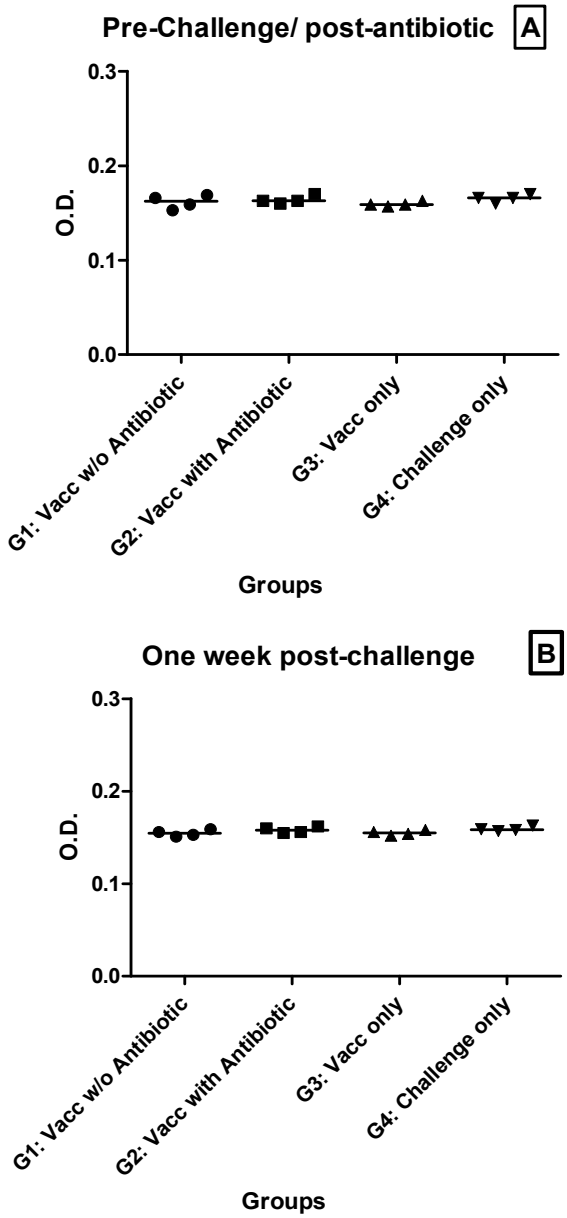

Fig 6.11. IgA antibody activity in cecal content of 28 days-old chickens (7 days post-boost and pre-challenge) (A) and 35 days-old chickens (7 days post-challenge) (B). Ceca were collected from 4 chickens from each group and processed as described in text. Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain *C. jejuni* K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination. G1, G2, and G4 were challenged on day 28 1 X 10^5 CFU/0.5 ml. Analysis using one-way ANOVA showed no significant difference among groups. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc only: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination.

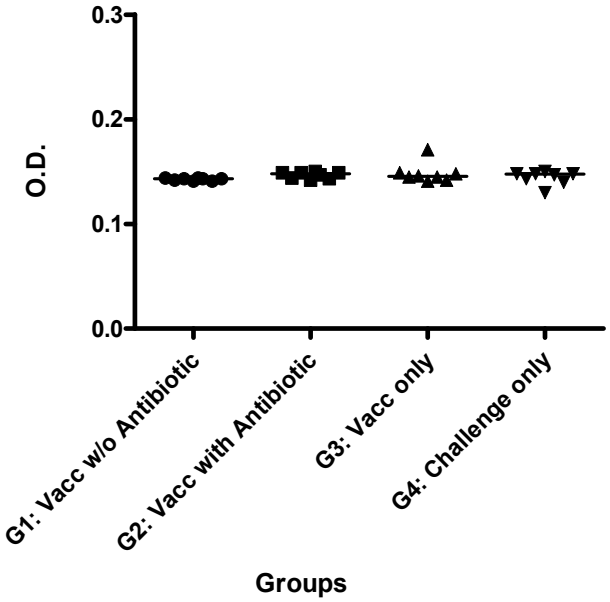

Fig 6.12. IgA antibody activity in the cecal contents of 40 day old chickens (12 days post-challenge). Ceca were collected from all remaining chickens in all groups and processed as described in text. Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain *C. jejuni* K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination. G1, G2, and G4 were challenged on day 28 1 X 10^5 CFU/0.5 ml. Analysis using one-way ANOVA showed no significant difference among groups. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc only: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination.

6.4.4. Evaluation of the ability of vaccination with *C. jejuni* K2-55 to protect chickens from *C. jejuni* NCTC11168V1 (heterologous) infection:

C. jejuni NCTC11168V1 has been used in several studies at VIDO as well as studies described in Chapters IV and V, making it an ideal strain to evaluate the ability of vaccination with *C. jejuni* K2-55 to protect against a heterologous challenge (Carrillo et al., 2004; Fernando et al., 2008; Fernando et al., 2007).

Chickens vaccinated with strain *C. jejuni* K2-55 were colonized throughout the experiment, at levels comparable to the challenge strain when groups were sampled on day 21 (Fig 6.13-A) with no significant difference among the 4 groups using one-way ANOVA. Birds in Groups 2 and 4 received streptomycin treatment in drinking water on days 25, 26, and 27 to reduce carriage of the vaccine strain *C. jejuni* K2-55. Birds sampled on day 28 (7 days after the boost) in Groups 1 and 3 remained colonized at the same level as day 21 (Fig 6.13-B). Chickens in Group 4 remained *C. jejuni*-free when cecal contents were plated on Karmali agar plates. Only two chickens in Group 2 were negative for *C. jejuni* K2-55, while the other two had an average of 2×10^4 CFU/g of cecal contents. On day 35 cecal contents from birds sampled from all groups were plated on Karmali-streptomycin agar plates indicated that birds were colonized with strain *C. jejuni* NCTC1168V1 (Fig 6.14-A). Cecal contents from birds sampled from Group 2 were additionally plated on Karmali agar plates with no streptomycin to test if *C. jejuni* K2-55 would grow. No significant difference in number of colonies growing on media with or without streptomycin. The ceca of birds remaining in Groups 1, 2, and 4 were colonized with *C. jejuni* 11168V1 on day 42 (Fig 6.14-B). *C. jejuni* K2-55 levels in the ceca of Group 3 (vaccinated twice and did not receive any challenge) were significantly lower ($P < 0.05$) than *C. jejuni* 11168V1 levels in ceca of other groups (Fig 6.14-B). No significant difference among groups 1, 2, and 4 was found using one-way ANOVA.

6.4.5. ELISA results of challenge with *C. jejuni* NCTC11168V1:

IgG titer levels in the sera of birds did not change significantly throughout the experiment (Fig 6.15-6.17). When birds were sampled one week post-challenge with wild-type IgG levels were higher in group 1 but not enough to be significant.

IgA levels in the cecal contents did not increase significantly throughout the experiment (Fig 6.17-B).

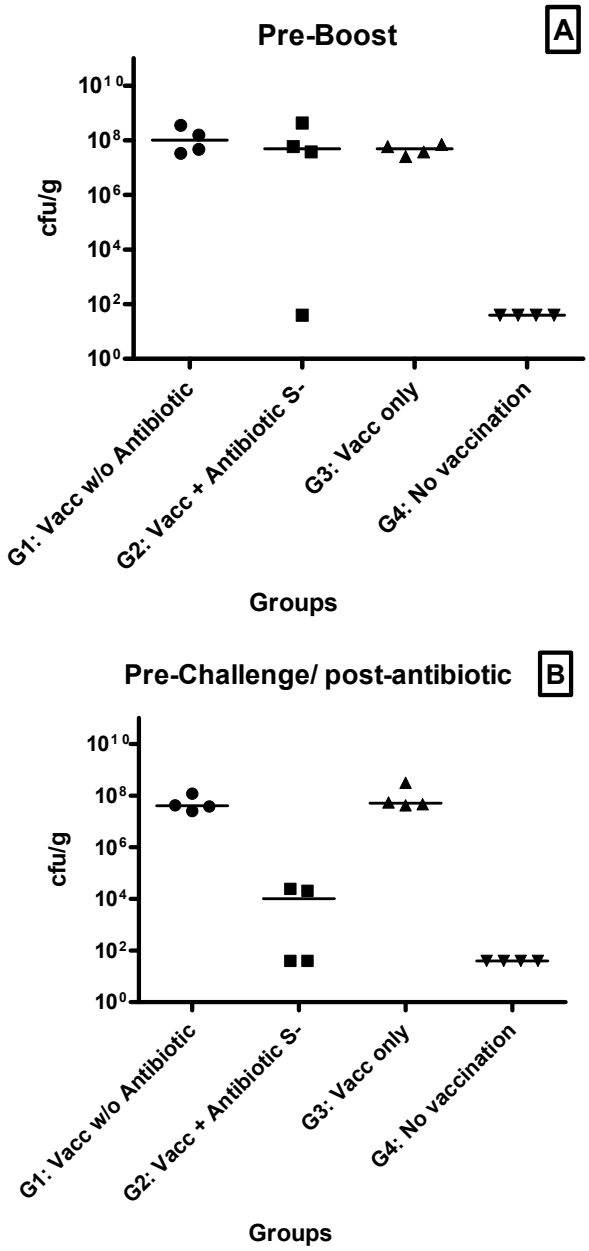

Fig 6.13. Colonization of broiler chickens by *C. jejuni* K2-55 on (A) day 21(Two weeks post vaccination, pre-boosting) and (B) on day 28 of the experiment (Day of challenge and one week post-boosting). Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain *C. jejuni* K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination, and G3 was not challenged with the wild-type. Cecal contents were collected from 4 birds from each group. Birds in groups 2 and 4 received streptomycin treatment on days 25, 26, and 27 (B). Birds were challenged on day 28 with *C. jejuni* NCTC1168V1. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc only: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.2 and Fig 6.1.

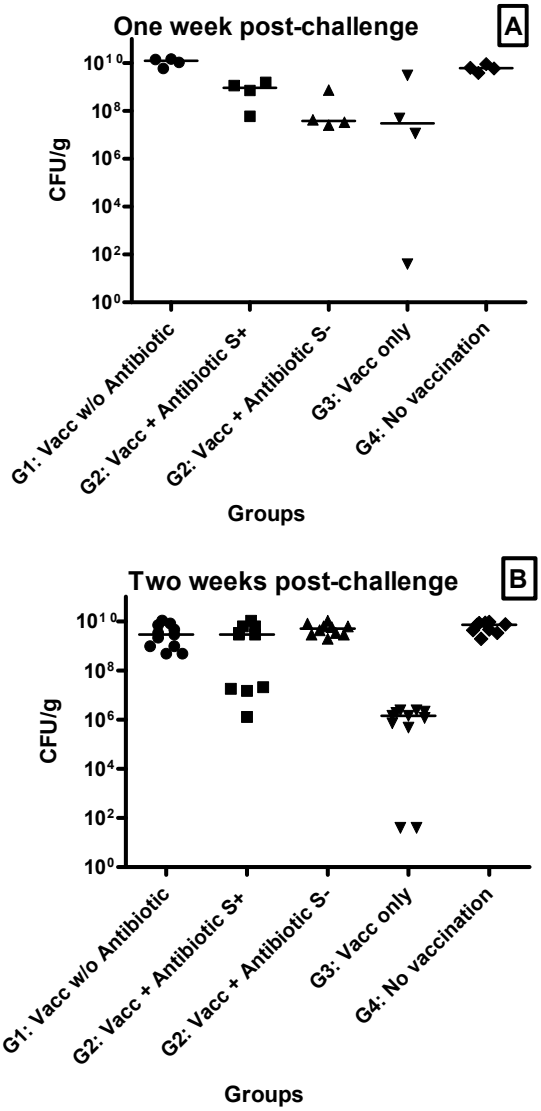

Fig 6.14. Colonization of broiler chickens by streptomycin-resistant *C. jejuni* NCTC1168V1 on (A) day 35 of the experiment (One week post-challenge) and (B) day 42 of the experiment (Two weeks post-challenge). Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using $1x10^5$ CFU/0.5 ml of vaccine strain *C. jejuni* K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination, and G3 was not challenged with the wild-type. Cecal contents were collected from 4 birds on day 35 from each group, and from all remaining birds on day 42 and plated on Karmali-SM. In addition, cecal contents from G2 were plated on streptomycin-free plates to allow for the vaccine strain to grow. No significant difference was found on day 35, but G3 had significant difference ($P < 0.05$) when analyzed using one-way ANOVA. G3 was colonized with K2-55 only. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic plated on Karmali-SM, G2: Vacc + Antibiotic S-: Vaccinated and treated with antibiotic plated on Karmali, G3:Vacc w/o challenge: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination.

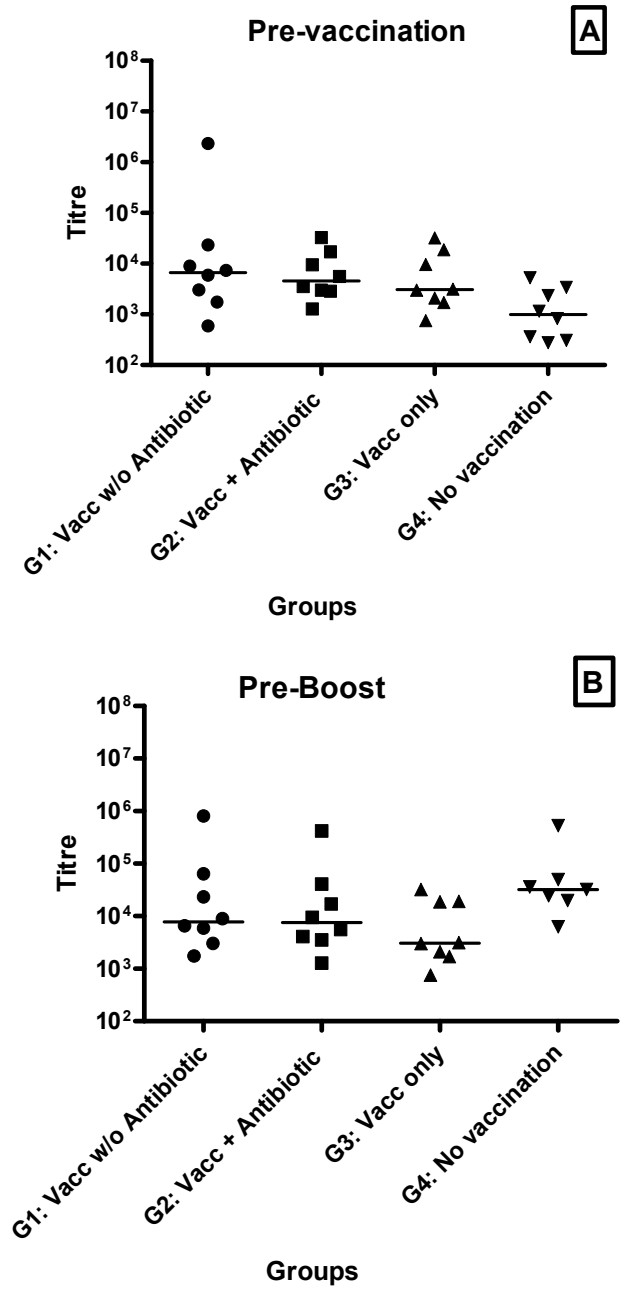

Fig 6.15. IgG antibody activity in the sera of (A) 7 days-old chickens (pre-vaccination), and (B) of 21 days-old chickens (two weeks post-vaccination, pre-boost). Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain *C. jejuni* K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination, and G3 was not challenged with the wild-type. Blood samples were collected from 8 birds. Analysis using one-way ANOVA showed no significant difference among groups. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc only: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.2 and Fig 6.1.

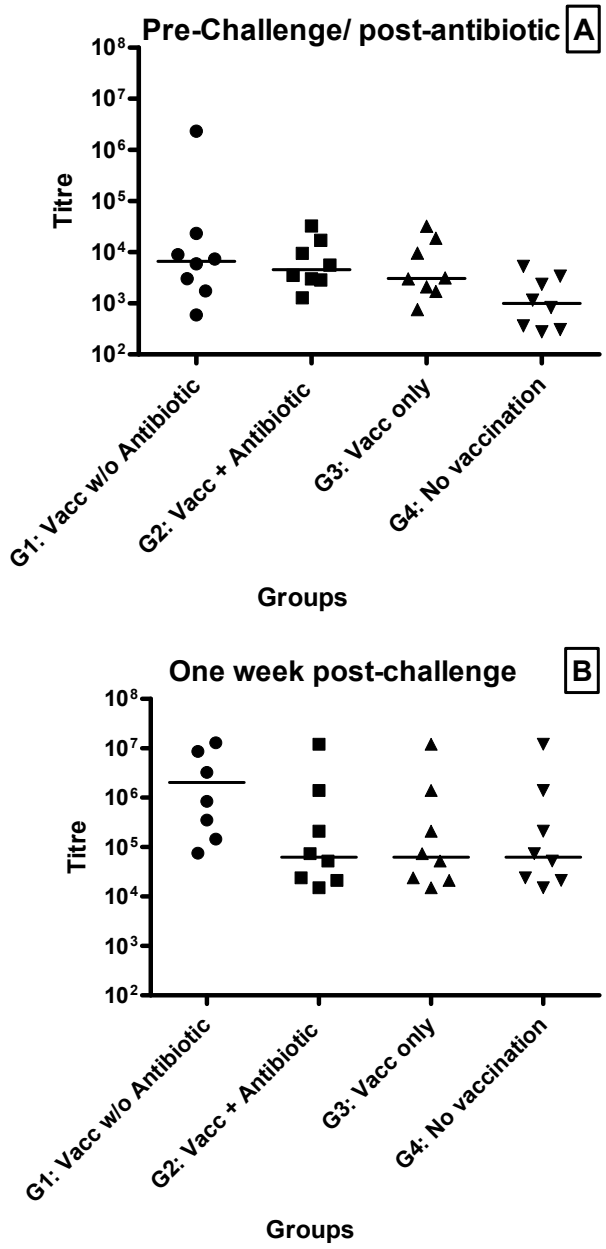

Fig 6.16. IgG antibody activity in the sera of (A) 28 days-old chickens (one week post-boost and day of challenge), and (B) 35 day old chickens (one week post-challenge). Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination, and G3 was not challenged with the wild-type. Blood samples were collected from 8 birds. Analysis using one-way ANOVA showed no significant difference among groups. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc only: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.2 and Fig 6.1.

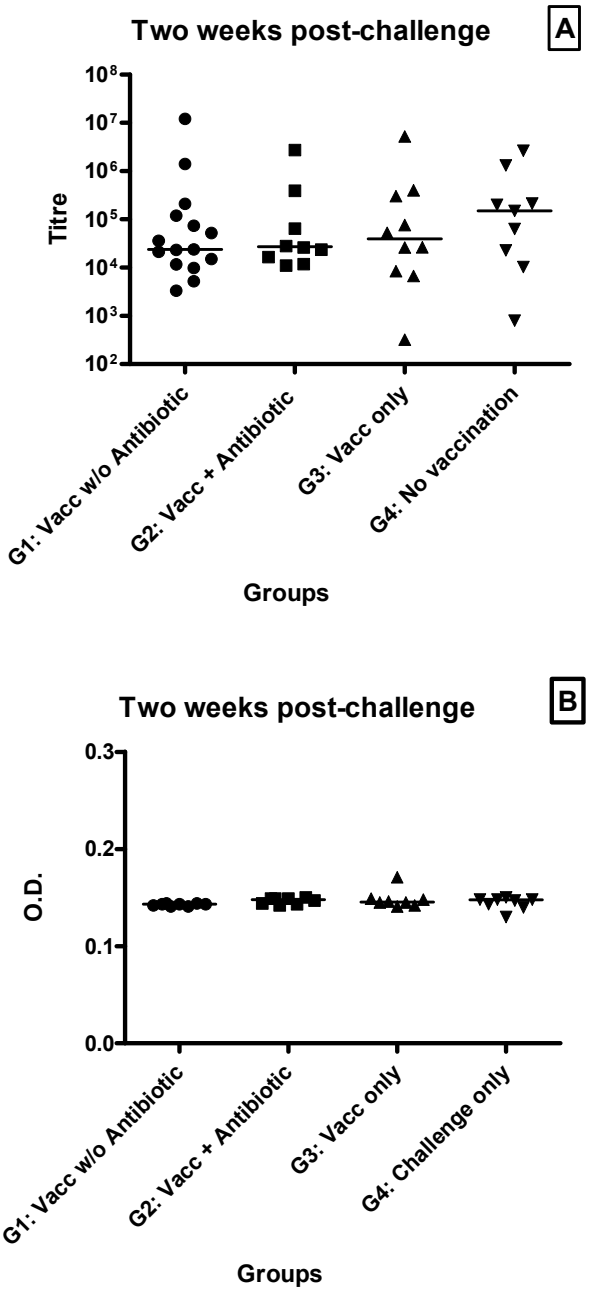

Fig 6.17. IgG (A) and IgA (B) antibody titers in the sera of 42 days old chickens (two weeks post-challenge). Group 1 (G1), group 2 (G2) and group 3 (G3) were vaccinated twice using 1 X 10^5 CFU/0.5 ml of vaccine strain K2-55 orally on day 7 of age and a booster on day 21 of age. Group 4 (G4) did not receive any vaccination, and G3 was not challenged with the wild-type. Blood samples and ceca were collected from all remaining birds in all groups. Analysis using one-way ANOVA showed no significant difference in IgG (A) or IgA (B) titers among groups. G1: Vacc w/o Antibiotic: Group 1 Vaccinated without antibiotic, G2: Vacc + Antibiotic: Group 2: Vaccinated and treated with antibiotic, G3:Vacc only: vaccinated only with no challenge, G4: No Vaccination: Group 4: No vaccination. Full details of the experiment are found in section 6.3.2 and Fig 6.1.

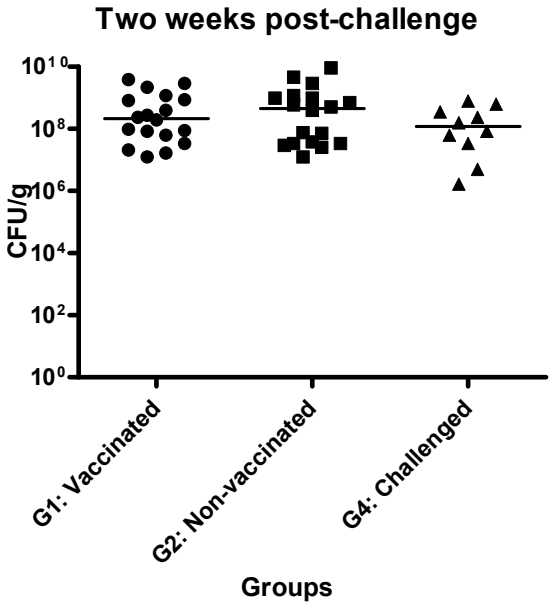

Fig 6.18. Colonization of broiler chickens by *C. jejuni* 81-176 on day 42 of the experiment in a seeder model. Birds in Group 1 (G1: Vaccinated) were vaccinated orally on day 7 and day 21 using 1 X 10^5 CFU of *C. jejuni* K2-55 suspended in 0.5 ml normal saline. Birds in Group 2 (G2: Non-vaccinated) did not receive any vaccination or challenge. Birds in Group 4 (G4: Challenged) were challenged orally on day 28 with 1 X 10^9 CFU/0.5 ml of normal saline. All birds were euthanized on day 42 and cecal contents were plated in Karmali-SM plates. Analysis using one-way ANOVA showed no significant difference among groups. Full details of the experiment are found in section 6.3.3 and Fig 6.2.

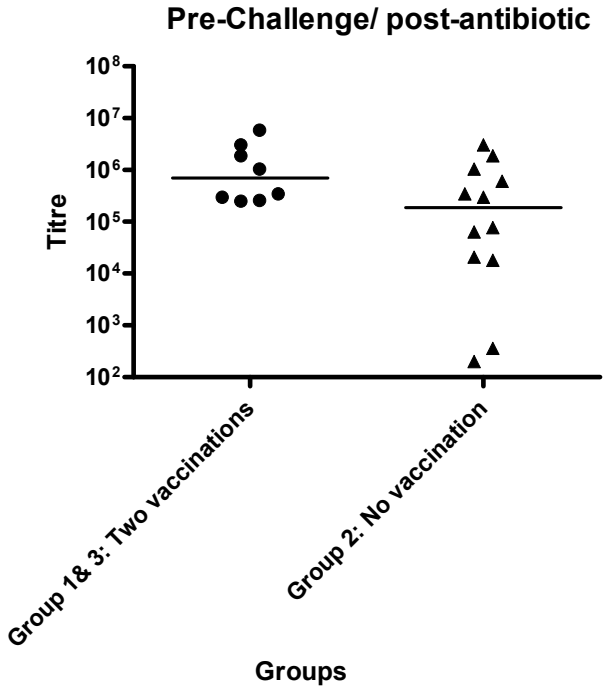

Fig 6.19. IgG antibody activity in the sera of 28 day old chickens (day of challenge) in a seeder model. Birds in group 1 and 3 have received two oral vaccinations. Each vaccine dose contained 1 X 10^5 CFU/ml. Birds in group 2 have received no vaccinations and remained *C. jejuni* free as detected by cloacal swabs. Analysis using one-way ANOVA showed no significant difference among groups. Full details of the experiment are found in section 6.3.3 and Fig 6.2.

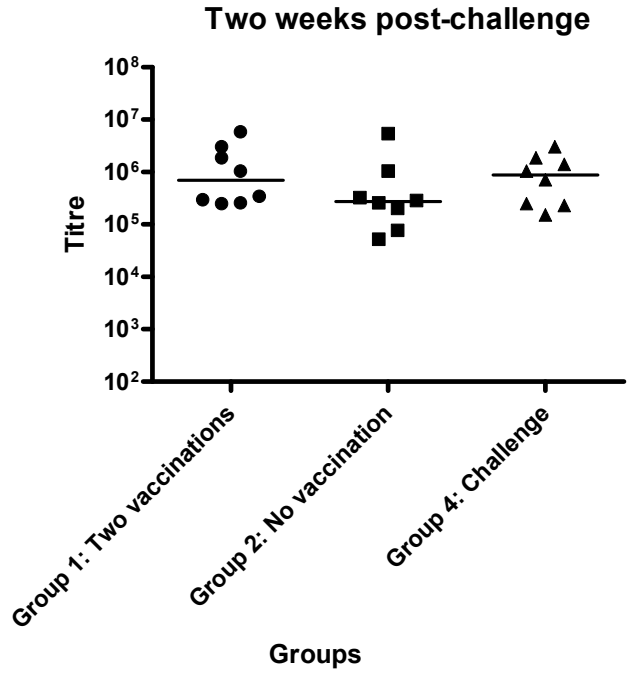

Fig 6.20. IgG antibody activity in the sera of 42 day old chickens (two weeks post-challenge). Group 1 birds have received two vaccinations on day 7 and 21. Group 2 birds did not receive any vaccinations or oral challenge. Group 4 birds were challenged on day 28 with 1 X 10^9 CFU/0.5 ml *C. jejuni* 81-176. Chickens were housed in the same room as of day 28. Blood samples were collected from all birds. Analysis using one-way ANOVA showed no significant difference among groups. Full details of the experiment are found in section 6.3.3 and Fig 6.2.

6.5. Discussion:

Despite *C. jejuni* being the leading cause of bacterial gastroenteritis in North America (Thomas et al., 2008b; WHO, 2001), and handling and consumption of poultry being the major source of infection (Stern et al., 2003; Vellinga and Van Loock, 2002), no vaccine is yet available for use in humans or chickens. The objective of this study was to evaluate the ability of paralyzed flagella mutant *C. jejuni* K2-55 to protect broiler chickens from infection with homologous wild-type *C. jejuni* 81-176 and heterologous strain *C. jejuni* NCTC11168V1. The data obtained from Chapter IV demonstrated that *C. jejuni* K2-55, which expresses a paralyzed flagellum and cannot produce *Campylobacter* invasion antigens (Cia), colonized chickens mucosal surfaces but failed to spread systemically to other organs. This balanced colonization ability made *C. jejuni* K2-55 an ideal candidate for a vaccine. The poor understanding of pathogenesis of *C. jejuni* in broiler chickens makes live attenuated vaccine, at the moment, a better choice than subunit vaccines since the protective antigens are not known.

The vaccine strain *C. jejuni* K2-55 was able to persist in broiler chickens' ceca throughout the experiment. However, the bacterial counts started to decline towards the end of the experiment, as the median was 2 logs lower on day 40 than on day 35 (2×10^6 versus 1.3×10^8 respectively), indicating that birds started clearing *C. jejuni* K2-55 from the ceca. Based on the data obtained from these sets of experiments, the specific cause of this decline is unknown, but the fact that IgG antibody titers have increased might have affected bacterial counts. Also, as birds grew, the physiology and microflora of their intestinal tract starts to change, and this might have affected the ability of this motility and Cia deficient vaccine strain to persist at a higher number (Rehman et al., 2007; Van Immerseel et al., 2005). Vaccine strain *C. jejuni* K2-55 CFU/g of cecal contents in samples collected on day 21 before the challenge were high, indicating persistent colonization ability. Boosting with another vaccination dose on day 21 might have extended the high levels of *C. jejuni* K2-55 persistence in the ceca. Future experiments are required to determine if this strain is capable of persisting throughout the lifespan of a broiler chicken if given once only. This mutant has a much better potential than mutants described in literature that failed to colonize chickens' ceca, as they were attenuated to a level that they could not persist (Ziprin et al., 2002b). To date, the only promising

vaccine candidate reported is a *Salmonella* strain expressing CjaA protein (Wyszynska et al., 2004).

To evaluate the ability of *C. jejuni* K2-55 to protect against homologous and heterologous challenge, two different bacterial challenge models were used. In the first model, every chicken was orally challenged using a specific dose of the wild-type strain, while in the second model, referred to as a seeder model, a small group of birds was challenged using the wild-type and then introduced to a larger group of chickens. Challenging individual chickens allowed for more accuracy, guaranteeing that every single chicken received the same amount of bacteria. The seeder model mimics the conditions found on a poultry farm, where the infection is thought to be introduced to a small group of birds before spreading throughout the flock (Conlan et al., 2007; Hald et al., 2007). A modification to the first challenge model was using streptomycin to eliminate the vaccine strain *C. jejuni* K2-55 (streptomycin sensitive) in one of the vaccinated groups. Including this group has allowed for discrimination between the role of a possibly protective immune response mounted by the bird, and the niche-filling role of *C. jejuni* K2-55 which might prevent the wild-type from colonization in the first place in a manner similar to competitive exclusion. When chickens were challenged with *C. jejuni* 81-176 (as described in section 6.3.1), birds receiving streptomycin treatment in drinking water had significantly lower *C. jejuni* 81-176 in their cecal contents on day 40 in comparison to the control group that received no vaccination, while birds that did not receive streptomycin treatment had no protection. The control group (Group 4), which also received streptomycin treatment, was colonized by a high number of CFU/g of cecal contents. Birds in Group 1, which received two vaccinations but no streptomycin treatment, also had high CFU/g of cecal contents, and were not significantly different from Group 4. The combination of vaccination and antibiotic treatment had significantly reduced bacterial counts in the cecal contents of Group 2, with 7 birds that had no detectable bacterial shedding. Based on the IgG titers observed in challenge with *C. jejuni* 81-176, no significant difference was observed among the median of 4 groups. However, the median of IgG titers in Group 1 (2 vaccination with no streptomycin), Group 2 (2 vaccination + streptomycin treatment), and Group 4 (received no vaccination

but was challenged with wild-type) were constantly lower than Group 3 as of day 21. On the other hand, IgA titers in cecal contents did not change throughout the experiment in any of the groups. Taken together, although *C. jejuni* K2-55 persistently colonized broiler chickens, it did not increase the levels of secretory IgA, and had a neutralizing effect on IgG antibodies, which was lower on day 21 in comparison to day 7 except for Group 3. This interference has been reported in studies targeting vaccination at an early age in chickens against *Salmonella*. Maternal antibodies are transferred passively into the egg yolk of the breeder chicken, and then absorbed by the growing embryo and deposited into the blood circulation (Kramer and Cho, 1970; Schiemann and Montgomery, 1991). During the first week of age, these maternal antibodies can still cross the intestinal barrier and interact with their target (Dohms et al., 1978; Schiemann and Montgomery, 1991). The short lifespan of broiler chickens and the early exposure of chickens to bacterial infections require vaccination of the birds as early as possible. The vaccine regime followed in this study consisted of an early vaccination on day 7 and a booster dose on day 21. The relatively high levels of IgG maternal antibodies in the sera of these birds may have reduced the efficacy of the first vaccination by interfering with colonization/replication of the vaccine strain (Hassan and Curtiss, 1996). This interference was reflected in the inability of the vaccine strain to prevent the colonization of the wild-type on day 28. In Group 2, the modification of the microflora caused by streptomycin, combined with the immune response to the vaccine strain was protective against homologous challenge. When chickens were challenged with *C. jejuni* NCTC11168V1, although the same experimental design was followed, the combination of streptomycin treatment and vaccination were not enough to protect. This is not unexpected, as live *C. jejuni* vaccines usually provide protection against homologous challenge (Abimiku et al., 1989; Scott, 1997; Widders et al., 1996).

Using the seeder model was an important step to evaluate the ability of *C. jejuni* K2-55 to prevent colonization of the homologous wild-type in a poultry industry setting. However, no significant difference was detected between vaccinated and non-vaccinated groups one week post-challenge. Since antibody titers obtained in this experiment were comparable to the ones obtained while chickens were housed separately (section 6.3.1), it is possible that streptomycin treatment in the experiment did not produce the same

changes in intestinal microflora that were previously protective. The streptomycin treatment regimen was enough to clear *C. jejuni* K2-55from the intestine of vaccinated birds sampled on day 28, indicating how significant the change to the microflora could be.

In summary, vaccination with paralyzed flagella mutant *C. jejuni* K2-55 combined with streptomycin treatment protected birds from colonization by wild-type *C. jejuni* 81-176. A modification to the experimental design should mainly apply to seeder model challenge, were chickens should be euthanized within 7 days of challenge. This might limit the exposure to *C. jejuni* and allow the protective effect of the vaccine to be demonstrated.

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Chapter VII: Chicken Macrophage Inflammatory Response to Wild-type and Paralyzed Flagella Mutant of *C. jejuni*

7.1. Introduction:

Campylobacter jejuni is a leading cause of bacterial food-borne diarrheal disease throughout the world (Allos, 2001). Ingestion of a small dose of 500 microorganisms is enough to cause disease in humans (Black et al., 1988). *C. jejuni* infections have been mainly associated with mishandling of poultry meat (Harris et al., 1986; Istre et al., 1984). Although *C. jejuni* frequently colonizes the gastrointestinal tract of chickens with up to 10^{12} colony forming units (CFU) per gram of cecal content (Meade et al., 2009), it does not cause any apparent disease (Soerjadi et al., 1982). The reason why *C. jejuni* causes disease in humans but not in chickens is still unknown.

Macrophages are a group of heterogeneous cells which are found throughout the body (Gordon and Taylor, 2005). The earliest description of macrophages was by Elie Metchinkoff, who described the process of clearance of foreign bodies (including micro-organisms) by a group of leukocytes which he named macrophages (Gordon, 2007). Today, the macrophage's vital role in both innate and adaptive immunity is well established. Through phagocytosis of invading micro-organisms and clearance of apoptotic and necrotic cells, macrophages secrete various signaling molecules that orchestrate the immune response (Gordon, 2007). Most of the limited knowledge available about chicken macrophages comes from the study of a macrophage-like laboratory cell lines available, mainly HD11, and not primary cells isolated from the bird, as it is difficult to isolate chicken macrophages due to their circulatory nature (Rose and Hesketh, 1974). The use of a laboratory cell line limits the conclusions that research can make about the role of chicken macrophages. The limited array of tools available for studying chicken's macrophages also contributes to the lack of information available.

The HD11 cell line was originally named LSCC-HD (MC/MA1) (Beug et al., 1979; Leutz et al., 1984). When first discovered, these cells were shown to have several macrophage characteristics, including bacterial phagocytosis, expression of antibody Fc receptors, and reaction with antiserum direct against avian macrophages (Beug et al., 1979). Over the years, many phenotypic and functional characteristics of the HD11 cell

line have been reported, including expression of several TLR's (TLR1, TLR2, TLR3, TLR4, TLR5, and TLR7 (Iqbal et al., 2005a). HD11 cells also produce a range of antimicrobial factors, including nitric oxide production (Sung et al., 1991), and oxidative burst (Chadfield and Olsen, 2001). HD11 cells express several cytokines such as IL-1β, IL-6, IL-8, and IL-10 (Smith et al., 2005).

C. jejuni invades, crosses and disrupts the polarized human epithelial cell monolayer barrier *in vitro* (Bras and Ketley, 1999). This invasive process induces the production of several immuno-modulatory signals. Human monocytes have been shown to produce several cytokines upon infection with *C. jejuni*, including IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), and IL-8 (Jones et al., 2003; Smith et al., 2005; Zilbauer et al., 2007). In chickens, *C. jejuni* has been shown to disseminate to the spleen and liver, and generate an antibody response upon infection, therefore interaction with macrophages, and stimulation of cytokine production is very likely during systemic spread (Meade et al., 2009; Smith et al., 2008). Cytokines are a group of peptides that regulate the immune system by acting as a signaling molecule. Cytokines are produced by almost all cell types, and they can both elicit and regulate immune response and are a means of inter-cellular communication (Eckmann and Kagnoff, 2001). The first cytokine discovered was interferon (IFN), and it was discovered in chickens' egg injected with Influenza A virus (Isaacs and Lindenmann, 1957), but most of the other cytokines were discovered first in mammals. Although many of the cytokines identified in mammals are found in chickens, they generally share only 25-35% amino acid identity (Asif et al., 2004; Staeheli et al., 2001). Cytokine classification is based on their activity and/or the cell producing or responding to them. Functions of several cytokines produced by chicken's cells are listed in Table 7.1.

C. jejuni stimulates the production of several cytokines *in vitro* upon interaction with HD11 cells and chicken kidney cells (CKC) (Smith et al., 2005), human dendritic cells (Pulimood et al., 2008), and intestinal epithelium (Johanesen and Dwinell, 2006). These studies did not investigate the impact of *C. jejuni* motility, flagellin, or *Campylobacter* invasion antigens (Cia) on cytokine gene expression. In this study, changes HD11 cells in expression of genes coding for IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12β, and TLR5 to *C. jejuni* wild-type strains and flagellin, motility and Cia

mutants were studied using real-time PCR over the period of 8 hours. The main functions of these genes' products are summarized in Table (1).

7.2. Materials and Methods:

7.2.1. Bacterial strains and growth conditions:

Four *C. jejuni* strains were used in this study. Strain *C. jejuni* 81-176 which has been well characterized and recently its genome sequenced has been published (Pearson et al., 2007). Strain *C. jejuni* K2-55, which was a generous gift from Dr. P. Guerry (Naval Medical Research Institute, Bethesda, Md.) shares the same genetic background with *C. jejuni* 81- 176 but has an insertion mutation in the *pflA* gene that maintains an intact flagellar structure although it abolishes the motility (Yao et al., 1994). Strain *C. jejuni* NCTC11168V1 which is a variant of *C. jejuni* NCTC11168 genetic background which has been fully sequenced and is proved to colonize chickens well(Carrillo et al., 2004; Parkhill et al., 2000), *C. jejuni* NCTC11168V1*flgK* mutant was generated by Dr. U. Fernando (VIDO, SK) using an insertion of kanamycin resistance cassette into *flgK* gene, which resulted the expression of the flagellar structure up to the hook level (Fernando et al., 2007). The *C. jejuni* NCTC11168V1*flgK* mutant also lacks the ability to secrete *Campylobacter* invasion antigens (Fernando et al., 2007). *Salmonella enterica* serovar Typhimurium strain SL1344 (SL1344) (Stocker et al., 1983) was used as a positive control for TLR5 gene expression. It is a wild-type *Salmonella* with no known mutations and is ampicillin resistant.

C. jejuni strains were cultured on Mueller-Hinton agar (MHA), (Difco), or Mueller-Hinton agar supplemented with 5% sheep blood (PML Microbiological, Richmond, BC) under microaerophilic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) at 42 °C. *Salmonella* strain SL1344 was grown on Luria-Bertani (LB) agar at 37 °C under aerophilic conditions.

Chicken macrophage (HD11) cells were maintained in 25 ml of RPMI 1640 medium containing 10% fetal bovine serum (FBS) using T-75 flasks (Corning) at 37 °C in a CO_2 incubator dedicated for tissue culture.

7.2.2. Incubation of bacteria with HD11 cells:

C. jejuni strains 81-176, NCTC1168V1, NCTC1168V1*flgK* mutant, and *C. jejuni* K2-55 were grown on MHA-blood as described above. Bacteria were harvested and re-suspended in RPMI 1640 medium containing 10% FBS. To obtain heat killed cells, *C. jejuni* 81-176 was harvested in normal saline, and incubated at 65 °C for 15 min. From each sample, 25 µl were serially diluted in normal saline and 10^{-4}, 10^{-5}, and 10^{-6} dilutions were plated on MHA to determine the actual colony forming unit counts (CFU).

To prepare HD11 cells for the experiment, cells grown in a T-75 flask were harvested using a plastic scraper (VWR) 24-48 hours before incubation with bacteria. Cells were transferred to sterile 50 ml centrifuge tubes (Corning) and pelleted at 300x *g* for 5 min. The supernatant was discarded and cells were re-suspended thoroughly using 30 ml of RPMI 1640 with 10% FBS. To each well of a 6-well plate, 5 ml of cell suspension was added and incubated to obtain 1 X 10^5 cells/well as determined by cell count using a hematocytometer. To each well, 1 X 10^7 CFU of a bacterial strain suspended in 200 µl RPMI 1640 with 10% FBS was added to obtain a multiplicity of infection (MOI) of 100. A duplicate from each sample was made. Plates were incubated under microaerophilic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) at 42 °C. Cells were harvested as described above at 2, 4, and 8 hours post infection. Immediately, RNA was extracted, and processed as described in the following section. Two samples were collected for each strain at each time point.

Gene	Functions of protein	Reference
β –actin	A house keeping gene, it is involved in the actin network and has been previously used as a reference gene for qRT-PCR reactions	1
IL-1β	Pro-inflammatory cytokine. Stimulates induction of IL-6 and TNF-α, leading to T-cell proliferation and triggering of acute phase response	2
IL-2	Involved in Th1immune response, development of T-regulatory cells, and immunological memory	3, 4
IL-4	Involved in Th2 immune response. Stimulates activated B- and T-cells proliferation.	3
IL-6	Stimulates Th2 responses, B-cell Ig production, and DC to macrophage conversion. Inhibits T1 responses.	5
IL-8	Renamed CXCL8. Secreted mainly by macrophages. Recruits polymorphonuclear cells (PMNs) heterophils and monocytes.	5
IL-10	Down-regulates Th1 responses and stimulates Th2 responses by affecting APCs, B, T, and NK cells.	2, 5
IL-12 β	Involved in Th1 response. Activates NK cells, stimulates IFN-γ production, and favors antigen specific Th1 cells proliferation while inhibiting Th2 specific cells	2, 5
TLR-5	Member of the Toll-like receptor (TLR) family, which recognize pathogen-associated molecular patterns (PAMPs). It mainly recognizes bacterial flagellin. It is expressed in a variety of tissues and cells including macrophages and HD11 cells	6

Table 7.1. The main function of the proteins of genes monitered by qRT-PCR. The gene β –actin was used as a control gene for qRT-PCR. Main functions of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12β listed here are derived from studies conducted human, mouse, and chicken. In chicken the Th1-Th2 cytokines exists but the structural and functional hallmarks are different than those of human and mouse (Degen et al., 2005).
References:
1: (Lambrechts et al., 2004), 2:(Giansanti et al., 2006), 3: (Degen et al., 2005) 4: (Malek, 2003), 5: (Staeheli et al., 2001), 6: (Iqbal et al., 2005a; Iqbal et al., 2005b)

7.2.3. RNA extraction:

RNA was extracted using an RNeasy mini kit (Qiagen). After cells were pelleted, the supernatant was removed completely and 350 µl of RNeasy Lysis Buffer (RLT) buffer were added and the pellet was disrupted by flicking the test tube. Cells were homogenized by passing them 5 times through 20-gauge needle. A volume of 350µl of 70% ethanol was added and mixed by pipetting. The sample was transferred to an RNeasy spin column and centrifuged for 15s at 9000 x *g*. DNase digestion was performed on column using Qiagen DNase kit (Qiagen). To each sample, a total of 30U of DNase suspended in RNase- and DNase-free water was added to the column and incubated at room temperature for 15 minutes. The column was washed twice to remove the DNase using 350µl washing buffer (RW1). Another 2 washes to remove any remaining DNA were done using 500µl of buffer RPE were performed. To elute RNA, a 100 µl RNase-free water was added to the column and centrifuged at 9000 x *g* for 1 minute. Samples were stored immediately at -20 °C. Concentration and purity of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Tech, Mississauga, ON).

7.2.4. Real-time reverse transcription polymerase chain reaction:

To produce cDNA, one µg of RNA was reverse transcribed using SuperScript III Platinum Two-Step qRT-PCR kit with SYPR GreenER (Invitrogen). The kit reaction mix contains both random hexamers and oligo dT primers. To obtain cDNA, 1µg of RNA from each sample was mixed with 10 µl of 2X RT reaction mix, 2 µl RT enzyme mix, and water was added according to RNA volume to bring the total reaction volume to 20 µl. Reaction mixture was mixed gently. The reverse transcription reaction was carried out in PCR machine using the following set of parameters: 25 °C for 10 minutes, 42 °C for 50 minutes, and finally 85 °C for 5 minutes. Reaction tubes were immediately chilled on ice. To each tube, 5 µl of deionized, diethylpyrocarbonate (DEPC)-treated water was added to bring the total volume to 25 µl. Reactions were stored at -20 °C until used.

All qRT-PCR reactions contained 1µl target cDNA, 0.2 µM each of forward and reverse primers, and 9 µl of qRT-PCR SuperMix (Invitrogen). Deionized, diethylpyrocarbonate-treated double distilled water was added to a final volume to 15 µl. Reactions were run in duplicate in 96-well RT-plates covered with optical quality tape

(Bio-Rad) using the iCycler iQ Real-Time PCR detection system (Bio-Rad). Amplification was performed using the following protocol: initial denaturation at 95 °C for 3 minutes (cycle one), followed by cycle two (95 °C, 15 seconds; 60 °C for 30 seconds; 76 °C for 30 seconds) which was repeated for 45 times. Cycle 3 ran at 95 °C for 1 minute. Cycle 4 was preset at 45 °C ramping to 95 °C with 1 °C increase each 30 seconds. The final hold temperature was 22 °C. Each plate had a negative template control that contained all the reagents except cDNA.

7.2.5. Real time RT-PCR primers:

Table 2 lists primers used for each gene and the size of expected cDNA product. Each primer spanned an intron to differentiate cDNA from genomic DNA by the product size. To confirm cDNA synthesis and to validate the primers in one step, 2 µl of the final cDNA reaction mixture were added to a PCR reaction as described in the general materials and methods section, with a pair of primers from Table 2 to amplify sequences corresponding to β-actin, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12β, and TLR5. For confirmation of the product size and the absence of genomic DNA contamination, two µl from the final PCR reaction were tested using 1% agarose gel and the product size was confirmed based on comparison to a molecular marker. Primers were adapted from Patel et al and Iqbal et al (Iqbal et al., 2005b; Patel et al., 2008) and were manufactured by Invitrogen (Burlington, ON). Before initial usage, primers were resuspended to a concentration of 10 µM/µl. A working solution of 1 µM/µl was made and used in PCR and qRT-PCR reactions. All resuspended primers were stored at -20 °C after each use.

Target gene	Primer	Primer sequence (5'→ 3')	cDNA product (bp)	Genomic product (bp)	Acc. No.
β-actin	F	GTACCCTGGCATTGCTAC	182	465	X00182
	R	CGGATTCATCGTACTCCTGC			
IL-1 β	F	GGCATCAAGGGCTACAAGC	296	386	AJ245728
	R	GTTGGAGCGGGCAGTCAG			
IL-2	F	GGAGCATCTCTATCATCAGC	347	2468	AJ224516
	R	AAGTTGGTCAGTTCATGGAG			
IL-4	F	GGCTGTGCCCACGCTGT	192	1283	AJ621249
	R	TGGCAGTGCTGGCTCTC			
IL-6	F	GTGCGAGAACAGCATGGAGA	312	1785	AJ250838
	R	GACTTCAGATTGGCGAGGA			
IL-8	F	CAGCTGCTCTGTCGCAAG	264	2248	AJ009800
	R	GGGGTCATCAGAATTGAGCT			
IL-10	F	AGCACCAGTCATCAGCAGA	240	818	NM_001004414
	R	GGCTCACTTCCTCCTCCT			
IL-12 β	F	CCGACTGAGATGTTCCTGGA	292	2346	AJ564201
	R	CCTGCACAGAGATCTTGTC			
TLR-5	F	TGCACATGTTTTCTCCTAGGT	262	2815	AJ626848
	R	CCACATCTGACTTCTGCCTTT			

Table 7.2. List of real time qRT-PCR primers, their corresponding cDNA and DNA product size, and GenBank accession number. Each primer spanned an intron to differentiate cDNA from genomic DNA by product size. F = Forward. R = Reverse.

7.2.6. Gene expression data and statistical analysis:

For each qRT-PCR reaction a cycle threshold value (Ct value) was determined. The Ct value was determined based on the cycle number at which there was an increase in detection of the fluorescence signal above base line as the PCR reaction reaches the exponential phase of amplification. The Ct values were expressed as the mean of duplicates ± standard deviation (SD). The relative difference in gene expression (ΔCt) was calculated by subtracting the mean Ct value for each gene from the corresponding endogenous control's mean Ct value ($\Delta Ct = Ct_{(sample)} - Ct_{(\beta\text{-actin})}$. The difference between the average $\Delta Ct_{(sample)}$ and the average $\Delta Ct_{(control)}$ was expressed as ΔΔCt and was used to calculate fold changes in gene expression using the following formula:

Fold change = $2^{-(\Delta\Delta Ct)}$.

Statistical significance among the different treatments for each gene at a given time point was confirmed using pairwise fixed reallocation randomization test (Pfaffl et al., 2002). Data was analyzed using the BioRad iQ5, Microsoft Excel and GraphPad prism software.

7.3. Results:

7.3.1. Purity and amounts of mRNA extracted:

To determine the amount and the purity of mRNA extracted from each sample, two µl of the mRNA were analyzed using an automated Bioanalyzer. All samples extracted were pure and no contamination was observed (Fig 7.1B). On average, each purification attempt produced 709 ng/µl of final elution volume.

7.3.2. Primer optimization:

Primer sequences were adapted from Patel et al (Patel et al., 2008) and Iqbal et al (Iqbal et al., 2005b). To validate primer sequences, two µl of cDNA synthesized from mRNA extracted from untreated HD11 cells were used as a template in PCR reactions. A separate reaction for each primer listed in Table 7.2 was carried and the primers amplified the selected gene product of the expected size. After each qRT-PCR reaction melt curve analysis was carried out to examine the specificity of amplification as all PCR products for each primer pair are expected to have the same melting temperature, unless there is contamination, mispriming, or primer-dimer artifact (Fig 7.3). None of the samples examined had any genomic DNA contamination.

7.3.3. Gene normalization:

The β-actin gene was selected as an internal standard (loading control) as it is expressed in HD11 cells. The Ct value (mean ±SD) for β-actin from all samples at 0 hours was 18.9 ±0.6, at 2 hours Ct value was 18.3±0.6, at 4 hours 17.3±1.1, and at 8 hours 16.9±1.3. No significant difference ($P < 0.5$) in β-actin gene expression was found between 2, 4, and 8 hours when cells were either stimulated or unstimulated. However, these values varied significantly ($P < 0.05$) when 0 hours the Ct values from unstimulated cells are compared with the Ct values after 4 and 8 hours stimulation with *C. jejuni* and *Salmonella* SL1344 (Fig 7.2).

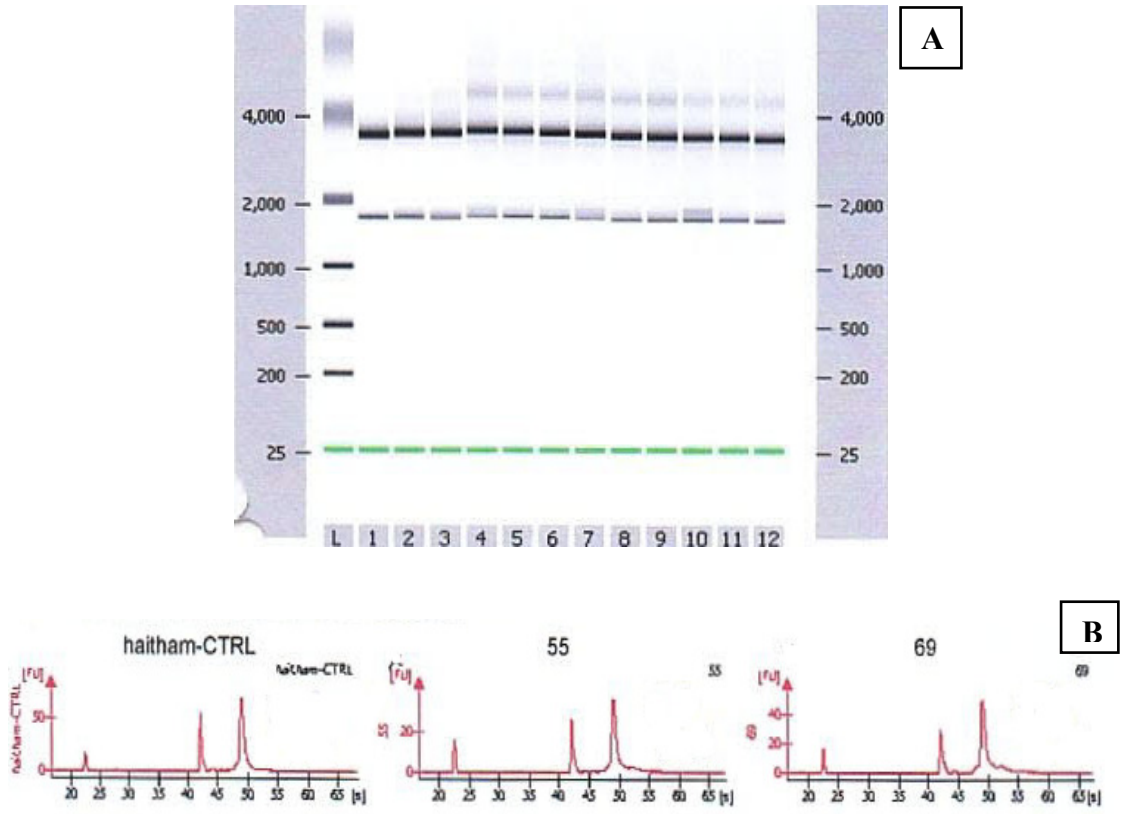

Fig 7.1. Analysis of purity of mRNA samples extracted from HD11 cells either incubated or non-incubated with *C. jejuni* wild-type strains or mutants. In **(A)** samples are loaded separately; mRNA samples in lanes 1-7 are from cells incubated for 2 hours, while samples from 8-12 were incubated for 8 hours. Samples represented in the figure are: 1: untreated HD11 cells (control), 2: *C. jejuni* 81-176, 3: HK *C. jejuni* 81-176, 4: K2-55, 5: *C. jejuni* NCTC11168V1, 6: *C. jejuni* NCTC11168V1*flgK* mutant, 7: SL1344, 8: untreated HD11 cells, 9: *C. jejuni* 81-176, 10: HK *C. jejuni* 81-176, 11: *C. jejuni* NCTC11168, and 12: *Salmonella* SL1344. **(B)** Purity of mRNA extracted is determined by the presence of only 3 spikes, a small spike at 23 seconds for positive control, spike at 43 seconds detects 18s ribosomal subunit, and at 49 seconds detects the presence of 28s ribosomal subunit. If any extra spikes are present mRNA is considered contaminated or degraded. Abbreviations: haitham-CTRL: mRNA extracted form unstimulated HD11 cells, 55: cells stimulated with *C. jejuni* K2-55, 69: cells stimulated with *C. jejuni* 81-176.

7.3.4. Gene expression levels of HD11 cells after incubation with *C. jejuni* 81-176, Heat-killed *C. jejuni* 81-76, and *C. jejuni* K2-55:

To determine if *C. jejuni* affects the level of expression of HD11 cytokine genes, HD11 cells were incubated with *C. jejuni* 81-176, *C. jejuni* K2-55. Heat-killed *C. jejuni* 81-176 was used as a control. Each strain was incubated with HD11 cells in duplicate. The expression of each gene from each sample at 0, 2, 4, and 8 hours was measured in duplicate, leading to a total of 4 wells/sample. Expression of IL-1β, IL-6, and IL- 8 genes showed increased levels indicating that living *C. jejuni* cells are not required to induce these cytokines (Table 7.3 and Fig 7.4, 7.5, and 7.6). Interlukin-4 was measured in all samples, and was not detected in any. Interleukin-2 was not expressed in any cells treated with the strains.

Expression level of IL-10 remained unchanged at the various time points (Fig 7.7), while IL-12β increased approximately 5, 4, and 3 fold in HD11 cells incubated with *C. jejuni* 81-176, heat-killed *C. jejuni* 81-176, and *C. jejuni* K2-55 respectively (Fig 7.8). Expression of TLR5 was detected in all samples and remained almost constant after 8 hours of exposure to the three strains (Fig 7.9).

7.3.5. Gene expression levels of HD11 cells after incubation with *C. jejuni* NCTC11168V1 and an isogenic *C. jejuni* NCTC11168V1*flgK* mutant:

A significant difference in HD11 cell gene expression of cytokines IL-1β, IL-6, and IL-8 in response to the presence of *C. jejuni* NCTC11168V1 or the isogenic *flgK* mutant was observed, as NCTC11168V1 increased expression of IL-1β, IL-6, and IL-8 by 17-, 20-, and 27-fold respectively, while the *C. jejuni* NCTC11168V1*flgK* mutant increased the expression by 6-, 3-, and 8- fold after 8 hours of incubation (Fig 7.4, 7.5, and 7.6). Levels of IL-10 gene expression remained unchanged for both strains (Fig 7.7), while IL-12β was upregulated slightly (Fig 7.8). As expected, the level of TLR5 gene expression did not change in the presence of either of the strains (Fig 7.9)

7.3.6. Gene expression levels of HD11 cells after incubation with *Salmonella* SL1344:

HD11 cell mRNA levels of IL-1β, IL-6, IL-8, and IL-10 increased after incubation with *Salmonella*SL1344 (Table 7.4) while IL-12β gene expression level was downregulated by 15 fold (Fig 7.8). Levels of TLR5 gene expression increased after 8

hours of incubation of HD11 cells with *Salmonella* SL1344 (Fig 7.9). Level of IL-2 expression was at a very low copy number (high Ct value) when levels of expression were measured at 8 hours after challenge with no significant fold changes.

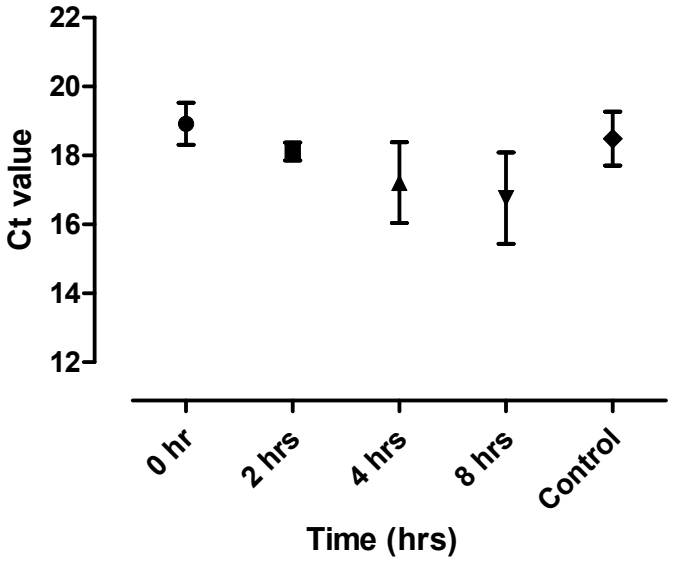

Fig 7.2. Mean Ct values ± SD for β-actin gene level of expression from all samples at 0, 2, 4, and 8 hours. Ct values were significantly ($P < 0.05$) lower after 4 and 8 hours of incubation with any of the *C. jejuni* strains and *Salmonella* SL1344 strain. Beta-actin gene level of expression in unstimulated HD11 cells remained unchanged (Control).

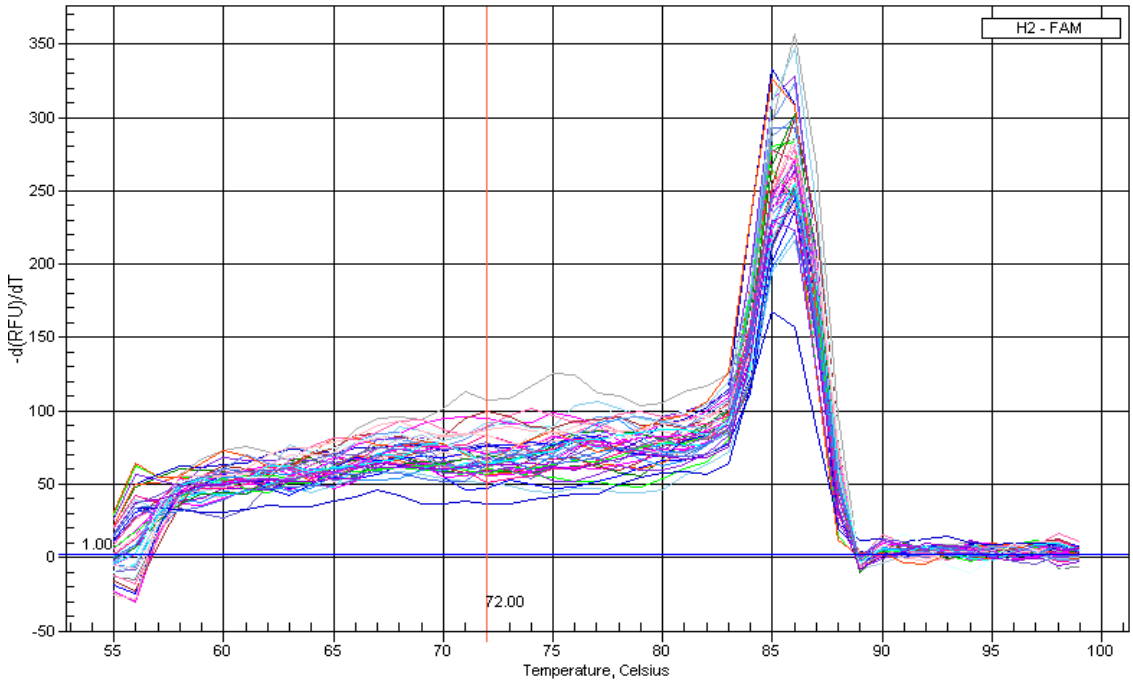

Fig 7.3. Real-time melting curves acquired after amplification of cDNA samples with IL-6 mRNA used as a template. For cDNA synthesis was extracted from HD11 samples treated with *C. jejuni* 81-176, heat killed *C. jejuni* 81-176, *C. jejuni* K2-55, *C. jejuni* NCTC11168V1, *C. jejuni* NCTC11168V1*flgK* mutant, and *Salmonella* SL1344 for 8 hours.

Fold change in gene expression over time												
Gene	0 hr			2 hrs			4 hrs			8 hrs		
	81-176	**K2-55**	**HK**	**81-176**	**K2-55**	**HK**	**81-176**	**K2-55**	**HK**	**81-176**	**K2-55**	**HK**
β-actin	1	1	1	1	1	1	1	1	1	1	1	1
IL-1β	1	1	1	0.7	3.5	2	3.4	4.3*	4.3*	16.7**	16.0**	15.5**
IL-6	1	1	1	1.5	1.3	0.8	29.7**	22.9**	22.9**	22.6**	17.8**	24.9**
IL-8	1	1	1	4.37*	5.5*	4.05*	19.97**	21.55**	21.55**	32.89**	30.27**	32**
IL-10	1	1	1	0.9	1	0.8	0.7	0.6	0.6	0.6	0.8	0.9
IL-12β	1	1	1	1.1	1.1	0.3	2.8	1.4	1.4	5.2*	3.6*	4.1*
TLR5	1	1	1	0.8	1.0	0.9	0.8	1.0	0.8	0.7	1.3	1.0

Table 7.3. Fold change in HD11 cell gene expression after incubation with *C. jejuni* 81-176, K2-55, and heat-killed *C. jejuni* 81-176. Untreated HD11 cells were used as controls. Gene expression values were normalized using β-actin gene expression value (reference gene) to obtain relative gene difference value (ΔCt). The difference between the average $\Delta Ct_{(sample)}$ and the average $\Delta Ct_{(control)}$ was expressed as ΔΔCt and was used to calculate fold changes in gene expression Fold change was calculated using the formula: fold change = $2^{-(\Delta\Delta Ct)}$. Fold change values of samples significantly different from control using pairwise fixed rellocation randomization test ($P < 0.05$) are marked with (*), while ($P < 0.01$) are marked with (**). The three treatments had induced a strong increase in IL-1, IL-6, and IL-8 expression, and a moderate IL-12β expression increase, but did not affect the expression of IL-10 or TLR5.

81-176: *C. jejuni* 81-176, K2-55: *C. jejuni* K2-55, HK: Heat-killed *C. jejuni* 81-176

Gene	Fold change in gene expression over time											
	0 hr			2 hrs			4 hrs			8 hrs		
	11168	***flgK***	**SL1344**	**11168**	***flgK***	**SL1344**	**11168**	***flgK***	**SL1344**	**11168**	***flgK***	**SL1344**
β-actin	1	1	1	1	1	1	1	1	1	1	1	1
IL-1β	1	1	1	0.8	1.3	1.3	3.8*	3.5*	4.2*	17.3**	5.7*	8.9**
IL-6	1	1	1	1.4	0.8	2	21**	2.3*	20.1**	20.3**	2.5*	15.3**
IL-8	1	1	1	4.4*	3.1*	1.13*	20.3**	8.57**	3.83*	26.90**	8.28**	23.75**
IL-10	1	1	1	0.6	0.6	1.7	0.8	1.4	1.1	0.8	0.9	4.3*
IL-12β	1	1	1	0.8	1	0.3	1.1	3.1*	0.08**	4.6*	2.8*	0.1**
TLR5	1	1	1	0.8	0.5	1.4	1.2	1.2	1.3	0.8	0.8	5.2*

Table 7.4. Fold change in HD11 cell gene expression after incubation with *C. jejuni* NCTC11168V1, *flgK* mutant, and *Salmonella* SL1344. Untreated HD11 cells were used as controls. Gene expression values were normalized using β-actin gene expression value (reference gene) to obtain relative gene difference value (ΔCt). The difference between the average $\Delta Ct_{(sample)}$ and the average $\Delta Ct_{(control)}$ was expressed as ΔΔCt and was used to calculate fold changes in gene expression Fold change was calculated using the formula: fold change = $2^{-(\Delta\Delta Ct)}$. Fold change values of samples significantly different from control using Turkey's test ($P < 0.05$) are marked with (*), while ($P < 0.01$) are marked with (**).*C. jejuni* NCTC11168 induced a strong expression of IL-1β, IL-6, and IL-8 cytokines genes, and a moderate increase in IL-12β gene expression, but did not affect the expression of IL-10 and TLR5. *flgK* mutant induced a moderate cytokine gene expression and did not affect TLR5 expression. *Salmonella* SL1344 induced a strong expression of IL-1β, IL-6, and IL-8 cytokines genes, a moderate expression of IL-10 and TLR5, and down-regulated the expression of IL-12β.

11168: *C. jejuni* NCTC11168V1, *flgK*: *C. jejuni* NCTC11168V1*flgK*, SL1344: *Salmonella* SL1344

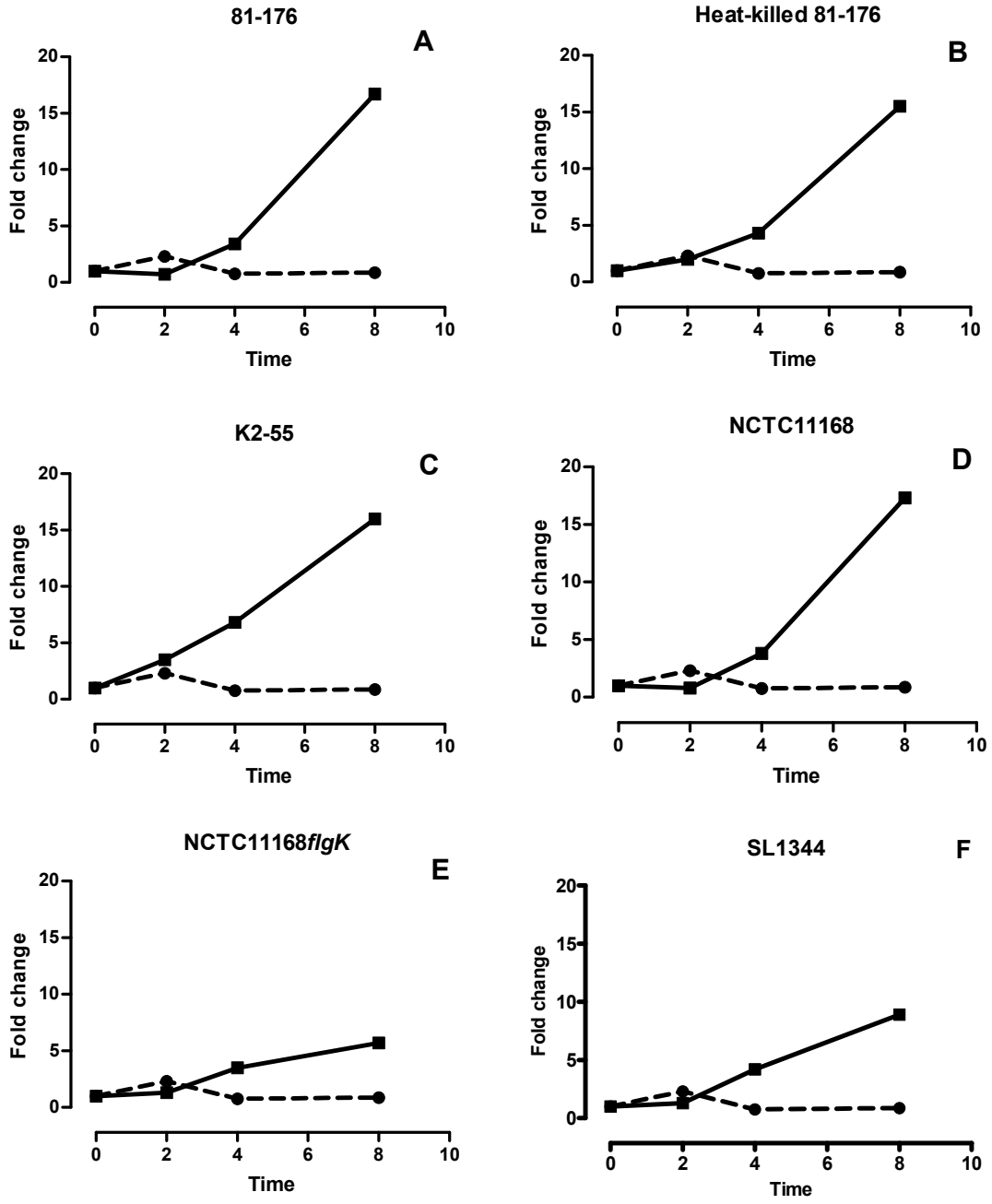

Fig 7.4. Fold changes in expression of IL-1β in HD11 cells at 0, 2, 4, and 8 hours post-exposure to: (A) *C. jejuni* 81-176, (B) heat killed (HK) *C. jejuni* 81-176, (C) *C. jejuni* K2-55, (D) *C. jejuni* 11168, (E) *flgK* mutant, and (F) *Salmonella* SL1344. Fold changes represented are calculated using mean Ct value of duplicate biological samples. The solid line represents samples and the dotted line represents the expression of IL-1β in control tissue culture. The NCTC11168*flgK* mutant induced a moderate increase in gene expression compared to other *C. jejuni* strains.
81-176: *C. jejuni* 81-176, heat-killed 81-176: heat-killed *C. jejuni* 81-176,
K2-55: *C. jejuni* K2-55, NCTC11168: *C. jejuni* NCTC11168V1, NCTC11168*flgK*: *C. jejuni* NCTC11168V1*flgK*, SL1344: *Salmonella* SL1344.

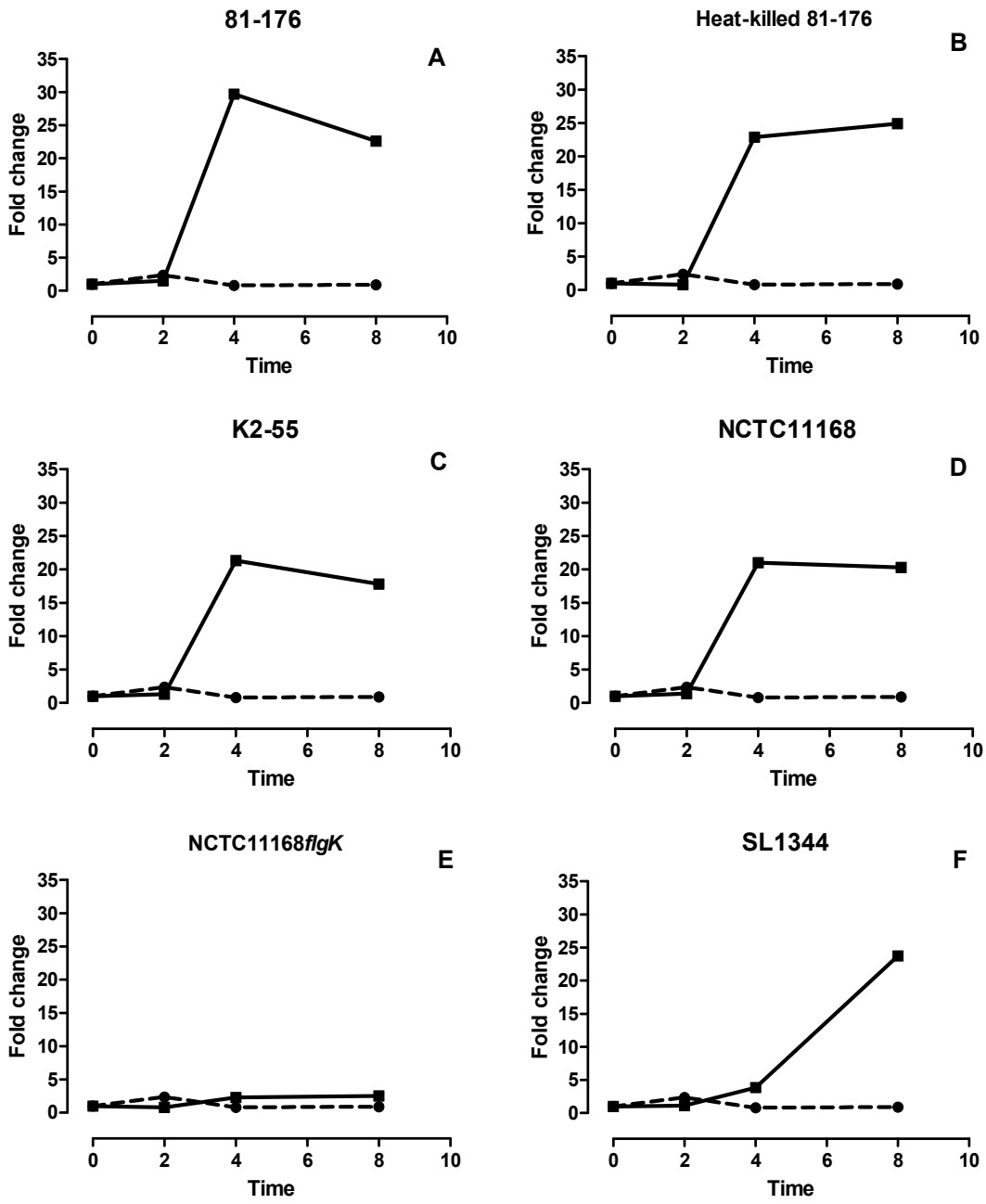

Fig 7.5. Fold changes in expression of IL-6 in HD11 cells at 0, 2, 4, and 8 hours post-exposure to: (A) *C. jejuni* 81-176, (B) heat killed (HK) *C. jejuni* 81-176, (C) *C. jejuni* K2-55, (D) *C. jejuni* 11168, (E) *flgK* mutant, and (F) *Salmonella* SL1344. The solid line represents samples and the dotted line represents the expression IL-6 in control tissue culture. *C. jejuni* NCTC11168*flgK* mutant induced a limited increase (2.5 fold) in IL-6 expression compared to other *C. jejuni* strains which induced more than 20 fold increase in expression. 81-176: *C. jejuni* 81-176, heat-killed 81-176: heat-killed *C. jejuni* 81-176, K2-55: *C. jejuni* K2-55, NCTC11168: *C. jejuni* NCTC11168V1, NCTC11168*flgK*: *C. jejuni* NCTC11168V1*flgK*, SL1344: *Salmonella* SL1344.

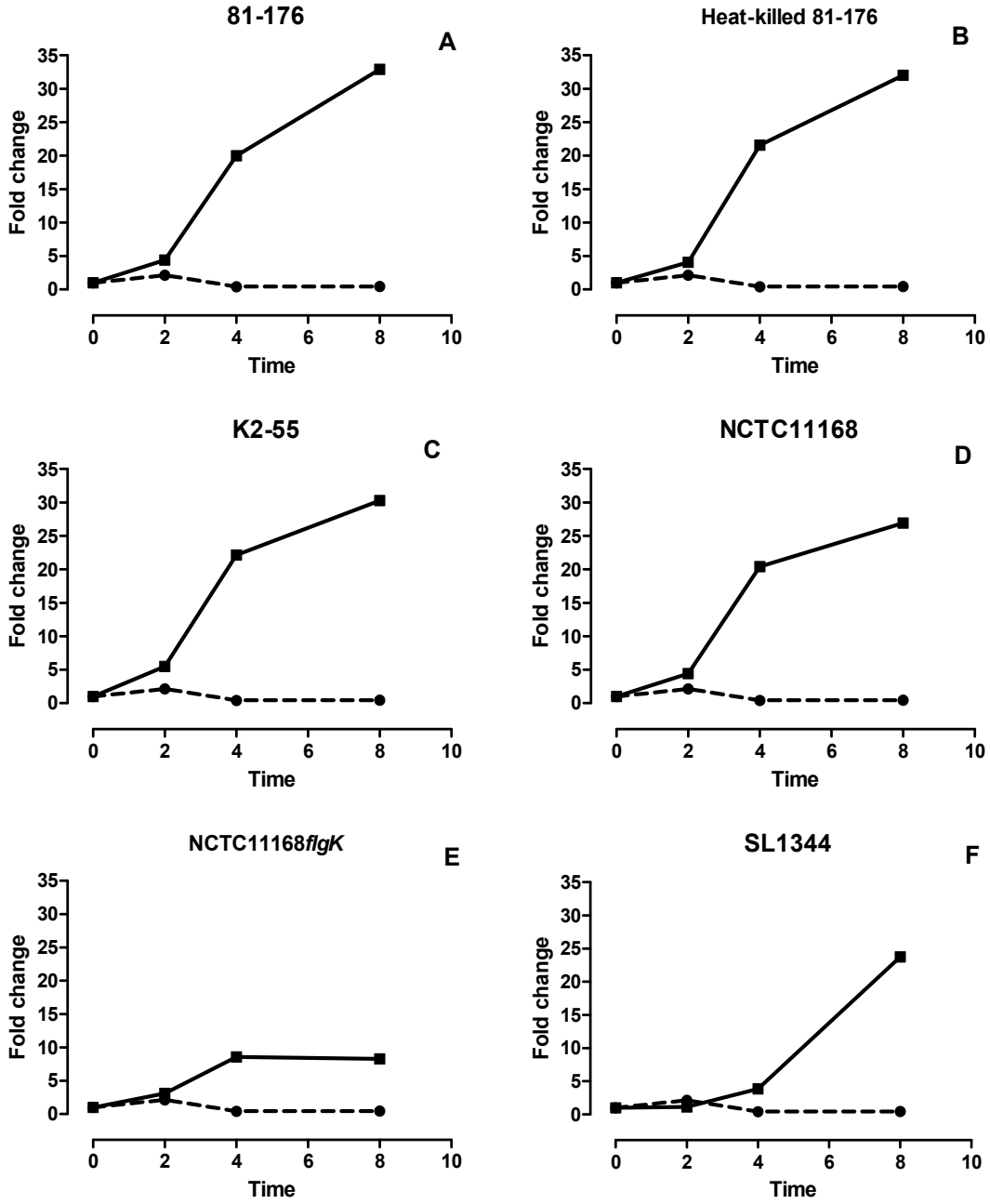

Fig 7.6. Fold changes in expression of IL-8 in HD11 cells at 0, 2, 4, and 8 hours post-exposure to: (A) *C. jejuni* 81-176, (B) heat killed (HK) *C. jejuni* 81-176, (C) *C. jejuni* K2-55, (D) *C. jejuni* 11168, (E) *flgK* mutant, and (F) *Salmonella* SL1344. The solid line represents samples and the dotted line represents the expression of IL-8 in control tissue culture. The *flgK* mutant induced a moderate 10 fold increase in IL-8 expression after 8 hours of incubation. Other *C. jejuni* strains induced more than 25 fold in expression of IL-8. *Salmonella* SL1344 induced a 4 fold increase in expression at 4 hours, and 24 fold increase by 8 hours. 81-176: *C. jejuni* 81-176, heat-killed 81-176: heat-killed *C. jejuni* 81-176, K2-55: *C. jejuni* K2-55, NCTC11168: *C. jejuni* NCTC11168V1, NCTC11168V1*flgK*: *C. jejuni* NCTC11168V1*flgK*, SL1344: *Salmonella* SL1344.

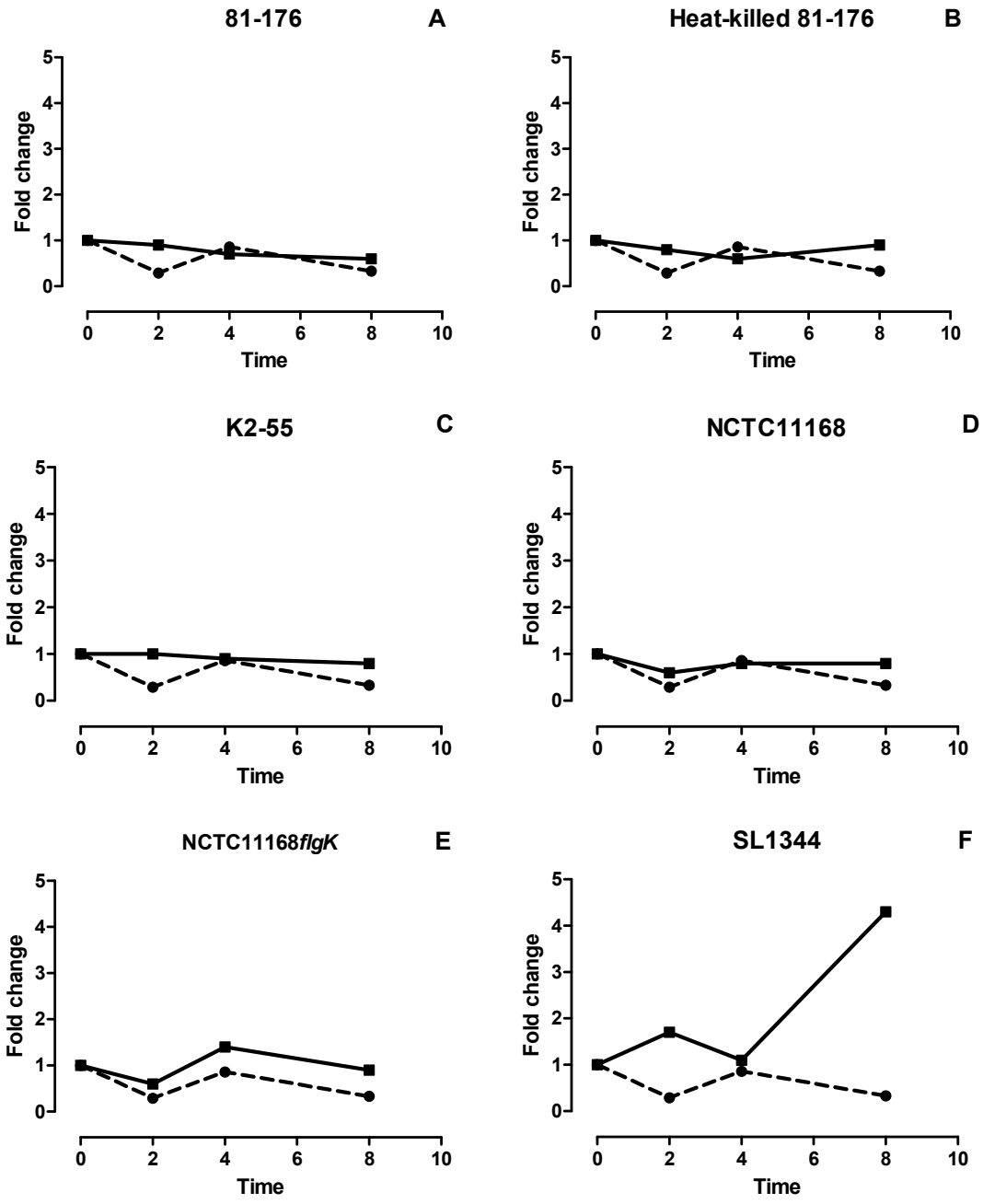

Fig 7.7. Fold changes in expression of IL-10 in HD11 cells at 0, 2, 4, and 8 hours post-exposure to: (A) *C. jejuni* 81-176, (B) heat killed (HK) *C. jejuni* 81-176, (C) *C. jejuni* K2-55, (D) *C. jejuni* 11168, (E) *flgK* mutant, and (F) *Salmonella* SL1344. The solid line represents samples and the dotted line represents the expression of IL-10 in control tissue culture. None of *C. jejuni* strains induced the expression of IL-10, while SL1344 induced a moderate increase by 4.3 fold after 8 hours of infection. 81-176: *C. jejuni* 81-176, heat-killed 81-176: heat-killed *C. jejuni* 81-176, K2-55: *C. jejuni* K2-55, NCTC11168: *C. jejuni* NCTC11168V1, NCTC11168V1*flgK*: *C. jejuni* NCTC11168*flgK*, SL1344: *Salmonella* SL1344.

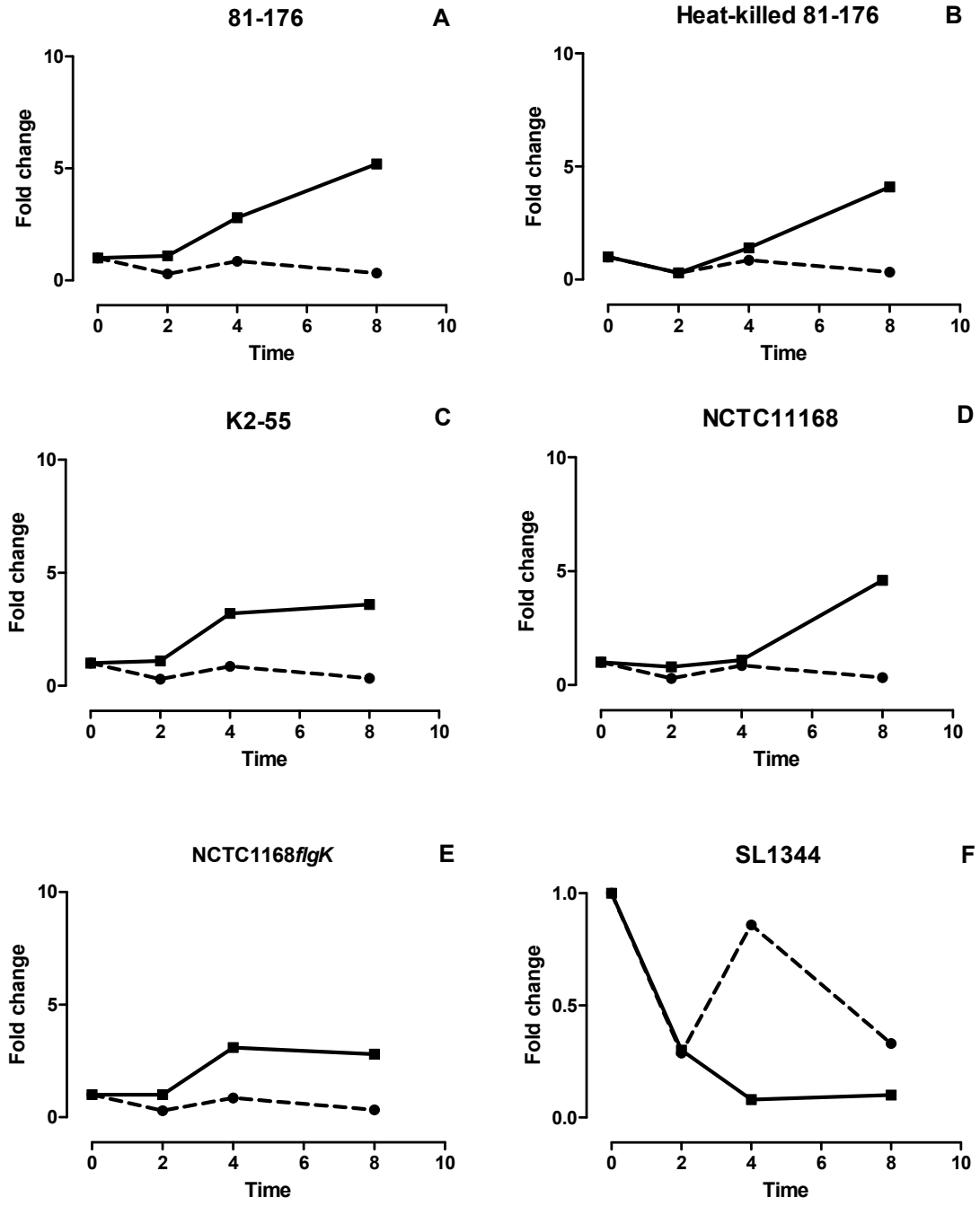

Fig 7.8. Fold changes in expression of IL-12β in HD11 cells at 0, 2, 4, and 8 hours post-exposure to: (A) *C. jejuni* 81-176, (B) heat killed (HK) *C. jejuni* 81-176, (C) *C. jejuni* K2-55, (D) *C. jejuni* 11168, (E) *flgK* mutant, and (F) *Salmonella* SL1344. The solid line represents samples and the dotted line represents the expression IL-2β in control tissue culture. *C. jejuni* strains induced a moderate (3-5 fold) increase in expression of IL-12β while *Salmonella* SL1344 down-regulated the expression by 15 fold after 8 hours of infection. 81-176: *C. jejuni* 81-176, heat-killed 81-176: heat-killed *C. jejuni* 81-176, K2-55: *C. jejuni* K2-55, NCTC11168: *C. jejuni* NCTC11168V1, NCTC11168*flgK*: *C. jejuni* NCTC11168V1*flgK*, SL1344: *Salmonella* SL1344.

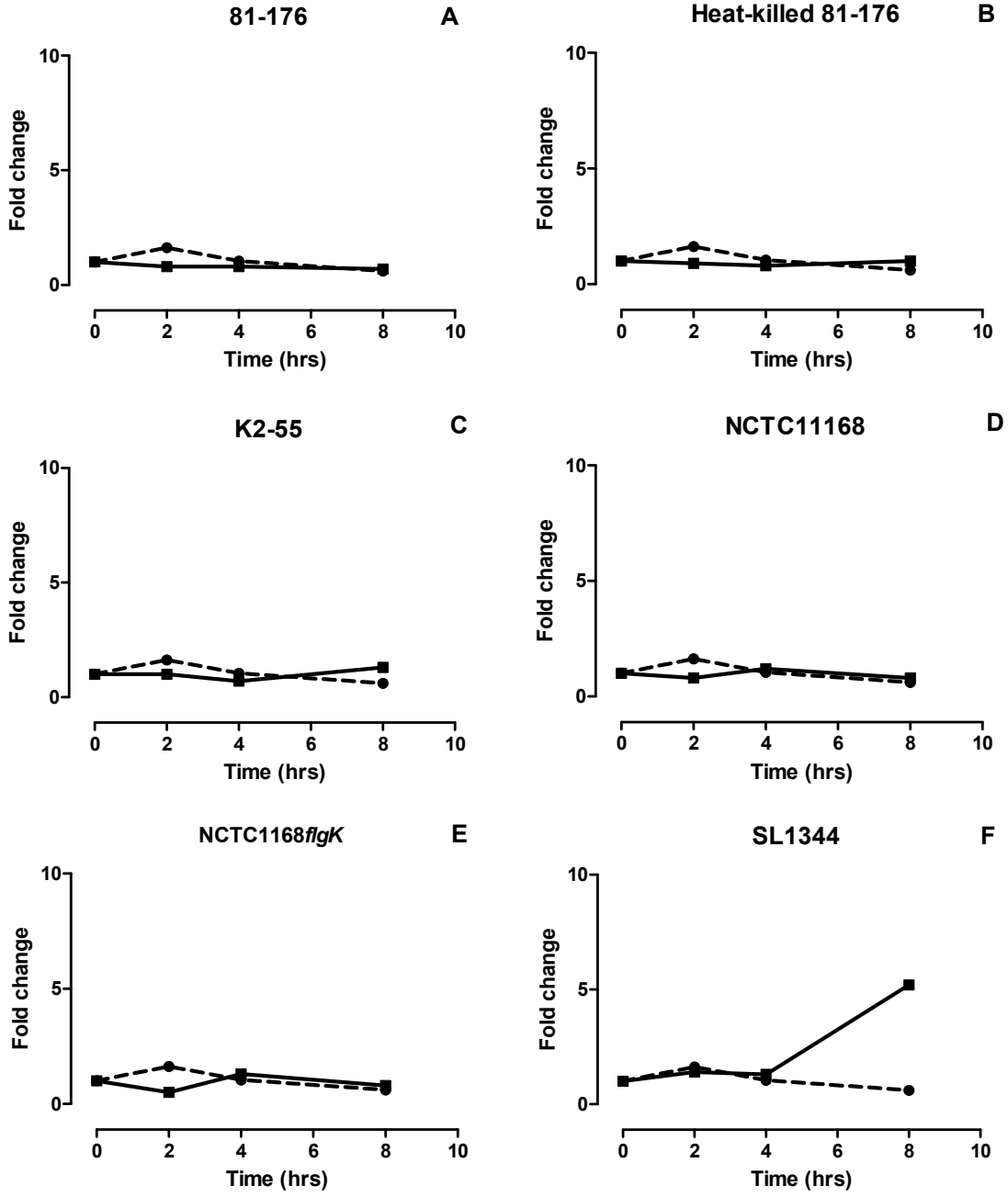

Fig 7.9. Fold changes in expression of TLR5 in HD11 cells at 0, 2, 4, and 8 hours post-exposure to: (A) *C. jejuni* 81-176, (B) heat killed (HK) *C. jejuni* 81-176, (C) *C. jejuni* K2-55, (D) *C. jejuni* 11168, (E) *flgK* mutant, and (F) SL1344. The solid line represents samples and the dotted line represents the expression of TLR5 in control tissue culture. *C. jejuni* strains did not affect the expression of TLR5 while SL1344 caused a moderate 5 fold increase after 8 hours of infection. 81-176: *C. jejuni* 81-176, heat-killed 81-176: heat-killed *C. jejuni* 81-176, K2-55: *C. jejuni* K2-55, NCTC11168: *C. jejuni* NCTC11168V1, NCTC11168V1*flgK*: *C. jejuni* NCTC11168*flgK*, SL1344: *Salmonella* SL1344.

7.4. Discussion:

The asymptomatic nature of *C. jejuni* infection in broiler chickens, despite the high level of colonization of the ceca (up to 10^{10} CFU/g) remains a major challenge for controlling the spread of infection to humans. The difference in the clinical outcome between chickens and humans is probably multi-factorial as no single factor has been identified to be solely responsible for the difference in outcome. Cytokines play a major role in orchestrating the immune response by either promoting or suppressing inflammation. A role for the pro-inflammatory cytokines in causing disease symptoms during gastro-intestinal disease has also been suggested (Enocksson et al., 2004). Cytokine induction by *C. jejuni* has been mainly studied in human cell lines (Al-Salloom et al., 2003; Bakhiet et al., 2004; Hickey et al., 2000; Jones et al., 2003), while only a few studies have investigated avian cytokine profiles (Smith et al., 2008; Smith et al., 2005; Watson and Galan, 2008). The development of an antibody response by chickens infected with *C. jejuni* indicates that inter-cellular signaling among the immune cells following the host-pathogen interaction has occurred, and *C. jejuni* presumably is exposed to the innate immune system during the infection (Meade et al., 2009; Smith et al., 2008). Macrophages are an integral part of the innate immune system, and may play a role in clearing systemic *C. jejuni* infection, as a macrophage-like HD11 cell kills *C. jejuni* within 24 hours of incubation *in vitro* (Watson and Galan, 2008). Several cytokines are induced during this process (Smith et al., 2005), and thus broiler chickens exposed to *C. jejuni* will have increased cytokine expression upon the colonization by *C. jejuni* (Smith et al., 2008).

One of the main antigenic components of *C. jejuni* is the flagellum, which is recognized by antibodies of chickens infected with *C. jejuni* (de Zoete et al., 2007). This study investigated the role that *C. jejuni* flagellum and Cia secreted proteins have in inducing an HD11 cytokine response. The results demonstrated that wild-type *C. jejuni* that has intact flagella induced a strong increase in expression of IL-1β, IL-6, and IL-8 in HD11 cells. These results are in agreement with the data previously reported (Smith et al., 2008; Smith et al., 2005; Watson and Galan, 2008). The response was moderate at 2 hours postinfection, and increased significantly ($P < 0.01$) after 4 and 8 hours of incubation as *C. jejuni* 81-176 and NCTC11168V1 caused an increase by more than 30

fold for IL-8, 16 fold for IL-1, and as much as 22 fold for IL-6 in comparison to control cells after 8 hours of incubation.

The absence of motility and *Campylobacter* invasion antigens secretion did not affect *C. jejuni* interaction with HD11 cells. *C. jejuni* K2-55, which has a paralyzed flagellum that is structurally intact and does not secrete Cia, had the same cytokine expression profile of wild-type *C. jejuni* 81-176. A moderate difference of IL-6 expression of 5 fold between the mutant and the wild-type, which might not be biologically significant as the response profile for both strains followed a similar trend. These findings are further confirmed by the fact that a similar response was induced by heat-killed *C. jejuni* 81-176, *C. jejuni*. This suggests that stimulatory antigens are not actively produced upon contact with the HD11 cells, but have already been synthesized by *C. jejuni*. These stimulatory antigens are probably classic innate immune system stimulators, such as lipooligosaccharides, DNA, and flagellin. When the flagellin-lacking *C. jejuni* NCTC11168V1*flgK* mutant, which expresses the flagellar structure up to the hook (Fernando et al., 2007), infected HD11 cells it stimulated only a moderate (though significant) increase in the expression of IL-1β and IL-8 (6 and 8 fold respectively), and approximately three-fold increase in IL-6 and IL-12β expression. The *C. jejuni flgK* mutant has a reduced ability to adhere to and invade HeLa cells *in vitro* and colonize day-old chickens (Fernando et al., 2007), which might have contributed to the limited response in gene expression. The increase in expression of IL-1β, a pro-inflammatory cytokine that stimulates T-cell proliferation, and IL-8, a chemokine that recruits heterophils, is an indicator that *C. jejuni* promotes an inflammatory response through recruitment of cells and promoting their replication. A similar response to the presence of *C. jejuni* by human epithelial cells and clinical samples has been observed (Enocksson et al., 2004; Hickey et al., 2000; Jones et al., 2003; Zheng et al., 2008), but the clinical outcome and the pathological findings are different than those found in chickens upon *C. jejuni* infection. Zheng and colleagues found that T84 human colonic epithelial cells produced IL-8 upon infection with any of 11 *C. jejuni* strains that varied in their adherence and invasion abilities (Zheng et al., 2008). Heterophils, which are equivalent to neutrophils in mammals, were recruited when two-week-old chickens were challenged with *C. jejuni* (Smith et al., 2008), but no lesions were found. The lack of lesion

formation is not unique to *C. jejuni*, as some *Salmonella* strains cause PMNs recruitment in pigs without any lesion formation (Foster et al., 2003).

C. jejuni did not affect the expression of IL-10, which is involved mainly in down-regulating Th1 response and stimulating B, T, and NK cells; while *Salmonella* caused a moderate 5 fold increase in HD11 cells. IL-10 expression was not detected when day-old and 2-weeks old chickens were challenged with *C. jejuni* (Smith et al., 2008), but was detected and remained unchanged in HD11 cells (Smith et al., 2005). These findings indicate a Th1-biased immune response. However, the presence of the Th1-Th2 biased response in chickens is still debatable as some of the hallmarks of Th2 response are absent in chickens. Interlukin-4, which induces proliferation of various cell types, was not expressed in HD11 cells before or after the challenge, supporting a Th1 biased response by HD11 cells infected with *C. jejuni*.

The absence of flagellin in *C. jejuni flgK* mutant clearly produced a lower expression of several cytokine by HD11 cells compared to wild-type *C. jejuni*, while the absence of motility and Cia secreted proteins had no effect on HD11 cells response to *C. jejuni* K2-55, indicating that the presence of flagellin has an immunomodulating effect, while Cia proteins did not affect cytokine gene expression in HD11 cells

The lack of pathology in birds infected with *C. jejuni* does not indicate a lack of pro-inflammatory response. The various *C. jejuni* strains used in this study were capable of inducing cytokine gene expression. The absence of motility or Cia did not alter cytokine expression in HD11 cell, but the lack of flagellin had a major impact on cytokine induction. It could be concluded that the presence of flagella itself is more detrimental to cytokine induction than motility or *Campylobacter* invasion antigens.

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Chapter VIII: General Discussion and Conclusions

Despite the importance of *C. jejuni* as a leading cause of bacterial gastroenteritis in humans, the pathogenesis of *C. jejuni* is poorly understood. When compared to the other two major bacterial pathogens, *E. coli* and *Salmonella*, several aspects of *C. jejuni*'s pathogenesis remain unknown. The study of *C. jejuni* pathogenesis in poultry has been hindered by the lack of tools that help to track the bacteria *in vivo*. This research is another step to understand the pathogenesis of *C. jejuni* in broiler chickens and the role that the flagella, *Campylobacter* invasion antigens, and motility play in mucosal and systemic spread of the bacteria.

By establishing a system using green fluorescent protein as a marker, we were able to track *C. jejuni* mucosally and systemically in broiler chickens. The plasmid pHG101was stable *in vitro* when subcultured several times in the absence or the presence of selective antibiotic pressure. Similar plasmid stability has been reported previously in *C. jejuni*, both *in vitro* and *in vivo* (Miller et al., 2000; Mixter et al., 2003). Miller and colleagues were unable to transform all the strains they tested with the plasmid they developed (Miller et al., 2000), while in our study all the variants we used were transformed. After transformation, no visible morphological changes were noticed in any of the *C. jejuni* strains when visualized using electron microscopy, and no significant changes, in the growth rate or morphology, were noticed when the bacteria were cultured or used to challenge poultry or mice. These findings are in agreement with the literature, as *Salmonella* expressing GFP constitutively, with a kanamycin marker similar to the one used in this study, had the same invasiveness in canine kidney cells as the wild-type (Clark et al., 2009). Mixter and colleagues detected GFP-expressing *C. jejuni* in mouse macrophages, neutrophils, spleenocytes, and hepatocytes after intraperitoneal challenge (Mixter et al., 2003). In our experiments, *C. jejuni* NCTC11168V1G remained fluorescent in the ceca of broiler chickens after 7 days of oral challenge, as detected by culture and fluorescence microscopy. The colonization level of *C. jejuni* NCTC11168V1G was similar to that of *C. jejuni* NCTC11168V1 (Biswas et al., 2007a; Carrillo et al., 2004), indicating that the ability to colonize the cecum was not affected by the expression of GFP. *C. jejuni* NCTC11168V1G and *C. jejuni* 81-176G remained fluorescent both mucosally and systemically, despite the different surrounding

environment in the host, which allowed for its detection in liver and spleen of broiler chickens. To date, there are no literature reports in about systemic detection of GFP-expressing bacteria delivered via an oral challenge. There are, however, reports of GFP-expressing *Lactobacillus* detected on mucosal surfaces and cecal contents (Yu et al., 2007). The use of confocal microscopy and immunohistochemistry allowed for confirmation of the presence of *C. jejuni* in positive tissues, and its absence in negative tissues. The GFP-based detection system developed in this study will be useful in studying the effect of mutations on colonization at mucosal surfaces and systemic spreading. When plasmid pHG101 was transformed into *C. jejuni* 81-176 and K2-55, the same plasmid stability was observed *in vitro* and *in vivo*. *C. jejuni* K2-55G colonized day-old chicks despite the absence of both motility and *Campylobacter* invasion antigens secretion, indicating that the presence of full-length flagella was the minimal requirement for mucosal colonization. The absence of motility and *Campylobacter* invasion antigens affected the ability of *C. jejuni* K2-55 to spread systemically in 21-day old broiler chickens, indicating that they are required for invasion/systemic spread, which is in agreement with our hypothesis that the flagella and flagella secreted proteins are important for *C. jejuni* colonization of broiler chickens.

C. jejuni 11168V1G, *C. jejuni* 81-176G, and *C. jejuni* K2-55G colonized the gastrointestinal tract of BALB/c mice, but were not detected in any of the spleens or livers examined. *C. jejuni* K2-55 was not as efficient as the wild-type *C. jejuni* 81-176 in colonizing the gastrointestinal tract of BALB/c mice, as it was detected in only 53.3% of mice challenged, compared to 66.6% of mice challenged with *C. jejuni* 81-176.

The lack of systemic spreading and *Campylobacter* invasion antigens secretion by *C. jejuni* K2-55 made this strain a potential live vaccine candidate. We investigated the vaccine potential of *C. jejuni* K2-55, and the difference the absence of *Campylobacter* invasion antigens and/or flagella may have on macrophage-like HD11 cell expression of cytokines.

Oral vaccination with *C. jejuni* K2-55 on days 7 and 21 of age resulted in a moderate antibody response, which was protective when combined with antibiotic treatment before the challenge with the homologous strain *C. jejuni* 81-176 when compared to non-vaccinated group. Vaccination was not protective against the challenge

with the homologous strain without the antibiotic treatment. The reason for the different outcome is not related to antibody response, as there was no significant difference between the median of antibody titers of these groups, but it could be due to the changes in the intestinal microflora after antibiotic treatment (Pedroso et al., 2006; Smirnov et al., 2005). *C. jejuni* K2-55 did not protect against a heterologous *C. jejuni* NCTC11168 challenge as well, as there was no significant difference between the colonization level (CFU/g) of the vaccinated and non-vaccinated groups. This limits the future applications of this vaccine (in its current form) to homologous protection only. The same vaccine failed to protect broiler chickens from colonization with *C. jejuni* 81-176 in a seeder model challenge despite the antibiotic treatment. The reason for the difference in the outcome remains unknown, but the dynamics of *C. jejuni* in a room that contains vaccinated and non-vaccinated birds that are shedding may be different than its dynamics in a room that contains vaccinated chickens only. The homologous protection and the lasting colonization of the paralyzed flagella *C. jejuni* K2-55 is a promising initial step that is still in early development.

The absence of motility and *Campylobacter* invasion antigens did not affect macrophage-like HD11 cell cytokine expression, while the absence of flagellin had a significant impact on cytokine expression levels under the same conditions. The absence of flagellin subunits in the *C. jejuni* NCTC11168*flgK* mutant resulted in a moderate (5-8 fold) stimulation of pro-inflammatory cytokines IL-1β, IL-6, and IL-8, compared to wild-type *C. jejuni*, heat-killed *C. jejuni*, and the *C. jejuni* K2-55 paralyzed flagella mutant which had a significant increase in expression of these cytokines (15-32 fold). None of the *C. jejuni* strains tested stimulated IL-10 or TLR5 expression, which is not a surprising finding (Iqbal et al., 2005b; Watson and Galan, 2005). There was no significant difference in stimulation of IL-12β expression among the different *C. jejuni* strains, indicating that the absence of flagellin or *Campylobacter* invasion antigens had no impact on IL-12β production. These results confirm our hypothesis that the flagella have a significant impact on cytokine expression, but indicate a limited role for either motility or secretion of *Campylobacter* invasion antigens in stimulation of cytokines by chicken macrophages. These findings do not rule out any impact motility or *Campylobacter* invasion antigens may have on expression of cytokines by intestinal cells.

The information obtained from the experiments described in this study, along with the current literature, were used to formulate a description of the major pathogenesis steps occuring during mucosal and systemic spread of *C. jejuni* in broiler chickens (Fig 8.1 and 8.2). After oral infection, *C. jejuni* passes through the gastrointestinal tract, and establishes its population mainly in the ceca of the chicken (Beery et al., 1988). The exact reason for preferential colonization of the cecum remains unknown, but it is probably a combination of abundant nutrients and the limited motility of the ceca (Rehman et al., 2007; Udayamputhoor et al., 2003). *C. jejuni* colonizes the large intestine as a second stage of infection, and subsequently establish infection in the small intestine. *C. jejuni* resides mainly in the mucus layer and in close proximity with the intestinal epithelium (Beery et al., 1988; Smith et al., 2008). *C. jejuni* was not observed in any of the submucosal layers in the intestinal tract, but it has to cross the intestinal epithelium to spread systemically. It is unclear if *C. jejuni* is internalized into intestinal epithelial cells or if it crosses the mucosal barrier by disrupting the intestinal tight junctions, but *C. jejuni* survives in intestinal cells (Watson and Galan, 2008), making intracellular route more probable than crossing between cells, as no lesions are observed in chickens colonized with *C. jejuni* (Smith et al., 2008). Immunomodulation by *C. jejuni* has two major indicators. Firstly, cecal cells produce a variety of cytokines in chickens colonized by *C. jejuni* (Smith et al., 2008). Secondly, expression of antimicrobial peptides is significantly reduced in *C. jejuni* infected chickens (Meade et al., 2009). Secreted cytokines, including pro-inflammatory cytokines, cause an influx of innate immune cells, mainly heterophils (Smith et al., 2008) and macrophages (Meade et al., 2009). *C. jejuni* probably uses macrophages to spread systemically, as the number of macrophages in chickens' blood increases after a challenge with *C. jejuni* (Meade et al., 2009). Once *C. jejuni* resides in the spleen or liver, it will survive for at least 3 days.

The adaptive immune response plays an important role in clearing *C. jejuni* infection in broiler chickens, as antibodies against *C. jejuni* help prevent or clear the cecal colonization (Sahin et al., 2003). In our vaccination study, chickens remained colonized as vaccinated groups did not develop a significant increase in antibody response when compared to an unvaccinated group.

The major finding from this study was that motility and *Campylobacter* invasion antigens mutations affected the ability of *C. jejuni* to colonize broiler chickens' mucosal surfaces, but their effect had a greater impact on systemic spread. Future experiments should investigate in depth the exact role of these two important characteristics in colonization of broiler chickens, as the exact role *Campylobacter* invasion antigens play at mucosal surfaces remains unknown. As well, the impact these antigens may have on immune system cells other than macrophages needs to be determined. Answering these questions will shed some light on pathogenesis of *C. jejuni* in broiler chickens, and lead to development of a protective vaccine that will reduce the *C. jejuni* carriage of broiler chickens, maybe to understanding the pathogenesis of these bacteria in humans.

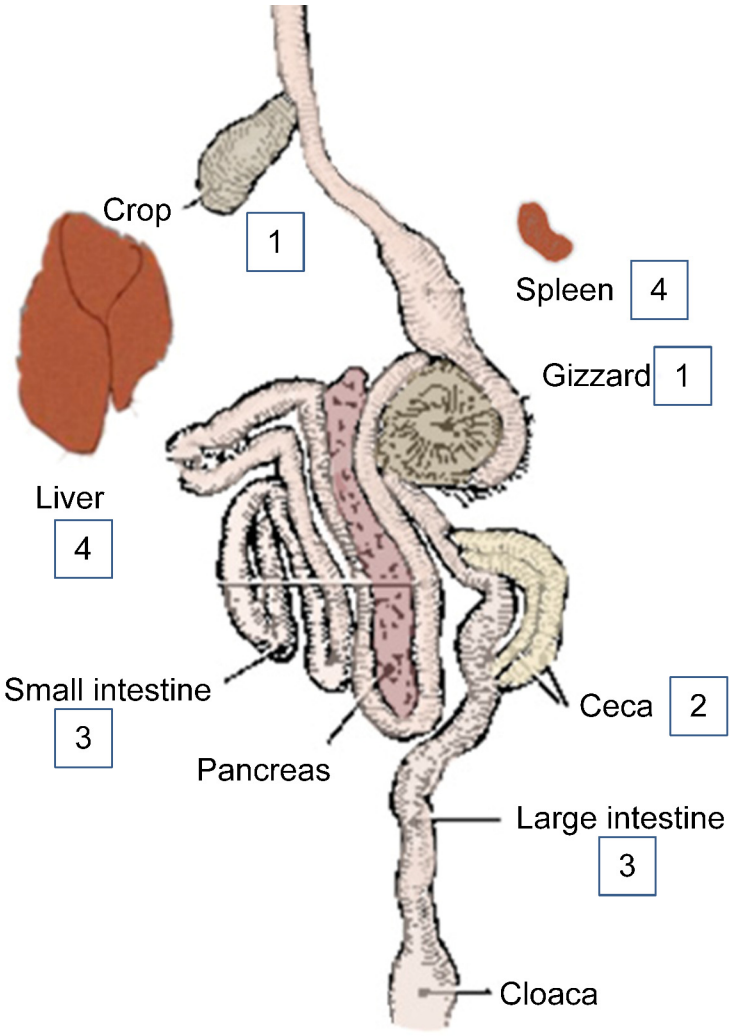

Fig 8.1. Colonization of broiler chickens by *C. jejuni*. The initial infection step for *C. jejuni* is to colonize the gastrointestinal tract of broiler chickens. The bacteria were not detected in the crop or the gizzard **(1)**, but the initial and main sites for colonization were the ceca of the chicken **(2)**, which provide abundant nutrients and a relatively reduced intestinal motility compared to other parts of the intestine. *C. jejuni* then spread to the large intestine **(3)**, and then spread to the small intestine shortly after **(3)**. After 72 hours of infection, *C. jejuni* was detected in the liver and spleen. The absence of *Campylobacter* invasion antigens prevented *C. jejuni* K2-55 from systemic spread. (Fig 8.1. is modified from an image courtesy of Dr. Bruce Hunter, University of Guelph).

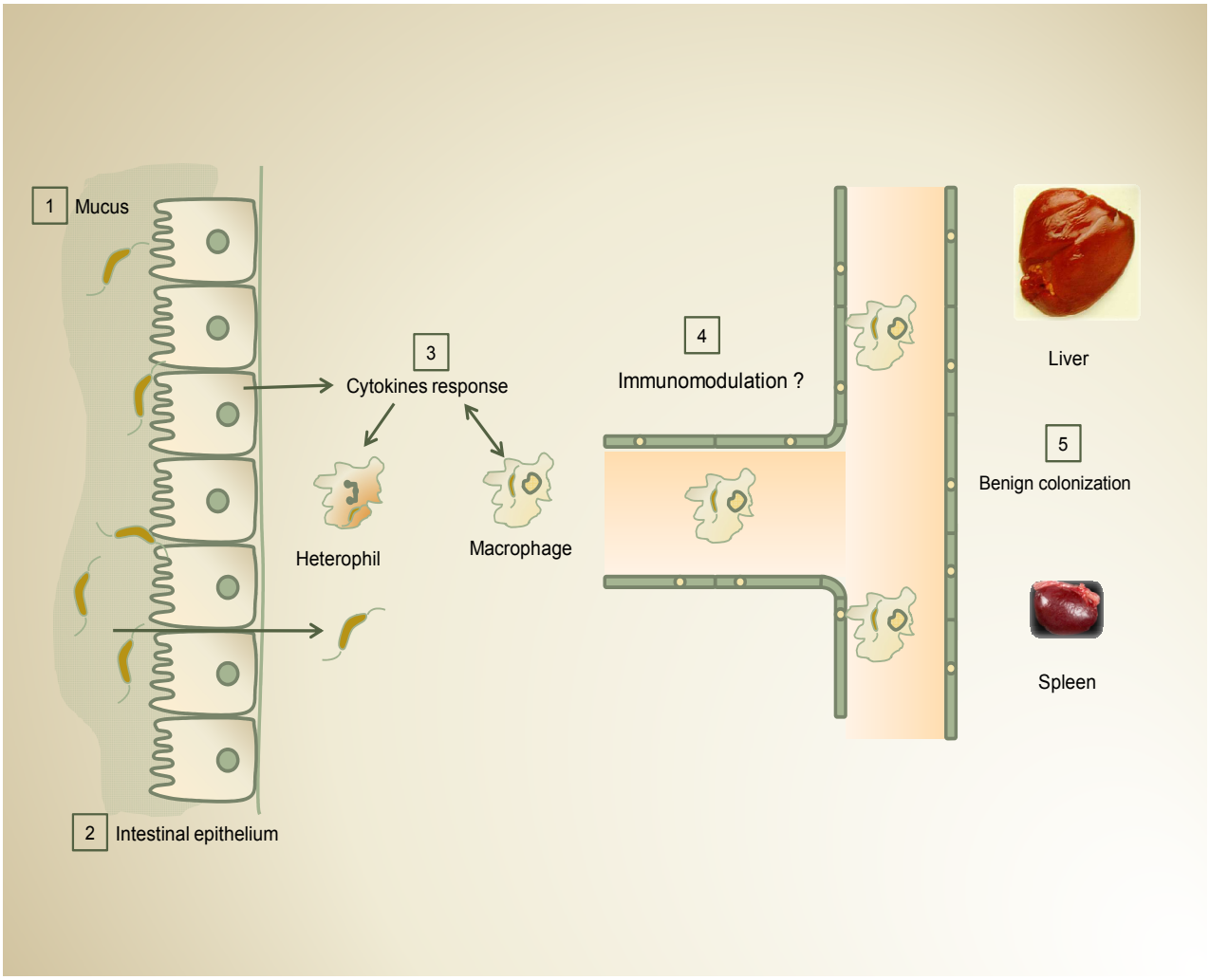

Fig 8.2. Mucosal and systemic dynamics of *C. jejuni* in broiler chickens. After establishing itself in the cecum, *C. jejuni* is associated mainly with the mucus layer **(1)**, but it must cross the intestinal epithelium barrier as it spreads systemically **(2)**. Crossing the epithelium will induce a cytokine response by epithelial cells regardless of the path followed by *C. jejuni*; i.e. para-cellular or intracellular **(3)**. Once cytokines are secreted, innate immune system cells will be recruited to the site, mainly macrophage and/or heterophils. At this stage *C. jejuni* is internalized, as there is no record of bacteremia in chickens. During the internalization process, *C. jejuni* immunomodulates the immune system, resulting in a delayed antibody response against the infection **(4)**. *C. jejuni* is transported to liver and/or spleen, where it colonizes benignly without any detectable lesion formation **(5)**. More details are provided in the text above.

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