CHARACTERISATION OF THE LIPOOLIGOSACCHARIDE BIOSYNTHESIS GENE CLUSTER IN *CAMPYLOBACTER* SPECIES

Submitted for the degree of

Doctor of Philosophy (Genetics)

At the University of Northampton

March 2019

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Declaration

I confirm that it is the result of work mainly done at the university during the period of registration. I confirm that the work presented in this thesis is of the author alone and it has not been submitted previously to qualify for any other academic award. I confirm that information, obtained from other sources, has been stated with its references in this thesis.

Word Count: 40,750

Abstract

The extensive genetic variation in the lipooligosaccharide (LOS) core biosynthesis gene cluster, a majority of which occurs in the LOS outer core biosynthesis gene content present between *lgtF* and *waaV*, have led to the development of a classification system; with 8 classes (I-VIII) for *Campylobacter coli* (*C. coli*) LOS region and four groups (1-4) with 23 classes (A-W) for *Campylobacter jejuni* (*C. jejuni*) LOS region. The aim of this work was to characterise the *C. jejuni* and *C. coli* LOS biosynthesis loci with special emphasis on their classes' distribution and also to determine the role of LOS in mediating the host immune response. Analysis of the LOS locus gene content in 50 *C. jejuni* clinical isolates and 703 publicly available *C. jejuni* genome sequences revealed that the class B (Group 1) was the most abundant LOS locus class in *C. jejuni*. Two novel *C. jejuni* LOS types were identified from the GenBank database which may have arisen due to interspecies and intraspecies LOS gene recombination. *In silico* analysis of LOS locus gene content in 564 publicly available *C. coli* genome sequences identified previously unknown LOS inner core biosynthesis genes; all were located between *waaF* and *gmhA* and occurred in 5 *C. coli* LOS locus types (I, II, III, V, VIII). It was also determined that class III is the most abundant LOS locus type in *C. coli* and the environmental niches are the major reservoirs of *C. coli*. Moreover, this work highlighted that live and heat killed cells of both *C. jejuni* and *C. coli*, as well as, extracted LOS activate the NLRP3 [Nucleotide binding oligomerisation domain (NOD) like receptors with pyrin domain–containing 3] inflammasome dependent signalling in a human monocytic cell line, THP-1. However, *C. jejuni* 11168 LOS mutant live cells and its modified LOS with altered lipid A and lack of LOS core oligosaccharides both stimulated significantly reduced Caspase-1 and IL-1β compared to the wild-type 11168 live cells and LOS, which indicated that variation in LOS structure can alter NLRP3 inflammasome activation. This work extends the understanding of the *Campylobacter* LOS locus classification system and determines that LOS plays an important role in the development of host immune response during *Campylobacter* infection.

Acknowledgements

I would first and foremost like to thank my supervisory team for accepting me as a Ph.D. student. I am very thankful to Dr Lee Machado for his constant support and encouragement over the duration of my Ph.D. course and especially for helping me with bioinformatics based analysis of LOS cluster. I am grateful to Dr Alex Woodacre whose expertise and assistance, particularly in the final stages of my Ph.D., led me in the right direction for carrying out the most important and successful experiments. I am also thankful to Dr Gemma Marsden for her advice on the project, guidance and support.

I am thankful to my director of studies, Dr Stuart Allen, who provided my immense support and encouragement in early stages of my Ph.D. The experience of my Ph.D. has enormously enhanced my potential to carry out the research independently, and for this I am indebted to him.

I am thankful to Andrea O'Connell (Northampton General Hospital, Northampton, UK) for providing me *C. jejuni* clinical isolates, Dr Mirko Rossi (University of Helsinki, Finland) for sending me strains of *C. jejuni* (4031) and *C. coli* (76339), and Khaloud Alarjani (University of Leicester, UK) for kindly giving me *C. jejuni* 11168 *flaA* mutant.

I wish to thank lab technical staff, Valerie Graham, Lin Clapham and Daniel Shaw for their assistance in laboratory aspects. Thank you to everyone in the lab past and present. I would also like to thank to Matt Lloyd and Tanya Hayes, laboratory staff members at the Institute for Creative Leather Technologies, University of Northampton, for helping me in electron microscopy.

Last, but not definitely least, I would like to thank my family, especially my mum, dad, and sister, for their great support through the last three and a half years. Finally, a big thanks goes to my husband who always encouraged and supported me through the good times and the bad! It was impossible to complete this Ph.D. without him. I would like to dedicate this thesis to my husband, Imran Ali Shah.

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ABBREVIATIONS

ACT	Artemis comparison tool
Amp	Ampicillin resistance gene
AMPs	Antimicrobial peptides
B	Beta
C. coli	*Campylobacter coli*
C. jejuni	*Campylobacter jejuni*
CDT	Cytolethal distending toxin
CFU	Colony forming unit
CPS	Capsular polysaccharide
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FCS	Fetal calf serum
FFT	Fast Fourier transform
g	Gram
x g	Times gravity; unit of relative centrifugal force
GBS	Guillain-Barré syndrome
hBD-2	Human beta-defensins 2
HSP	High scoring segment pair
IECs	Intestinal epithelial cells
IFN-γ	Gamma interferon
IL-1β	Interleukin-1 beta
KDO	3-Deoxy-D-manno-oct-2-ulosonic acid
L	Litre
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAFFT	Multiple alignment using fast Fourier transform
MAMPs	Microbe associated molecular patterns
MFS	Miller Fisher syndrome
μg	Microgram
μL	Microlitre
μM	Micromolar
MIC	Minimum inhibitory concentration
mL	Millilitre
MOI	Multiplicity of infection
MSP	Maximal-scoring segment pair
NF-κB	Nuclear factor kappa light-chain-enhancer of activated B cells
NLRP3	NLR family, pyrin domain–containing 3
NOD	Nucleotide-binding oligomerisation domain
OD	Optical density
Ori	Origin of replication
P	Phosphate
PAGE	Poly-acylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid

Rpm	Revolutions per minute
RPMI	Roswell park memorial institute
SDS	Sodium-dodecyl-sulphate
Sn	Sialoadhesin
SNP	Single nucleotide polymorphism
SOC	Super optimal broth with catabolite repression
strep^R	Streptomycin resistant
strep^S	Streptomycin sensitive
THP-1	Human acute monocytic leukaemia cell line
TLR-2	Toll-like receptors
TNF-α	Tumour-necrosis factor alpha
v/v	volume/volume
WG	Whole-Genome
WT	Wild-type
w/v	weight/volume

CHAPTER 1

Introduction

1.1. *Campylobacter*

Campylobacter, initially known as "*Vibrio*", was observed for the first time in the large intestine of a child (Escherich, 1886) and subsequently, in an aborted sheep (McFaydean and Stockman, 1913). Later, *Vibrio* was reported as a cause of diarrhoea in humans (Levy, 1946; King, 1957). In 1963, Sebald and Veron found basic differences in the genomic DNA (gDNA) of *Vibrio*-like microorganisms and bacterial strains belonging to the *Vibrio* genus and therefore classified these *Vibrio*-like microorganisms in a new genus "*Campylobacter*" (Greek word; meaning: curved rod). Currently, this genus consists of 25 *Campylobacter* species and 8 sub-species (Man, 2011).

1.1.1. Physical aspects

Campylobacter are Gram-negative rods (1.5-6.0 µm long and 0.2-0.5µm wide) which are either spiral curved or straight in shape with thin ends (Sebald and Veron, 1963; Man, 2011), as it can be seen in the microscopic images of *Campylobacter jejuni* (*C. jejuni*) 11168 and *Campylobacter coli* (*C. coli*) RM1875 (Figure 1.1). These rods have flagella either at a single or both tapering ends for a cork-screw like motion (Yamamoto *et al*., 2013; Baldvinsson *et al*., 2014).
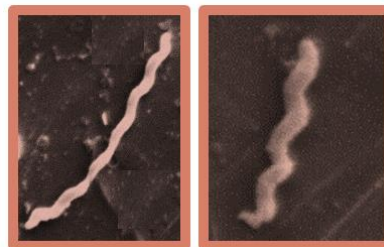
C. jejuni 11168   *C. coli* RM1875

Figure 1.1. Scanning electron micrographs showing the cell shape of *C. jejuni* 11168 and *C. coli* RM1875 (Current study)

Campylobacter grow well in nutrient rich media in a microaerobic atmosphere (5-10% CO_2, 3-10% O_2, and 85% N_2) and at temperatures between 34 and 44 °C (Skirrow, 1977). *Campylobacter* are unable to grow below 30 °C and grow best at 42 °C (Konkel *et al*., 1998; Apel *et al*., 2012). In the presence of limited-oxygen, *Campylobacter* can utilise various compounds from the environment including fumarate, nitrate, nitrite, sulphite, trimethylamine-N-oxide, dimethyl sulfoxide, and hydrogen peroxides. Adaptation to use various compounds from the environment instead of oxygen for electron accepter dependent respiration help *Campylobacter* to survive in an oxygen-limited environment (Sellars *et al*., 2002; Myers and Kelly, 2005; Cameron *et al*., 2012). *Campylobacter* use these respiratory processes for energy preservation as well and do not oxidise or ferment carbohydrates for energy purposes (Mohammad *et al*., 2004). *Campylobacter* generally are non-spore forming and oxidase-positive microorganisms (Barrett *et al*., 1988).

Under unfavourable growth conditions, spiral form *Campylobacter* cells may convert into coccoid forms and exhibit a viable, but non-cultureable state (Ng *et al*., 1985; Cappelier *et al*., 1999, Ziprin *et al*., 2003). Under strict anaerobic or hyperosmotic (2 % sodium chloride) conditions, *Campylobacter* cells appear as thin filaments with inhibition of DNA synthesis and growth (Sellars *et al*., 2002; Cameron *et al*., 2012). In addition, long-term storage and frequent sub-culturing in the laboratory can also affect *Campylobacter* physiology and motility traits. This is supported by the characterisation of a Whole Genome (WG) sequenced variant of *C. jejuni* 11168, where this variant was straight in shape and had reduced motility in comparison to the spiral-shaped and highly motile original strain (Gaynor *et al*., 2004).

1.1.2. Genetic features

C. jejuni 11168 in *C. jejuni* species (1.64 MB; Parkhill *et al*., 2000) and RM2228 in *C. coli* species (1.68 MB; Fouts *et al*., 2005) were the first sequenced isolates. Subsequently, *C. jejuni* RM1221 (1.77 MB), *C. jejuni* 81-176 (1.62 MB), *C. coli* 15-537360 (1.7MB), *C. coli* N29710 (1.67 MB) and other *Campylobacter* species related strains such as *C. upsaliensis* RM3195 (1.66 MB), *C. lari* RM2100 (1.53

MB), *C. fetus* 82-40 (1.77 MB), and *C. geochelonis* RC7 were also WG sequenced (Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Miller *et al.*, 2008; Pearson *et al.*, 2013; Chen *et al.*, 2013; Fitzgerald *et al.*, 2014; Piccirillo *et al.*, 2016). All of these sequenced *Campylobacter* strains had a small genome size (~1.5-1.8 MB) in comparison to other enteropathogens such as *Escherichia coli* (*E. coli)*, whose genome size is ~4.5 MB (Casale *et al.*, 2018). The WG sequencing of *Campylobacter* strains has revealed many unique features which are commonly present in the genome of almost every species of the genus "*Campylobacter*". The genome of almost all *Campylobacter* species contains a few phage-associated and repeat sequences, but many phase variable genes (Parkhill *et al.*, 2000; Dorrell *et al.*, 2001; Prendergast *et al.*, 2004; Fouts *et al.*, 2005; Bayliss *et al.*, 2012). It has low GC content (28-38 %) and may harbour pseudogenes as well as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) sequences (Park *et al.*, 1991; Fouts *et al.*, 2005; Pearson *et al.*, 2013; Ghatak *et al.*, 2017). It consists of highly variable gene regions and most of them are involved in the biosynthesis of cell-surface carbohydrates containing structures, [lipooligosaccharide (LOS) and capsular polysaccharide (CPS)], flagellum, and iron-uptake system (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006). The absence of transposable inserted sequence (IS) elements in the *C. jejuni* 11168 genome (Parkhill *et al.*, 2000) and the presence of four large IS elements in the *C. jejuni* RM1221 genome (Fouts *et al.*, 2005) suggest that the number of genomic IS elements varies between strains, even in those, which belong to the same *Campylobacter* species. Exceptionally, a fragment of plasmid sequence was found inserted in the gDNA of *C. jejuni* 81-176 next to the leucine tRNA genes (Hofreuter *et al.*, 2006).

The presence or absence of plasmids differs among *Campylobacter* strains. For example, *C. jejuni* 11168 and RM1221 do not harbour a plasmid, but *C. jejuni* 81-176 contain two plasmids, pVir and pTet. *C. coli* RM2228, *C. coli* 15-537360, *C. lari* RM2100, and *C. upsaliensis* RM3195, all have one plasmid (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2013). *Campylobacter* plasmids may harbour genes for drug resistance. The plasmid pN29710 in *C. coli* N29710 contains multi-drug resistance genes, and gives resistance for gentamicin, kanamycin, streptomycin, streptothricin, and

tetracycline (Chen *et al*., 2013). Similarly, pCC178 plasmid in *C. coli* RM2228 confers resistance against kanamycin, neomycin, tetracycline, oxytetracycline, and minocycline (Fouts *et al*., 2005). *Campylobacter* plasmids may also contain genes linked to the type-IV protein secretion system. This is evidenced by the presence of type-IV secretion system related proteins encoding genes in *C. jejuni* 81-176 pVir plasmid and *C. coli* 15-537360 cryptic plasmid (Hofreuter *et al*., 2006; Pearson *et al*., 2013).

1.1.3. Reservoirs

Campylobacter species including *C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, *C. upsaliensis, C. hypointestinalis*, *C. helveticus*, *C. lanienae*, and *C. mucosalis* are mostly found in warm-blooded animals (Table 1.1). However, some *Campylobacter* species, such as, *C. fetus* and *C. geochelonis* can also occur in cold-blooded reptiles (lizard, tortoise, and snake) (Wang *et al*., 2015; Piccirillo *et al*., 2016). Interestingly, the most common species of *Campylobacter*, *C. jejuni*, has also been found in Antarctic penguins and fur seals (Broman *et al*., 2000). Chicken is the main reservoir of *Campylobacter* who colonise *Campylobacter* in its intestines after hatch, usually at the age of 2-5 weeks (Neill *et al*., 1984; Humphrey *et al*., 1993; Berndtson *et al*, 1996). In addition to livestock, *Campylobacter* isolates can be also be present in non-livestock niches (such as the environment), which may be agricultural or non-agricultural (Champion *et al*., 2005; Wilson *et al*., 2008; Sheppard *et al*., 2010, 2013).

Table 1.1: Known Reservoirs of *Campylobacter* spp.

Campylobacter spp.	Reservoirs	Reference
C. jejuni	Chicken, dog, wild-birds (duck, American crow, gull), monkey, cat, turkey, cow, sheep, Antarctic penguin, and fur seal	Broman *et al*., 2000; Moore *et al*., 2004; Workman *et al*., 2005; Inglis *et al*., 2005; Rahimi *et al*., 2010; Weis *et al*., 2014
C. coli	Chicken, dog, wild-birds, monkey, pig, turkey, cow, sheep	Workman *et al*., 2005; Rahimi *et al*., 2010; Weis *et al*., 2014
C. lari	American crow, gull	Moore *et al*., 2004; Weis *et al*., 2014
C. fetus	Duck, turtle, lizard, snake	Luechtefeld *et al*., 1980; Wang *et al*., 2015
C. upsaliensis	Dog, cat	Fouts *et al*., 2005; Workman *et al*., 2005
C. geochelonis	Western Hermann's tortoise	Piccirillo *et al*., 2016
C. hypointestinalis	Pig, cow	Gebhart *et al*., 1985
C. helveticus	Cat	Workman *et al*., 2005
C. lanienae	Cow	Inglis *et al*., 2005
C. mucosalis	Pig	Roberts *et al*., 1980

1.1.4. Transmission

Soil and surface water at farm houses, contaminated with faeces of *Campylobacter* colonised animals or birds, are the two major environmental factors, which are considered responsible for animal-to-animal or animal-to-bird *Campylobacter* transmission (Ross and Donnison, 2006; Denis *et al.*, 2011; Weis *et al.*, 2014; Smith *et al.*, 2016). Chicken flocks have mostly been found colonised with *Campylobacter* when other farm animals, such as cow and sheep, are present in the close proximity of poultry houses and old litter of these animals remain dispersed in the soil of poultry farms (Neill *et al.*, 1984; Ahmed *et al.*, 2013). The farm environment does not only facilitate *Campylobacter* transmission from farm animals to chicken flocks, but also to other animals (dog, cat) and wild-birds (gulls, crow) (Wilson *et al.*, 2008; Whiley *et al.*, 2013; Bronowski *et al.*, 2014). Subsequently, circulation of *Campylobacter* contaminated surface water in drinking water (typically untreated or non-chlorinated) can transmit *Campylobacter* to humans (Kuusi *et al.*, 2004; Uhlmann *et al.*, 2009; Rosef *et al.*, 2010), but at a low rate (Denis *et al.*, 2011).

Poultry flocks contaminated with approximately 10^9 *Campylobacter* are considered as a major source of *Campylobacter* transmission to humans (Atanassova and Ring, 1999; Newell and Fearnley, 2003). At slaughter time, *Campylobacter* present in the chicken intestine comes in contact with meat and further survives during retail meat processing. *Campylobacter* can then transmit to humans if this contaminated meat remains partially cooked or is consumed in its raw state (Zhao *et al.*, 2001; Rahimi *et al.*, 2010). It is considered, that a whole chicken can contain *Campylobacter* cells in the range of 350-10^7 (Hood *et al.*, 1988) and 500-800 cells can be a sufficient infectious dose for humans (Black *et al.*, 1988). Feeding of raw meat to pets (specifically dogs) can also transmit *Campylobacter* from farm animals to pets and then pets to humans (Lenz *et al.*, 2009; Gras *et al.*, 2013). In addition to meat, consumption of dairy products including unpasteurized milk from these animals can also contribute in *Campylobacter* transmission to humans (Levy, 1946; Robinson, 1981). Currently, no evidence of human-to-human *Campylobacter* transmission is available (Nichols, 2005).

The rate of *Campylobacter* colonisation in poultry flocks as well as *Campylobacter* infection in humans remains high during the summer (May to June) (Louis *et al*., 2005; Meldrum *et al*., 2005), which may occur due to increased fly populations in summer (Berndtson *et al*, 1996; Nichols, 2005). The presence of flies in the environment is another contributor to *Campylobacter* transmission between animals and humans (Nichols, 2005). The following figure 1.2 represents the routes of *Campylobacter* transmission, important for infection in humans.
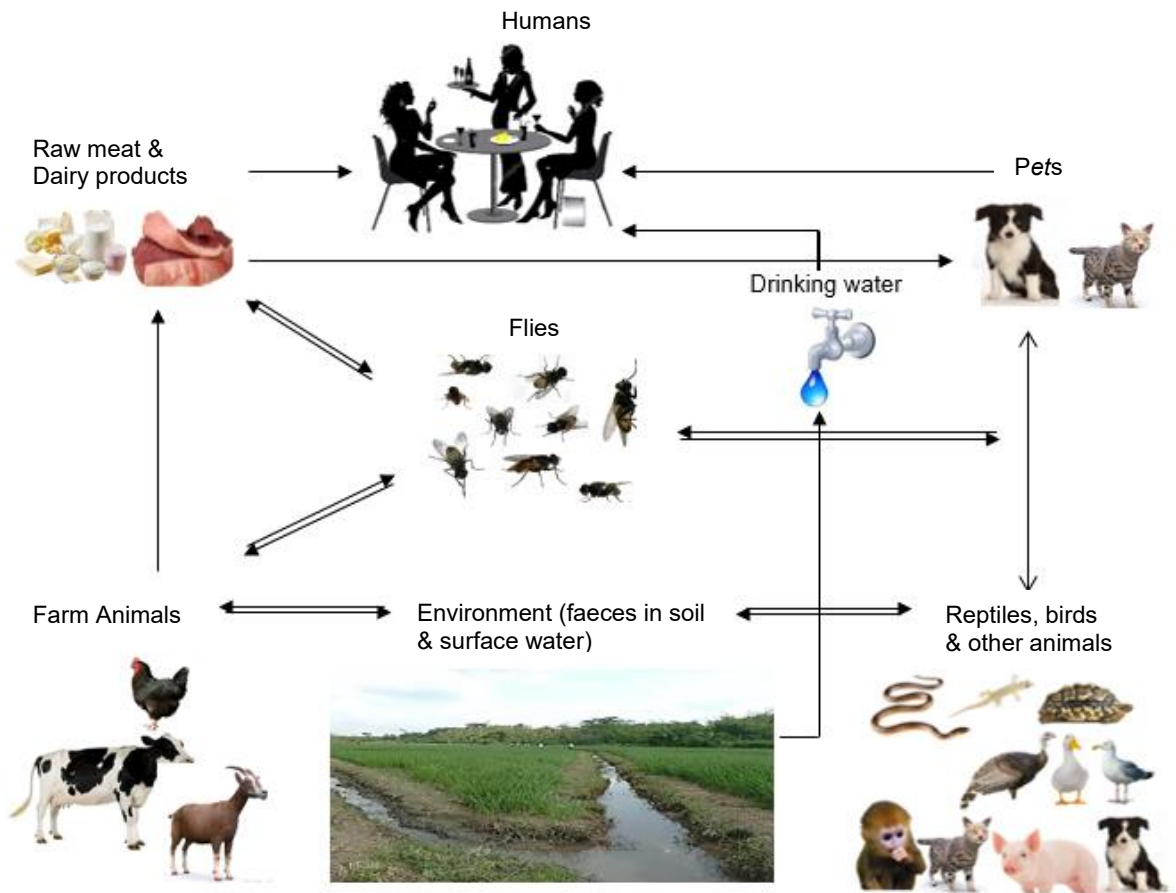
Figure 1.2: *Campylobacter* transmission routes; linking *Campylobacter* transmission from different *Campylobacter* reservoirs to humans.

1.2. *Campylobacter* Infection

Campylobacter is a worldwide foodborne pathogen, associated with human gastroenteritis (EFSA, 2010; CDC, 2011; WHO, 2018). In comparison to other *Campylobacter* species, *C. jejuni* and *C. coli* are highly prevalent in *Campylobacter* reservoirs, specifically in chickens, and account for approximately 99% of all *Campylobacter* infections in UK and USA (Zhao *et al*., 2001; Louis *et al*., 2005; Rahimi *et al*., 2010). Other *Campylobacter* species such as *C. hyointestinalis* and *C. fetus* can also cause infection in humans (Gebhart *et al*., 1985; Wang *et al*., 2015).

Campylobacter infection commonly presents itself as an acute, self-limiting gastroenteritis with various non-specific symptoms including watery or bloody diarrhoea, abdominal pain, headache, fever, chills, and dysentery (van Spreeuwel *et al*., 1985; Black *et al*., 1988; Perkins and Newstead, 1994). Interestingly, *Campylobacter* infection has a different clinical manifestation in developed and developing countries and the reasons behind this geographical difference are unknown. In developed countries, illness is characterised by bloody diarrhoea with mucus and occurs mostly in young adults, whereas, in developing countries, it causes watery diarrhoea and occurs mostly in children of age < 5 years (van Vliet and Ketley, 2001; Masanta *et al*., 2013). Symptoms begin to develop after 24-72 hours of infection and last for 5 to 7 days (van Spreeuwel *et al*., 1985; Black *et al*., 1988; van Vliet and Ketley, 2001). After 72 hours, stools containing leukocytes and erythrocytes together with 8-10 bowel movements are the two major signs of severe *Campylobacter* infection (Black *et al*., 1988; Samie *et al*., 2007).

1.2.1. Post-infection effects

It is hypothesised that Guillain-Barré Syndrome (GBS), Miller Fisher Syndrome (MFS), Reiter's arthritis, and irritable bowel syndrome are the post-infectious, long-term consequences of *Campylobacter* infection (Endtz *et al*., 2000; McCarthy and Giesecke, 2001; Janseen *et al*., 2008). In GBS, cranial nerves extending from brain to various areas of the head and neck are affected, which

further develop difficulty in walking, muscle weakness, and muscle pain. MFS, a variant of GBS, is characterised mainly by paralysis of eye muscles and problems with balance and coordination (Nachamkin *et al*., 1998).

Almost 25-40% of GBS patients have been found with *C. jejuni* infection 1-3 weeks prior to the onset of illness. This is supported with the high expression of Penner heat-stable serotypes, HS: O19 and HS: O41, in GBS patients. High numbers of these serotypes have also been observed in non-GBS patients or patients with gastroenteritis only (Endtz *et al*., 2000). Therefore, it has been suggested that *Campylobacter* infection is not a sole contributor to GBS onset. Other host/bacterial factors, for instance, host immune status and concurrent infections in the host, are also involved in GBS progression (Janseen *et al*., 2008). In addition, vaccines administrated for rabies, oral polio, influenza, measles, tetanus toxoid, and hepatitis B administration can also contribute to the GBS development (Baxter *et al*., 2012). These post-infection complications occur rarely in humans (~1 to 8 per 1000 individuals) and typically appear in immune compromised individuals, such as, individuals with HIV infection (McCarthy and Giesecke, 2001; Janseen *et al*., 2008).

1.2.2. Epidemiology

The annual estimated number for *Campylobacter* infection cases is 400-500 million worldwide and 71 per 100,000 population in the European Union alone (Jeon *et al.,* 2010; Magana *et al*., 2017). As *Campylobacter* infection is self-limiting, reported incidences are likely to be under-estimates of the true disease burden of infection (Allos, 2001). The actual incidence rate is thought to be 10-100 times higher than the reported cases of *Campylobacter* infection (Guerry *et al.,* 2012). The estimates of reported incidences of *Campylobacter* infection cases per 100,000 of the population of several developed and developing countries are given in figure 1.3, indicating epidemiology of *Campylobacter* infection varies from region to region.
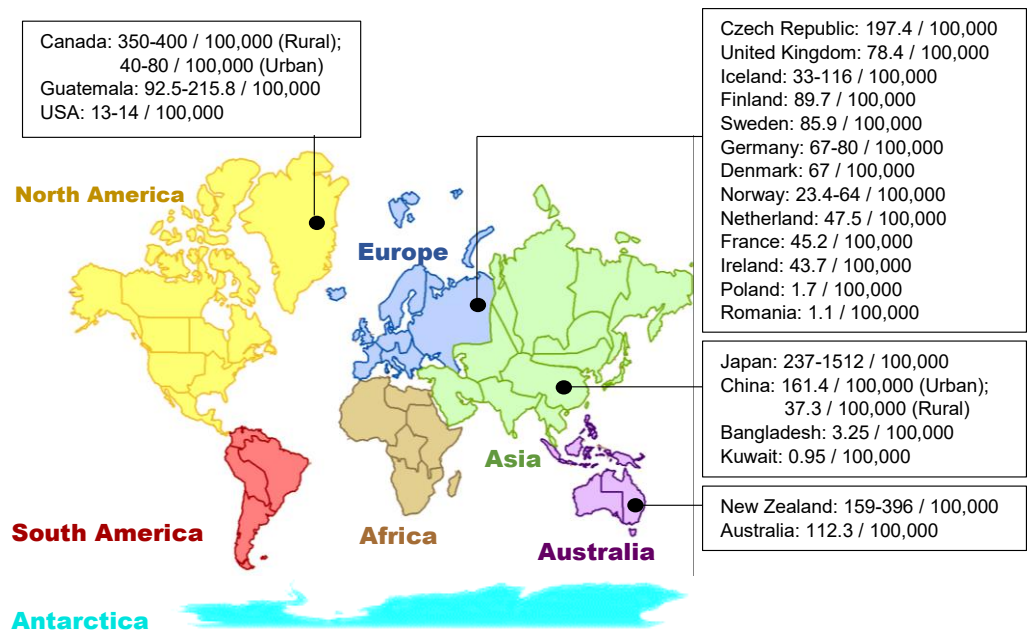
Figure 1.3: An overview of worldwide epidemiology of *Campylobacter* infection

Average, reported incidences of gastroenteritis cases in Canada (Le´vesque *et al*., 2013), United Kingdom (Louis *et al*., 2005), Iceland (Stern *et al*., 2003), Germany (Schielke *et al*., 2014), Norway (Sandberg *et al*., 2006), Ireland (Foley and Mckeown, 2006), China (Jun *et al*., 2013), Bangladesh (Islam *et al*., 2011), Kuwait (Ismail *et al*., 1998), and other countries (Pitkanen and Hanninen, 2017).

1.2.3. Prevention measures and treatment

Public awareness related to food handling, cooking of meat at appropriate temperatures, and the avoidance of drinking unpasteurized milk or untreated water is crucial to decrease the incidence rate of *Campylobacter* infection. Maintenance of food hygiene measures in the kitchen, slaughter houses, and food processing units can also reduce the potential cross-contamination of *Campylobacter* (Humphrey *et al*., 2001; Zhao *et al*., 2001; Humphrey, 2006; Rahimi *et al*., 2010).

Campylobacter infection is a self-limiting disease with an incubation period of 5-7 days and therefore, clinical treatment is not required (van Spreeuwel *et al*., 1985; Black *et al*., 1988; Perkins and Newstead, 1994). A homemade solution of table sugar with water to patients with mild diarrhoea and commercially available oral solutions to patients with severe diarrhoea can be given to overcome the loss of body fluids and electrolytes (Mackenzie & Barnes, 1988). Antibiotics are recommended mostly for very young children (< 2 years of age), pregnant, and immunocompromised patients, but a risk of antibiotic resistance development in *Campylobacter* always remains present (Funke *et al*, 1994; Mamelli *et al*, 2003; Eiland and Jenkins, 2008).

Various flagellum-secreted proteins based vaccines (*C. jejuni* 81-176 FlaC, *C. jejuni* 81-176 FspA1, and *C. jejuni* CG8486 FspA2) and a conjugated capsule polysaccharide vaccine, CPS (81-176)-CRM (197), have been experimentally tested in mice (Baqar *et al*., 2008; Monteiro *et al*., 2009). A recombinant protein vaccine, ACE 393, was tested in volunteers (phase-II clinical trials), but remained unsuccessful as it did not provide the adequate immunity. Despite these experiments and efforts, no commercial vaccine has been developed for *Campylobacter* to date and this is largely due to the versatile and diverse nature of *Campylobacter* physiology and genomics (Riddle and Guerry, 2016).

1.3. *Campylobacter* Virulence in Humans

1.3.1. Colonisation in the GI tract

The main areas of *Campylobacter* localisation in humans is the lower GI tract, which includes the small intestine (ileum and jejunum), caecum, and colon (van Spreeuwel *et al*., 1985; Black *et al*., 1988). The GI tract in humans structurally consists of four cell layers: the mucosa, submucosa, muscularis externa, and serosa. The mucosa is the innermost layer which is further divided into the epithelium [a single layer of mucosal epithelial cells or intestinal epithelial cells (IECs)], lamina propria (a layer of connective tissues), and muscularis mucosae (a thin layer of smooth muscles). The mucosal epithelium of the small intestine consists of villi or crypts (finger-like projections), enriched with mucus secreting goblet cells. *Campylobacter* cells colonise the mucus layer and crypts in high numbers, rather than the intestinal lumen, due to the nutrients availability for maximal growth and low concentration of oxygen in this layer (Apel *et al*., 2012; Stahl and Vallance, 2015). The highly viscous mucus layer is mainly composed of mucin glycoproteins; proteins attached with L-fucose, galactose, sialic acid, *N*-acetyl galactosamine, *N*-acetyl glucosamine and mannose (Tu *et al*., 2008, Bäumler and Sperandio, 2016). *Campylobacter* utilises the mucin components (L-serine and L-fucose) by putative mucin-degrading enzymes as a source of energy (Tu *et al*., 2008; Stahl and Vallance, 2015; Bäumler and Sperandio, 2016).

1.3.2. Motility and chemotaxis

Campylobacter flagellum filaments act as adhesins, develop strong host-bacterial interaction, and help bacteria to colonise the viscous mucus layer of the human GI tract (Wösten *et al*., 2010; Yamamoto *et al*., 2013; Baldvinsson *et al*., 2014). *C. jejuni* mutant cells (with paralysed flagella) were found only at 10^3/gram cecal content in comparison to the wild-type (WT) cells, which colonised up to 10^9/gram cecal content (Baldvinsson *et al*, 2014).

Campylobacter cells sense chemoattractants (bile, L-fucose of mucin, amino acids, and salts of the organic acids) and chemorepellents within their surroundings via chemoreceptors. *Campylobacter* chemoreceptors (Tlp1, Tlp4, Tlp7, and Tlp10) have been divided into three groups (A. B, and C) based on their structural differences and affinity for ligands (chemoattractants) (Yamamoto *et al*., 2013; Baldvinsson *et al*, 2014). Binding of ligands to *Campylobacter* chemoreceptors initiate signal transduction in chemoreceptors, which further develops an interaction between chemoreceptors and flagellum. Consequently, *Campylobacter* flagellum receives a signal to move either clockwise or anti-clockwise in order to achieve movement or chemotaxis, which may be towards the favourable environment or away from the unfavourable conditions (Tareen *et al*., 2010; Rahman *et al*., 2014; Reuter *et al*., 2019).

1.3.3. Adherence and invasion

Campylobacter crosses the mucus layer and adheres to the microvilli present on the apical surface of the epithelium cell layer (Monteville *et al*., 2003; O Cróinín and Backert, 2012). Following adherence, *Campylobacter* invades into the epithelial cells without changing the transepithelial electrical resistance and migrates either via transcellular translocation (apical endocytosis) or paracellular translocation (Hu *et al*., 2008; Backert *et al*., 2013). During transcellular translocation, disruption and subsequent accumulation of the host cell cytoskeletal proteins (microtubule-associated dynein and microfilament actin proteins) is a prerequisite of the *Campylobacter* entry process (Monteville *et al*., 2003; Hu *et al*., 2008). In contrast, simultaneous cell membrane engulfment of bacteria into an endosome is thought to occur in order to initiate paracellular translocation (Hu *et al*., 2008). The actual mechanism behind endocytosis before the paracellular migration is not known (O Cróinín and Backert, 2012).

Transcellular translocation is achieved generally via two mechanisms, zipper and trigger. The zipper-mechanism is more common in *Listeria* in which bacterial adhesins or invasins bind with the host cell receptors and disrupt the cytoskeletal proteins. The cytoskeleton protein-mediated zippering of host cell membrane causes the engulfment of interacting bacterial cells. In contrast, the trigger-

mechanism is more common in *Salmonella,* where a bacterial cell releases many proteins into a host cell via type-III and type-IV protein secretion systems to trigger the disruption of host cell cytoskeletal proteins (O Cróinín and Backert, 2012). The triggered signalling pathways in the host cell subsequently induce membrane ruffling for the internalisation of in-contact bacterial cell (Watson and Galan, 2008; O Cróinín and Backert, 2012).

Campylobacter utilise both types of mechanisms, zipper and trigger, for its transcellular translocation or apical endocytosis (Figure 1.4) (O Cróinín and Backert, 2012). The *Campylobacter* adhesins including JlpA (Jejuni lipoprotein A; 42.3 kDa lipoprotein), Fn (Fibronectin; 220kDa glycoprotein), FlpA (Fn-binding protein), CadF (Cadherin-Fn binding protein; 37 kDa), Cbf-1 (Cell binding factor 1; 28 kDa)**,** KpsE and KpsM (capsule biosynthesis proteins), and glycans (specifically LOS) use the zipper mechanism as they bind to the specific host cell membrane proteins and induce endocytosis (Bacon *et al*., 2001; Jin *et al*., 2001; Monteville *et al*., 2003; O Cróinín and Backert, 2012; Rubinchik *et al*., 2012). It is considered that *Campylobacter* may decorate adhesins onto the cell surface in the form of pili that come in contact with host cells (Jin *et al*., 2001). An example of utilisation of the trigger-mechanism in *Campylobacter* is the secretion of several virulence associated, mainly *Campylobacter* invasion antigens (Cia; CiaB, CiaC, CiaI), by the flagellum into the host cells in order to facilitate the invasion process (Guerry, 2007; Hu *et al*., 2008; Wösten *et al*., 2010; Baldvinsson *et al*., 2014). The mechanism linking flagellum structural elements to endocytosis has not been fully explored yet. However, it has been observed that *C. jejuni* flagellum secreted CiaC recruits Rac1, a Rho GTPase, responsible for the cytoskeletal disruption in a host cell (Konkel *et al*., 2013).
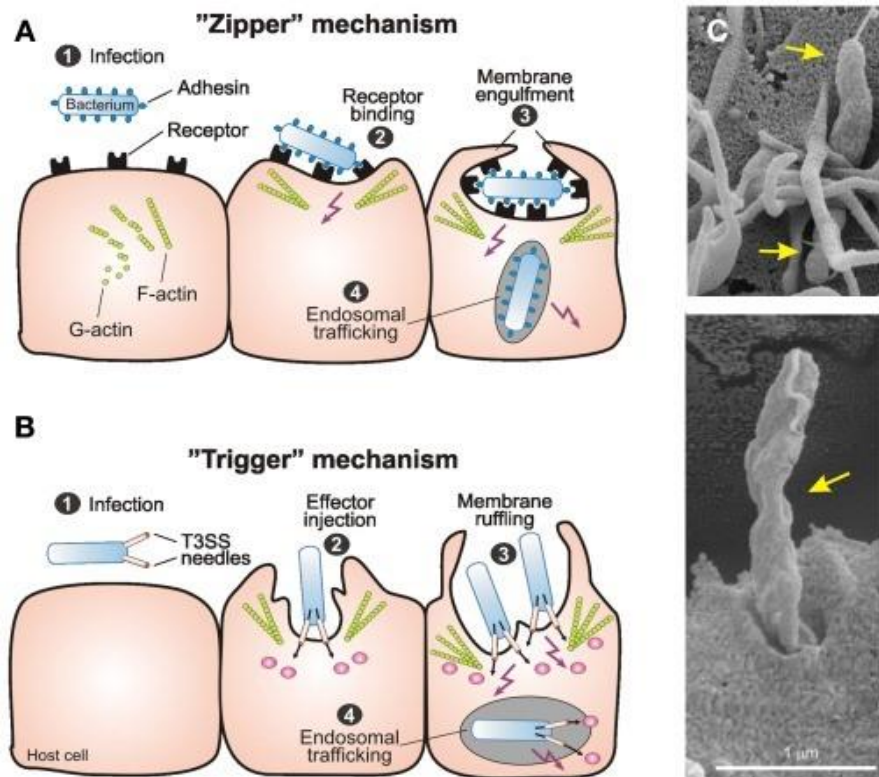
Figure 1.4: A representation of zipper (A) and trigger (B) routes in *C. jejuni*, causing its translocation into a host cell (Reproduced from O Cróinín and Backert, 2012).

Yellow arrows in electron micrograph in figure 1.4 (A) represent invasion of *C. jejuni* cells into host cells with "zipper" mechanism. Yellow arrow in electron micrograph in figure 1.4 (B) shows invasion of a *C. jejuni* cell into host cells with "trigger" mechanism.

1.3.4. Intracellular survival

After internalisation, *Campylobacter* cells reside in the vacuolar compartment or *Campylobacter*-containing vacuoles (CCVs) within the host cells (Hu *et al*., 2008; Watson and Galan, 2008). Golgi vacuolar compartments contain an acidic mixture to lyse the bacterial cell (Watson and Galan, 2008). CCVs are structurally and functionally different from the Golgi-vesicles and protect the enclosed bacteria from the action of lysosomes (Watson and Galan, 2008). CCVs fuse with the epithelial basolateral layer and consequently, bacteria exit from the host cell (basolateral exocytosis) subepithelially (Hu *et al*., 2008; Watson and Galan, 2008). Inflammation and destruction of epithelial cells is an important aspect of *Campylobacter* infection which causes an imbalance in fluid transport across the GI tract and develops severe diarrhoea in host (Friis *et al*., 2009).

1.3.5. Toxin production

Toxins are defined as high molecular weight proteins which are secreted by *Campylobacter* cells inside the host IECs to cause cellular damage (Guerrant *et al*., 1987; Whitehouse *et al*., 1998). All *Campylobacter* strains do not produce the Cytolethal Distending Toxins (CDTs) (AbuOun *et al*., 2005), and their expression and activity may also vary among strains for unknown reasons (Wassenaar, 1997). *Campylobacter* CDTs share structural similarity with the *Vibrio cholerae* toxin and *E. coli* heat-labile toxin. Generally, these types of toxins enter into the host cells via cell receptors and induce signalling to increase the intracellular level of cAMP (Wassenaar, 1997). Increase in cAMP affects the intracellular ion instability which leads to excess secretion of fluid from the damaged IECs (Wassenaar, 1997). Accumulation of fluid in intestinal cells has been proposed as a reason for diarrhoea development during *Campylobacter* infection (Guerrant *et al*., 1987; Whitehouse *et al*., 1998).

Campylobacter CDTs treated HeLa cells have been observed with cell apoptosis, chromatin condensation, and nuclear fragmentation after 2-3 days of treatment. This is due to inactivation of a cyclin-dependent kinase (CDK-1) by its phosphorylation at a tyrosine-15 residue, which further arrests the cell in G2-

phase and induces apoptosis. This mechanism can be employed in the non-differentiated IECs and crypts where Cdc-2 inactivation can affect the cells' maturation into fully functional epithelial cells (Whitehouse *et al.*, 1998). Production of immature and non-functional cells with less absorptive properties can then majorly contribute to the progression of diarrhoea in humans (Whitehouse *et al.*, 1998; van Vliet and Ketley, 2001). In addition to inducing ion instability and apoptosis in a host cell, *Campylobacter* toxins with haemolysin domains and phospholipase activity are also thought to be responsible for pore formation in erythrocytes and haemolysis (Guerrant *et al.*, 1987; Grant *et al.*, 1997).

1.3.6. Other virulence factors

As discussed above, endocytosis and exocytosis of *Campylobacter* as well as *Campylobacter* CDTs facilitated ion instability, cell apoptosis, and pore formation in host cells are the key virulence factors. Iron acquisition, flagellum glycosylation, and stress regulating mechanisms are the other virulence factors, which help *Campylobacter* to survive inside the human body. The actual free iron (Fe^{2+}) bioavailability in mammalian cells is approximately 10^{-18} M to 10^{-24} M, which is insufficient for optimum bacterial cell growth (Palyada *et al.*, 2004). *Campylobacter* have evolved various mechanisms to uptake iron (Fe^{2+}) from the host iron (Fe^{3+})-binding compounds (siderophores: enterochelin, ferrichrome, and rhodotorulic acid) and iron (Fe^{3+})-containing compounds (haem and ferri-transferrins) in order to attain the required concentration of iron (10^{-7} M) (Miller *et al.*, 2009). In addition, the post-translational modifications or O-linked glycosylation of flagella filaments and other flagellar components also contribute to the *Campylobacter* virulence (Guerry, 2007; Baldvinsson *et al.*, 2014). Moreover, oxidative stress decreasing proteins, such as, superoxide dismutase (SodB), catalase (KatA), alkyl hydroperoxide reductase (AhpC), ferredoxin regulator protein (Fdx), thiol peroxidases, and cytochrome C peroxidases as well as heat shock proteins including GroESL, DnaJ, DnaK, and ClpB regulate the stress response and help *Campylobacter* to cope with the stressful environment inside the host (Konkel *et al.*, 1998; Palyada *et al.*, 2004, 2009; Apel *et al.*, 2012).

1.4. Immune Responses against *Campylobacter* Infection

1.4.1. Chicken immune responses

Campylobacter is commensal in poultry because it modifies its physiological state during colonisation in the chicken intestine and does not produce any disease symptoms in chickens (Woodall *et al*., 2005). *Campylobacter* is not a harmless commensal in chickens as it stimulates the innate and adaptive immune responses in almost all types of chicken breeds. However, the extent of harm posed by *Campylobacter* may vary among different breeds of chickens (Humphrey *et al*., 2014). Chickens become infected with *Campylobacter* at the age of 2-3 weeks. Due to a lack of a fully developed adaptive immune system at this age, maternal antibodies, already passed from hens to chicks, provide protection against *Campylobacter* (Sahin *et al.*, 2003; Shoaf-Sweeney *et al*., 2008). Maternal antibodies against the *Campylobacter* flagellar proteins, outer membrane proteins and LOS were observed in new-born chicks (Shoaf-Sweeney *et al*., 2008). After developing an adaptive immune system at the age of 6-7 weeks, chickens produce antibodies against *Campylobacter* cellular components, such as outer membrane proteins and flagellum (Cawthraw *et al*., 1994; Shoaf-Sweeney *et al*., 2008). However, circulation of maternal antibodies as well as development of adaptive immune B-cells play a limited role in the clearance of *Campylobacter* cells from the chicken intestines (Sahin *et al*., 2003; Lacharme-Lora *et al*., 2017). It is proposed that *Campylobacter* avoids rapid clearance in the chicken intestine due to the adaptation to a novel colonisation mechanism, where it continues short-term invasion of chicken IECs followed by escape from these cells (van Deun *et al.,* 2008).

1.4.2. Human innate immune responses

The nucleotide-binding oligomerisation domain (NOD) proteins are the IECs intracellular pattern recognition receptors (PRRs) which directly recognise the microbe associated molecular patterns (MAMPs) of *Campylobacter* and induce the release of antimicrobial peptides (AMPs) including human beta-defensins 2 (hBD-2) (Zilbauer *et al*., 2007). The hBD-2 are bactericidal as they disrupt the

Campylobacter cell wall integrity (Zilbauer *et al*., 2005). In addition, NOD-1 binding to *Campylobacter* MAMPs (*Campylobacter* toxins or adhesins) also promotes the secretion of a chemokine, interleukin- 8 (IL-8) from the human IECs (Hickey *et al*., 2000; Jin *et al*., 2003; Zilbauer *et al*., 2007) by the MAPK signalling pathway (Watson and Galan, 2005; John *et al*., 2017). The secreted IL-8 recruits the innate immune cells, specifically neutrophils, macrophages, and dendritic cells (DCs) at the site of infection. The accumulated DCs internalise the *Campylobacter* cells and become mature after the expression of cell surface co-stimulatory molecules (CD40, CD80, and CD86). Subsequently, mature DCs produce different pro-inflammatory cytokines, including IL-1, IL-6, IL-8, IL-10, IL-12, gamma interferon (IFN-γ), and tumour necrosis factor α (TNF-α) (Hu *et al*., 2006; Murphy *et al*., 2011, Malik *et al*., 2014).

Toll-like receptor (TLR)-2 are other IECs cell-surface PRRs which recognise the *C. jejuni* MAMPs and consequently, induce IL-6 secretion from IECs (Friis *et al*., 2009). Different cellular constituents of *Campylobacter* such as lipoproteins (bind TLR-1/2/6), LOS (bind TLR-4), DNA, capsule, cell wall polysaccharides, flagella, and CDT can bind to TLRs in human immune cells to activate them (Hickey *et al*., 2000; Jin *et al*., 2003; Andersen-Nissen *et al*., 2005; Hu *et al*., 2006; de Zoete *et al*., 2009; Stephenson *et al*., 2013; Stahl *et al*., 2014). TLR signalling leads to the activation and translocation of NFκß to the nucleus to induce the transcription of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β (Wesche *et al*., 2001; Verstak *et al*., 2009). In addition to NOD-1 and TLR dependent signalling, human serum or complement proteins also play an important role in *C. jejuni* infection (Bar, 1988; Keo *et al*., 2011).

Human monocytes undergo apoptosis following infection with *Campylobacter* (Hickey *et al*., 2005; Wassenaar *et al*., 1997), however, macrophages rapidly kill *Campylobacter* cells subsequent to their internalisation (Wassenaar *et al*., 1997; Watson and Galán, 2008; Heikema *et al*., 2013). *Campylobacter* viability is not very important for macrophage infection as *Campylobacter* cellular components detached from killed *Campylobacter* cells can also bind to macrophage receptors (Stephenson *et al*., 2013; Bouwman *et al*., 2014; Korneev *et al*., 2018), which further induce cell signalling pathways and the secretion of pro-inflammatory

cytokines. For example, *C. jejuni* lipoproteins induce the production of TNF-α and IL-6 in macrophages (Jin *et al*., 2001; Shang *et al*., 2016). In addition to cytokines, protein complexes or NLRP3 [Nucleotide binding oligomerisation domain (NOD) like receptors with pyrin domain–containing 3] inflammasomes, also accumulate in the cytosol of human macrophages in response to the *Campylobacter* infection (Bouwman *et al*., 2014).

1.4.3. Human adaptive immune responses

During *Campylobacter* infection, DCs-derived cytokines, IL-12 and IL-10 in particular, stimulate the proliferation of CD4$^+$ T-cells and their secretion of IFN-γ, IL-22 and IL-17 from these T-cells. In addition, DC-derived cytokines also contribute to the development of B lymphocytes in a T cell-independent manner (Hu *et al*., 2006; Edwards *et al*., 2010; Fimlaid *et al*., 2014; Malik *et al*., 2014). Antibodies against the *Campylobacter* toxins, flagella, LOS, and major outer membrane proteins, have been observed previously in human serum (Blaser *et al*., 1984; Kirimat *et al*., 1989; Godschalk *et al*., 2007). In the acute phase of infection (7 days post-infection), the level of serum antibodies, IgA and IgM, increase in serum (Strid *et al*., 2001). In the convalescent phase of infection (1 week – 2 months), IgG also begins to circulate in the blood (Cawthraw *et al*., 2000; 2002). These serum antibodies are detectable in the serum and faeces of *Campylobacter* infected patients (Lane *et al*., 1987). IgA and IgM decline over time. In contrast, IgG present in serum as well as serum IgG expelled into saliva, remain persistent inside the host for long time period (~ 1 year) and provide protection against subsequent *Campylobacter* infection (Cawthraw *et al*., 2000; 2002).

1.5. *Campylobacter* LOS

The lipopolysaccharides (LPS) present in the outer-cell membrane of Gram-negative bacteria generally contain lipid A, core saccharides, and O-chains (a set of repeating saccharides). However, Gram-negative bacteria related to *Neisseria, Haemophilus, and Campylobacter* species lack LPS in the outer-cell membrane. Instead, they possess LOS with the composition of lipid A and core saccharides only (Mandrell *et al*., 1992; Moran, 1997; Duncan *et al*., 2009). In comparison to LPS, LOS are low-molecular weight biological molecules with the lack of O-chains (Moran, 1997). Other *Campylobacter* cell-surface structures include CPS, *O*-linked glycosylated flagellum, and *N*-linked glycoproteins. LOS, CPS, and *O*-linked glycans (mainly flagellar glycans) are variable among different strains, while, *N*-linked glycoproteins remain conserved (Karlyshev *et al.*, 2005). The glycome comprising these four types of carbohydrates containing conjugate molecules are synthesised by more than 8 % of the genome in *C. jejuni* 11168 (Parkhill *et al*., 2000).

1.5.1. The biosynthesis of LOS in *Campylobacter* and its comparison to LPS biosynthesis in *E. coli*

Acyl transferases (encoded by genes, *lpxA*, *lpxD*) facilitate the attachment of two acyl chains to Uridine Di-Phosphate *N*-acetyl glucosamine (UDP-GlcNAc) and initiate the biosynthesis of LPS-lipid A in *E. coli*. The acylated UDP-GlcNAc is then deacylated and phosphorylated respectively by UDP-3-O-acyl-*N*-acetyl glucosamine deacetylase (*lpxC*) and UDP-2, 3-diacylglucosamine diphosphatase (*lpxH*) to form lipid X. Two lipid X molecules combine by lipid A disaccharide synthase (*lpxB*) and the produced disaccharide complex is then phosphorylated by tetraacyldisaccharide 4' kinase (*lpxK*) to form lipid IVa. This lipid IVa is known as the lipid A backbone (Figure 1.5) (Emiola *et al*., 2015). The lipid A backbone in *C. jejuni* contains a 3-diamino-2, 3-dideoxy-D-glucopyranose linked to 2-amino-2-deoxy-D-glucose (GlcN), whereas, *C. coli* lipid A backbone consists of two GlcN (Culebro *et al*., 2016). Subsequently, in *E. coli*, two 3-deoxy-D-manno-octulosonic acid (abbreviated KDO) molecules are joined to the lipid A

backbone by 3-deoxy-D-manno-octulosonic acid transferase (*waaA*) (Figure 1.5 & 1.6A; Emiola *et al.*, 2015). Similarly, lipid A biosynthesis lauroyl acyltransferase (*waaM*) in *Campylobacter* adds one KDO molecule to the backbone of lipid A (Figure 1.6B; Karlyshev *et al.*, 2005). *E. coli* LPS-lipid A generally consists of 4 hydroxyl-linked acyl chains and 2 amide-linked acyl chains, whereas, the LOS-lipid A in most of the *Campylobacter* strains consists of 2 hydroxyl-linked acyl chains and 4 amide-linked acyl chains (Moran, 1997).

The O-antigen of *E. coli* LPS is synthesised in the cytoplasmic side of the inner cell membrane by the addition of 3-5 monosaccharides to undecaprenol-PP (Emiola *et al.*, 2015). Following synthesis, it is translocated to the periplasmic side of the inner membrane where a chain of repeating monosaccharide units is attached to already ligated 3-5 monosaccharides. Similarly, *E. coli* LPS lipid A and core are synthesised in the cytoplasmic side of inner cell membrane and later, translocate to the periplasmic side. In the periplasm, the lipid A-core assembles with O-antigen by O-antigen polymerase and finally, a complete synthesised LPS structure is decorated on the outer-cell membrane (Figure 1.5). Likewise, *Campylobacter* LOS lipid A and core are synthesised in the cytoplasmic side of inner cell membrane from where they are translocated to the periplasm and finally, to the outer cell membrane. The process of LOS biosynthesis and translocation is accomplished to further complete the synthesis of outer cell membrane (Whitfield and Trent, 2014; Simpson *et al.*, 2015).

Cell-surface LPS

Outer Membrane

O-antigen

Transport

LPS

Periplasm

LPS lipid A and core saccharides

O-antigen polymerase

UDP-3-O-acyl-N-acetylglucosamine deacetylase (lpxC)
UDP-2,3-diacylglucosamine diphosphatase (lpxH)

Lipid IVa

Lauroyltransferase (lpxL)
Lipid A biosynthesis myristoyltransferase (lpxM)

Inner Membrane

Lipid A disaccharide
Synthase (lpxB)

Tetraacyldisaccharide
4' kinase (lpxK)

Lipid IVa 3-deoxy-D-
manno-octulosonic acid
transferase (waaA)

Acyl transferases (lpxA, lpxD)

UDP

Synthesis of inner
core

Synthesis of outer
core

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Figure 1.5: Biosynthesis of *E. coli* LPS and its translocation from inner membrane to outer membrane.

O-antigen backbone (undecaprenol-PP with 3-5 saccharides), as well as, lipid A-core saccharides are synthesised in the cytoplasmic side of the inner cell membrane. Then these complex biological molecules flip towards the periplasmic side of the inner cell membrane, where they join together to form a complete LPS structure. LPS after synthesis are translocated to the outer cell membrane to build this membrane.

The core biosynthesis gene clusters involved in the biosynthesis of *E. coli* K12 LPS core and *C. jejuni* 11168 LOS core have been demonstrated respectively (Figures, 1.6A and 1.6B). The inner core of *C. jejuni* 11168 LOS has two heptose and two glucose molecules in contrast to three heptose containing inner core of *E. coli* K12 LPS. The process of addition of first two heptoses to lipid A-KDO is similar in both *E. coli* and *C. jejuni*, where heptosyltransferase-I (*waaC)* adds the first heptose (Hep-I) to KDO and heptosyltransferase-II (*waaF*) catalyses the addition of a second heptose (Hep-II) to Hep-I (Klena *et al*., 1998; Kanipes *et al*., 2004; 2006; Emiola *et al*., 2015). In *C. jejuni* 11168, two glucose molecules are added to Hep-I and Hep-II by *lgtF* (*cj1135*) encoded putative two-domain glucosyltransferase (Gilbert *et al*., 2002). Hep-1 and Hep-II in *C. jejuni* 11168 are synthesised by four LOS genes including *gmhA* (phosphoheptose isomerase), *waaE* (D-glycero-beta-D-manno-heptose-7-phosphate kinase), *waaD* (ADP-L-glycero-D-manno-heptose-6-epimerase), and *gmhB* (dephosphatase) that add into the inner core of LOS. Unlike to the inner core, the outer core of LPS and LOS varies among *E. coli* as well as *C. jejuni* strains (Parker *et al*., 2005; Emiola *et al*., 2015). The outer core of *C. jejuni* 11168 is synthesised by glycosyltransferases (*cj1136*, *cj1137*, and *cj1138*), *N*-acetyl galactosaminyl transferase (*cgtA*/*neuA1*), sialyltransferase (*cst-III*), and galactosyltransferase (*wlaN*) (Gilbert *et al*., 2000; Linton *et al*., 2000; Gilbert *et al*., 2002; Guerry *et al*., 2002; Karlyshev *et al*., 2005; Javed *et al*., 2012). The presence of *cst-III*, *neuA1*, *neuB1*, and *neuC1* in the LOS biosynthesis cluster leads to the development of sialic acid containing LOS structural epitopes or human gangliosides mimics in *C. jejuni* 11168 (Gilbert *et al*., 2000; Gilbert *et al*., 2002; Guerry *et al*., 2002). Finally, the figure 1.6 (A) and figure 1.6 (B) demonstrate that each LOS biosynthesis gene produces an individual enzyme either for the monosaccharide biosynthesis or addition of a particular monosaccharide to the LOS structure, explaining that LOS structures are synthesised at the genetic level (Karlyshev *et al*., 2005; Parker *et al*., 2005, 2008; Iwata *et al*., 2013).
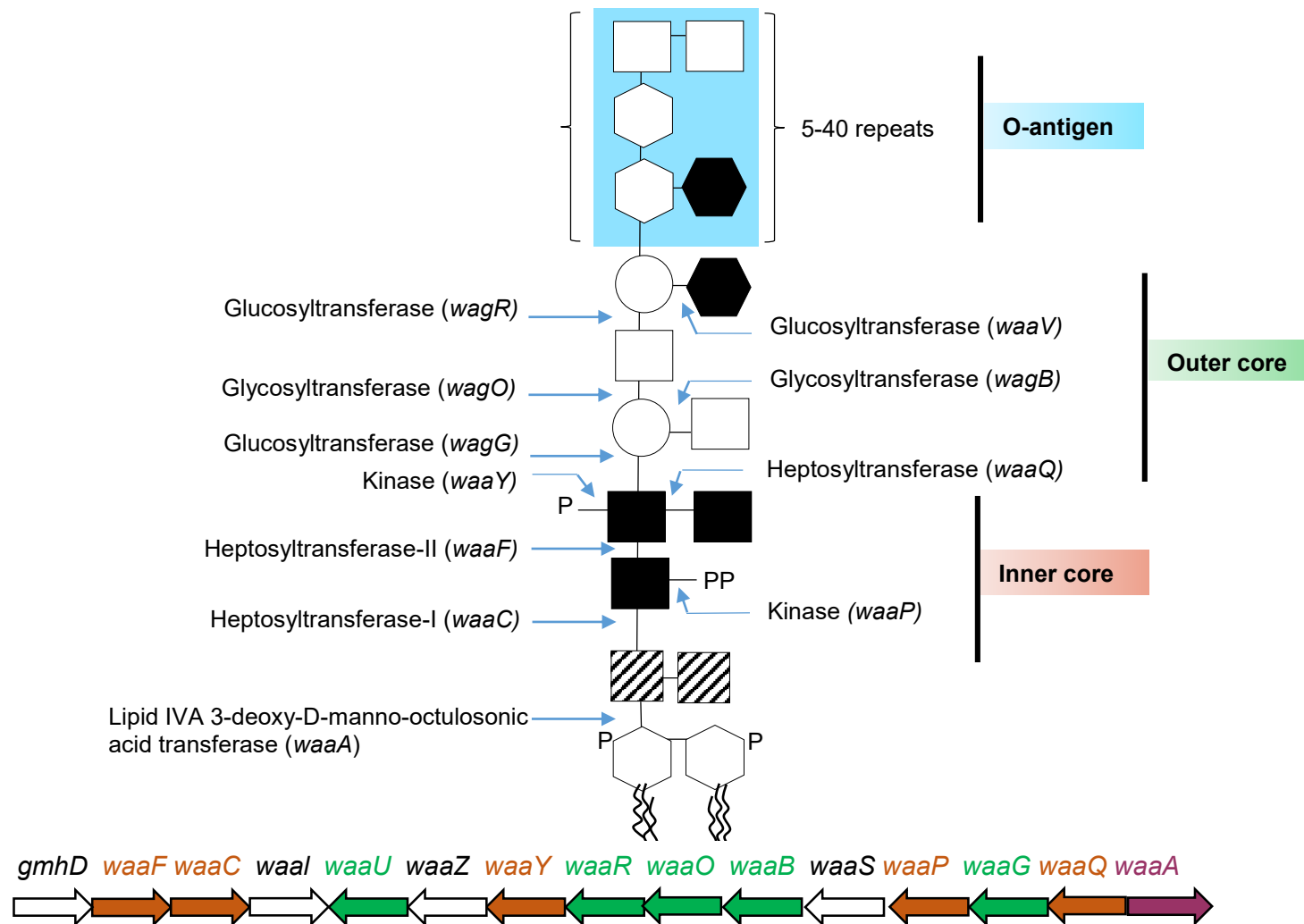
Figure 1.6 (A): A representation of *E. coli* K12 LPS core biosynthesis gene cluster and its LPS structure. Each arrow represents an individual LPS core biosynthesis gene and its direction indicates the direction of gene transcription. A purple coloured gene catalyses the addition of KDO molecules to lipid A. Each orange coloured gene encoding enzyme catalyses the addition of a specific monosaccharide to LPS inner core structure. Each green coloured gene encoding enzyme catalyses the addition of sugars to LPS outer core structure.

■, Heptose; □, Galactose; ○, Glucose; ⬡, *N*-Acetyl galactosamine; ▨, 2-keto-3-deoxy octulosonic acid (KDO); ⬢, *N*- Acetyl glucosamine
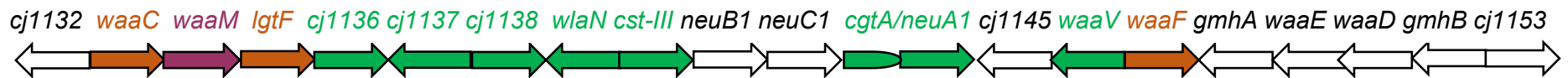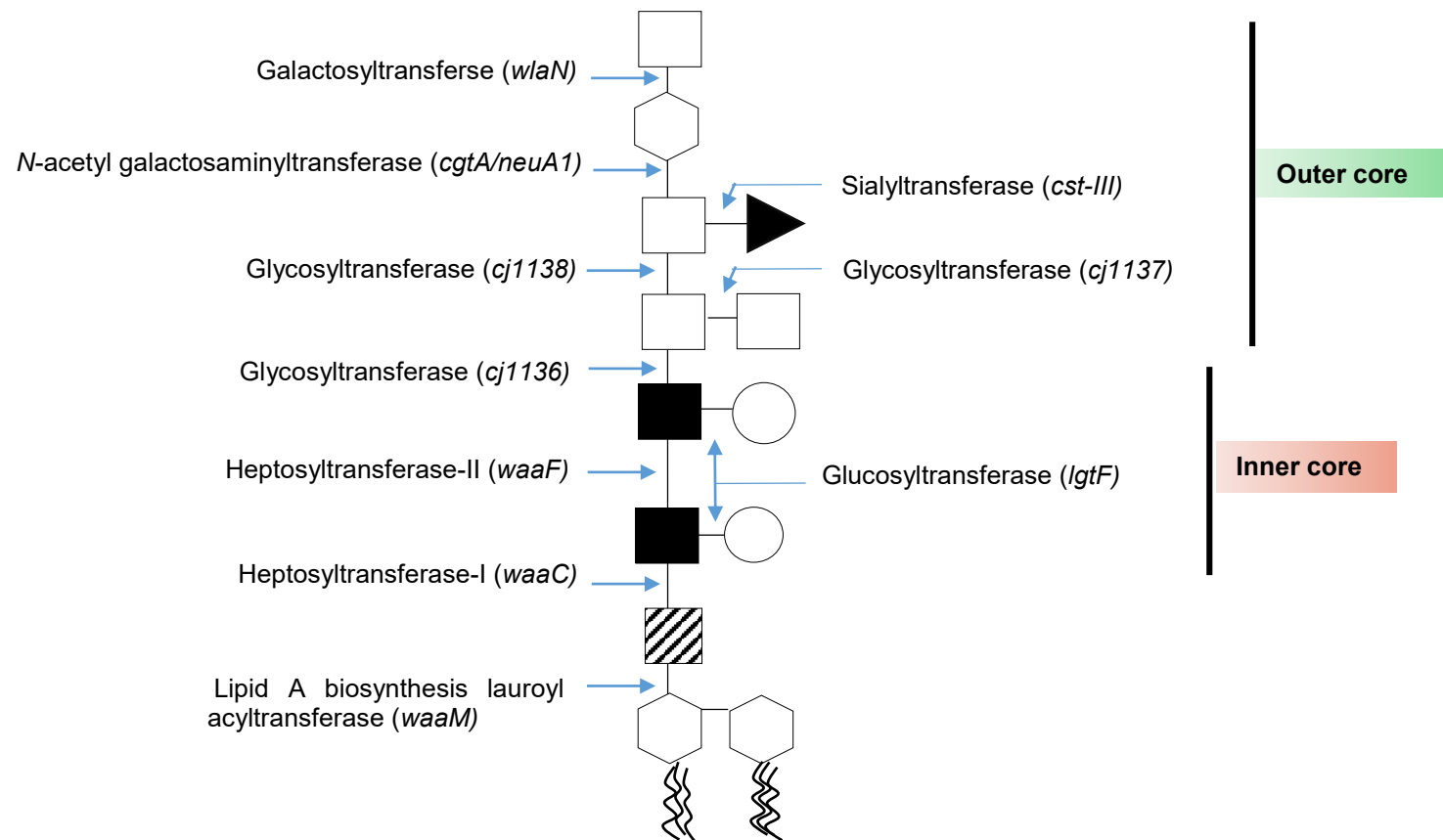
Figure 1.6 (B): A representation of *C. jejuni* 11168 LOS core biosynthesis gene cluster and its LOS structure. Each arrow represents an individual LOS core biosynthesis gene and its direction indicates the direction of gene transcription. A purple coloured gene catalyses the addition of a KDO molecule to lipid A. Each orange coloured gene encoding enzyme catalyses the addition of a specific monosaccharide to LOS inner core structure. Each green coloured gene encoding enzyme catalyses the addition of sugars to LOS outer core structure. LOS genes, *gmhA*, *waaE*, *waaD*, and *gmhB*, synthesise heptoses for inner core, whereas, *neuB1*, *neuC1* and *cgtA/neuA1* synthesise sialic acid to incorporate in the outer core.

■, Heptose; □, Galactose; ◯, Glucose; ⬡, *N*-Acetyl galactosamine; ▨, 2-keto-3-deoxy octulosonic acid (KDO); ▶, Sialic acid

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1.5.2. Classification of *C. jejuni* and *C. coli* LOS biosynthesis gene clusters

The LOS inner core biosynthesis genes present upstream (*waaC, waaM,* and *lgtF*) and downstream (*waaV, waaF, gmhA, waaE, waaD, gmhB,* and *cj1153)* within the LOS biosynthesis gene cluster occur in the same order in almost all *C. jejuni* and *C. coli* strains. This is the reason that the inner LOS core is structurally similar in almost all *Campylobacter* strains (Gilbert *et al*., 2002; Karlyshev *et al*., 2005; Richard *et al*., 2013). However, the LOS biosynthesis gene cluster extending from *lgtF* and *waaV* varies among *C. jejuni* and *C. coli* strains as this region may incorporate new genes or delete the existing genes. This region of the LOS biosynthesis cluster is involved in the biosynthesis of LOS outer core, hence, variations in this region cause modifications in the LOS outer core structure. The gene insertion or deletion events in this LOS biosynthesis region give rise to a new locus organisation or type in *Campylobacter* strains (Parker *et al.*, 2005, 2008). As an example, the development of *C. jejuni* LOS type F derives from type D following a gene deletion event and type L derived from type G after insertion of two genes (Figure 1.7). A specific organisation of LOS genes named with an alphabetical letter is known as one *C. jejuni* class, and a Roman numeral is known as one *C. coli* LOS class. Previously known *C. jejuni* LOS classes (A through W) and *C. coli* LOS classes (I-VIII) are highlighted, respectively, in Table 1.2 and Table 1.3 (Gilbert *et al*., 2002; Parker *et al*., 2008; Richard *et al*., 2013).
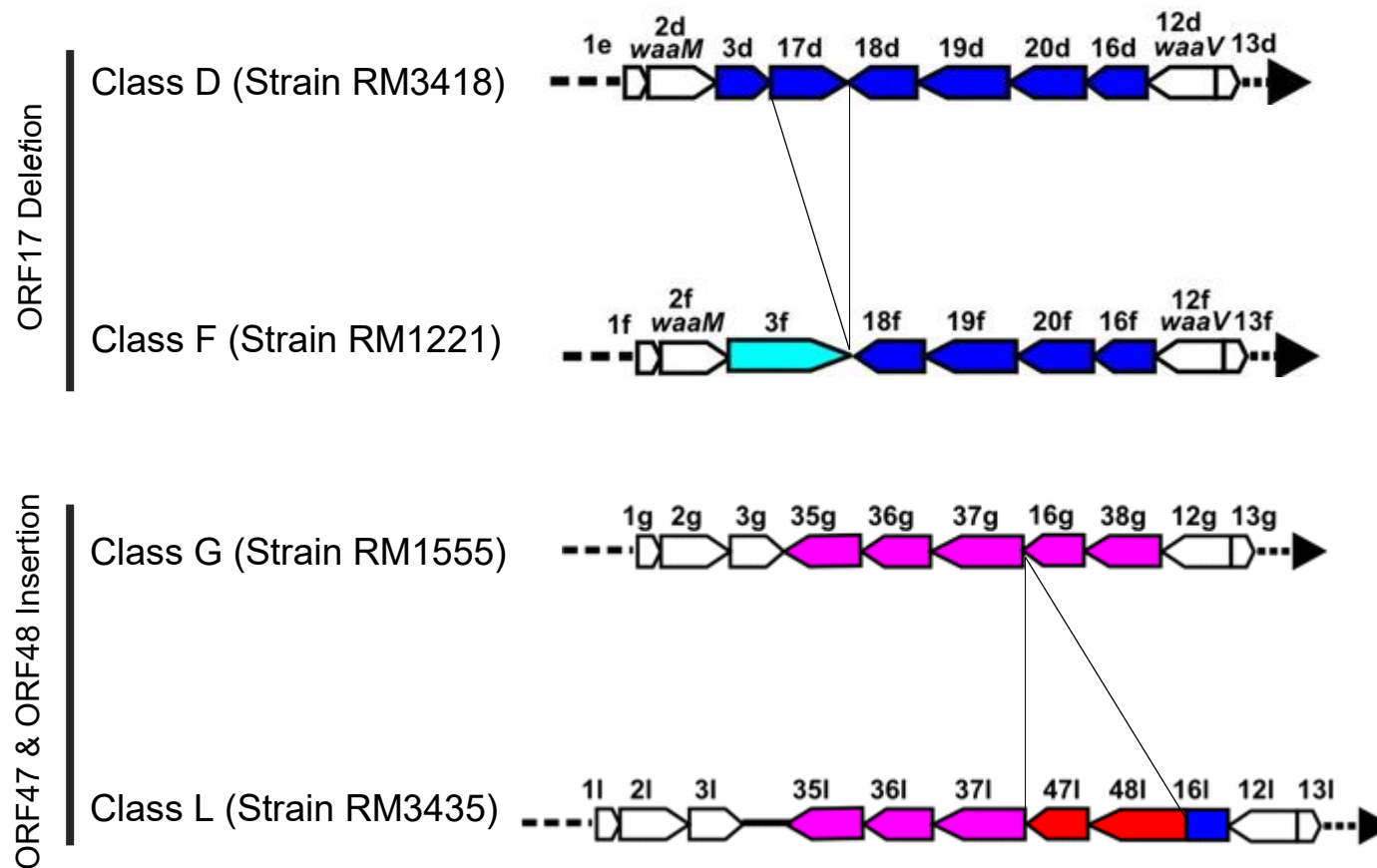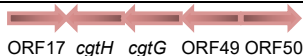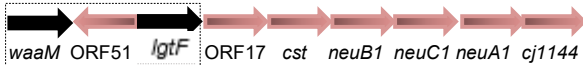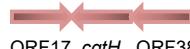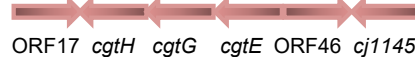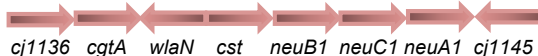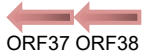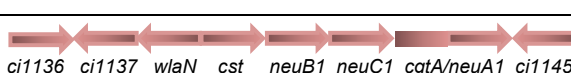
Figure 1.7: Development of new *C. jejuni* LOS types following the occurrence of gene deletion or insertion events in the LOS biosynthesis gene cluster.

Deletion of ORF17 from *C. jejuni* LOS biosynthesis gene cluster type D establishes LOS class F and insertion of ORF47 and ORF48 in LOS class G leads to the development of LOS class L. Coloured boxes: LOS genes, likely to vary between *C. jejuni* strains. White boxes: LOS genes, commonly present in *C. jejuni* strains (Reproduced from Parker *et al*., 2008)

Table 1.2: Previously known *C. jejuni* LOS types or classes (Gilbert *et al*., 2002; Parker *et al*., 2005, 2008; Richard *et al*., 2013)
LOS genes commonly present in *C. jejuni* are illustrated with black colour and LOS genes, likely to vary among strains, are presented with pink colour.

LOS class	Reference *C. jejuni* strain	Accession no. (Sequencing level)	Gene content present between *lgtF* and *waaV* LOS biosynthesis genes
			cj1132 waaC waaM *lgtF* // waaV waaF gmhA waaE waaD gmhB
A	RM1048/ ATCC43432 (A1) RM1556/ ATCC43438 (A2)	AF215659 (Partial) AF400048 (Partial)	cj1136 cgtA wlaN cst neuB1 neuC1 neuA1 cj1144 / cj1136 cgtA wlaN cst neuB1 neuC1 neuA1 cj1144
B	RM1050/ ATCC43449 (B1) RM1052/ ATCC43456 (B2)	AF401529 (Partial) AF401528 (Partial)	cj1136 cgtA wlaN cst neuB1 neuC1 neuA1 cgtA-II cj1144 / cj1136 cgtA wlaN cst neuB1 neuC1 neuA1 cgtA-II cj1144
C	11168	AL 111168 (WGS)	cj1136 cj1137 cj1138 wlaN cst neuB1 neuC1 cgtA/neuA1 cj1145
D	RM3418	EU404109 (Partial)	ORF17 cgtH cgtG cgtE cj1145
E	81116	CP000814 (WGS)	ORF21 ORF22 ORF23 ORF24 ORF25 ORF26 ORF27 ORF28 ORF29 ORF30 ORF31 ORF32 ORF33 ORF34
F	RM1221	CP000025 (WGS)	cgtH cgtG cgtE cj1145
G	RM1555/ ATCC 43437	AY436358 (Partial)	ORF35 ORF36 ORF37 *cj1145* ORF38
H	RM1553/ ATCC43435	EU404106 (Partial)	ORF21 ORF22 ORF23 ORF24 ORF25 ORF26' ORF27 ORF29 ORF30 ORF31 ORF32 ORF33 ORF34
I	RM1850	EU 404107 (Partial)	ORF17 cgtH cgtG cgtE ORF40 ORF41 ORF42 ORF43 ORF44 ORF45
J	RM1508	EU 404104 (Partial)	cgtH cgtG cgtE ORF40 ORF42 ORF43 ORF44 ORF45

	Strain	Accession	Gene organization
K	RM1861	EU410350 (Partial)	ORF17 *cgtH* *cgtG* ORF49 ORF50
L	RM3435	EU404111 (Partial)	ORF36 ORF37 ORF47 ORF48 *cj1145*
M*	RM1503	EF140720 (Partial)	*waaM* ORF51 *lgtF* ORF17 *cst* *neuB1* *neuC1* *neuA1* *cj1144*
N	RM2095	AY816330 (Partial)	ORF17 *cgtH* ORF38
O	RM3423	EF143352 (Partial)	ORF21 ORF22 ORF23 ORF24 ORF25 ORF26 ORF27 ORF29 ORF30 ORF31 ORF32 ORF33 ORF34
P	GB4	AY943308 (Partial)	ORF21 ORF22 ORF23 ORF24 ORF25 ORF26' ORF27 ORF28 ORF29 ORF30 ORF31 ORF32 ORF33 ORF34
Q	RM3437	EU 404112 (Partial)	ORF17 *cgtH* *cgtG* *cgtE* ORF46 *cj1145*
R	GC149	AY962325 (Partial)	*cj1136* *cgtA* *wlaN* *cst* *neuB1* *neuC1* *neuA1* *cj1145*
S	RM3419	EU 404110 (Partial)	*cgtH* *cgtG* ORF40 ORF41 ORF42 ORF43 ORF44 ORF45
T	LMG23223	AIOC00000000 (WGS; contig 1)	ORF37 ORF38
U	2008-979	AIOU00000000 (WGS; contigs 12, 88)	*wlaN* ORF37 ORF48 *cj1145* ORF38
V	2008-1025	AIOP00000000 (WGS; contigs 26, 57, 134)	*cj1136* *cj1137* *wlaN* *cst* *neuB1* *neuC1* *cgtA/neuA1* *cj1145*
W*	M1	CP001900 (WGS)	ORF21 ORF22 ORF23 ORF24 *M1118* *M1119* *M1120* ORF24 ORF27 ORF29 ORF30 ORF31 ORF32 ORF33 ORF34 *waaV* *waaF* *M1130* *M1131*

* Class M and Class W possess LOS genes exceptionally outside the defined LOS outer core biosynthesis gene region (*lgtF-waaV*).

WGS: Whole-genome sequence

Table 1.3: Previously known *C. coli* LOS classes (Richard *et al*., 2013)

LOS genes, commonly present in *C. coli*, are illustrated with black colour and LOS genes, likely to vary among strains, are presented with brown colour.

LOS class	*C. coli* reference strain	Accession no. (Sequencing level)	Gene content present between *lgtF* and *waaV* LOS biosynthesis genes
			cj1132 waaC waaM *lgtF* // waaV waaF gmhA waaE waaD gmhB
I	LMG2336	AINM01000000 (WGS; Contig 29)	ORF4 ORF5 ORF6 ORF7 ORF8
II	202/04	AINH01000000 (WGS; Contig 4)	ORF4' ORF5' ORF6' ORF7' ORF8' ORF9 ORF10 ORF11 ORF12
III	LMG23341	AINN01000000 (WGS; Contig 20)	ORF4 ORF5 ORF6'' ORF7'' ORF8'' ORF9' ORF10' ORF11'
IV	1948	AINE00000000 (WGS; Contig 24)	ORF4' ORF5' ORF6''' ORF7''' ORF8''' ORF9'' ORF10'' ORF11'' ORF12' ORF13 ORF14 ORF15
V	1957	AINF01000000 (WGS; Contig 1)	ORF4' ORF5' ORF6''' ORF7''' ORF8''' ORF9'' ORF30 ORF10'' ORF11'' ORF12' ORF13 ORF14 ORF15
VI	1148	AIMX00000000 (WGS; Contig 1)	ORF4' ORF5' ORF6''' ORF7''' ORF8''' ORF9'' ORF10'' ORF11'' ORF12' ORF31 ORF32 ORF14 ORF15
VII	LMG9853	AINR00000000 (WGS; Contig 1)	ORF4' ORF5' ORF6''' ORF7''' ORF8''' ORF9'' ORF10'' ORF11'' ORF33 ORF34 ORF35 ORF36 ORF13 ORF14 ORF15
VIII	H9	AINV01000000 (WGS; Contig 11)	ORF4 ORF5 ORF6* ORF7* ORF8* ORF9'''ORF10'''ORF11'''ORF12''ORF13' ORF14' ORF15' ORF16 ORF17

1.5.3. *Campylobacter* LOS as a virulence determinant

LOS is an integral part of the outer cell membrane. It does not only maintain the integrity of the cell membrane structure, but also acts as a barrier for those molecules which are transported through the cell membrane (Karlyshev *et al.*, 2005). For example, antibiotic permeability into the cell increases due to alteration in LOS structures, possibly because LOS structural changes decrease the cell membrane hydrophobicity. This is the reason that mutants of *Campylobacter* LOS genes are highly susceptible to some antibiotics, specifically to erythromycin (Kanipes *et al.*, 2004; Jeon and Zhang, 2009; Marsden *et al.*, 2009). In addition to providing a barrier to antibiotics, LOS also confers resistance to *Campylobacter* cells against the human serum proteins including polymyxin B, α-defensins, cathelicidins and bactericidal/permeability-increasing proteins (Marsden *et al.*, 2009; Keo *et al.*, 2011). DNA uptake into a bacterial cell is an outer cell membrane-dependent process. Therefore, LOS modification in the outer cell membrane may also affect *Campylobacter*'s ability to uptake foreign DNA or its characteristic of natural transformation (Jeon and Zhang, 2009; Marsden *et al.*, 2009).

Campylobacter LOS mediate the interaction between the host and bacterial cell. The *Campylobacter* LOS terminal *N*-acetyl galactosamine residues bind with the human macrophage galactose-type lectin receptors in order to enhance the bacterial interaction with human macrophages (van Sorge *et al.*, 2009). Similarly, *Campylobacter* LOS sialic acid residues bind to TLR-4 and sialoadhesin receptors present on the human macrophage cell surfaces (Klaas *et al.*, 2012; Heikema *et al.*, 2013; Stephenson *et al.*, 2013). *Campylobacter* LOS sialic acid residues are also ligands of Sialic-acid binding immunoglobulin-like lectins (Siglecs) present on human monocytes and natural killer cells (Avril *et al.*, 2006). Mutants of *Campylobacter* LOS genes (*galE*, *cj1136*, and *waaF*), in comparison to respective WT strains have showed reduced adherence and invasion into host IECs (Fry *et al.*, 2000; Kanipes *et al.*, 2004; Javed *et al.*, 2012), which might be due to reduced interaction between host cell receptors and altered LOS structures. A mutant of *C. jejuni* 11168, lacking the core oligosaccharides in its LOS structures, was unable to invade Caco-2 cells, indicating the importance of

LOS in *Campylobacter* invasion into host cells (Marsden *et al*., 2009). Thus, LOS structures present on the *Campylobacter* cell surfaces develop interaction with host cells to further facilitate the adherence, invasion, endocytosis and translocation of *Campylobacter* into host cells (Fry *et al*., 2000; Marsden *et al*., 2009; Louwen *et al*., 2012).

Campylobacter LOS are stimulators and mediators of human immune cells. DC-expressed Siglec-7 receptor interaction with α-2, 8-linked sialylated LOS induces the type-1 helper T cells (Th1) polarisation, whereas, its interaction with α-2, 3-linked sialic acid induces a type-2 helper T cells (Th2) development. Hence, LOS structures mediate the DCs-dependent differentiation of Th$_o$ cells into Th1 or Th2 cells (Hu *et al*., 2006; Bax *et al*., 2011). Moreover, variable LOS-outer core structures (displayed in figure 1.8) help *Campylobacter* to escape from the host immune system as they mimic the GM1, GM2, GM3, GD3 and GD1a containing human gangliosides (Ang *et al*., 2002). For this reason, antibodies produced against the LOS structural epitopes do not only bind to LOS structures, but also to human gangliosides. The cross-reactivity of anti-LOS antibodies with human gangliosides leads to the development of GBS or MFS in humans (Nachamkin *et al*., 1998; Endtz *et al*., 2000; McCarthy and Giesecke, 2001). It is proposed that *C. jejuni* LOS triggers the NFκß pathway via TLR signalling in CD14+ DCs and macrophages to stimulate cytokine production from these cells (Huizinga *et al*., 2013; Stephenson *et al*., 2013). Increase in the expression of DC-derived cytokines, such as IFN-ß, TNF-α, and IL-12, induces the proliferation of human mucosal B-lymphocytes in a T cell-independent manner. Subsequently, B-lymphocytes then produce the cross-reactive antibodies in GBS patients post-infection with *Campylobacter*. This links *C. jejuni* sialylated LOS structures with B-cell mediated autoimmunity in GBS patients (Kuijf *et al*., 2010; Huizinga *et al*., 2013).

Structure of *C. jejuni* LOS   Corresponding ganglioside-mimic

Pen O:1 — GM2
Pen O:2 — GM3
Pen O:4 & O:19 — GD1a
— GM1
Pen O:10 — GD3
Pen O:23 & O:36 — GM2

Legend:
- ○ Glucose
- □ Galactose
- ⬡ *N*-Acetyl galactosamine
- ◇ (Phosphoryl)ethanolamine
- ▨ 2-keto-3-deoxy octulosonic acid (KDO)
- ● Dideoxyglucose
- ■ Heptose
- ⬢ *N*-Acetylglucosamine
- ◆ 2-aminoethyl phosphate
- ▼ Sialic acid
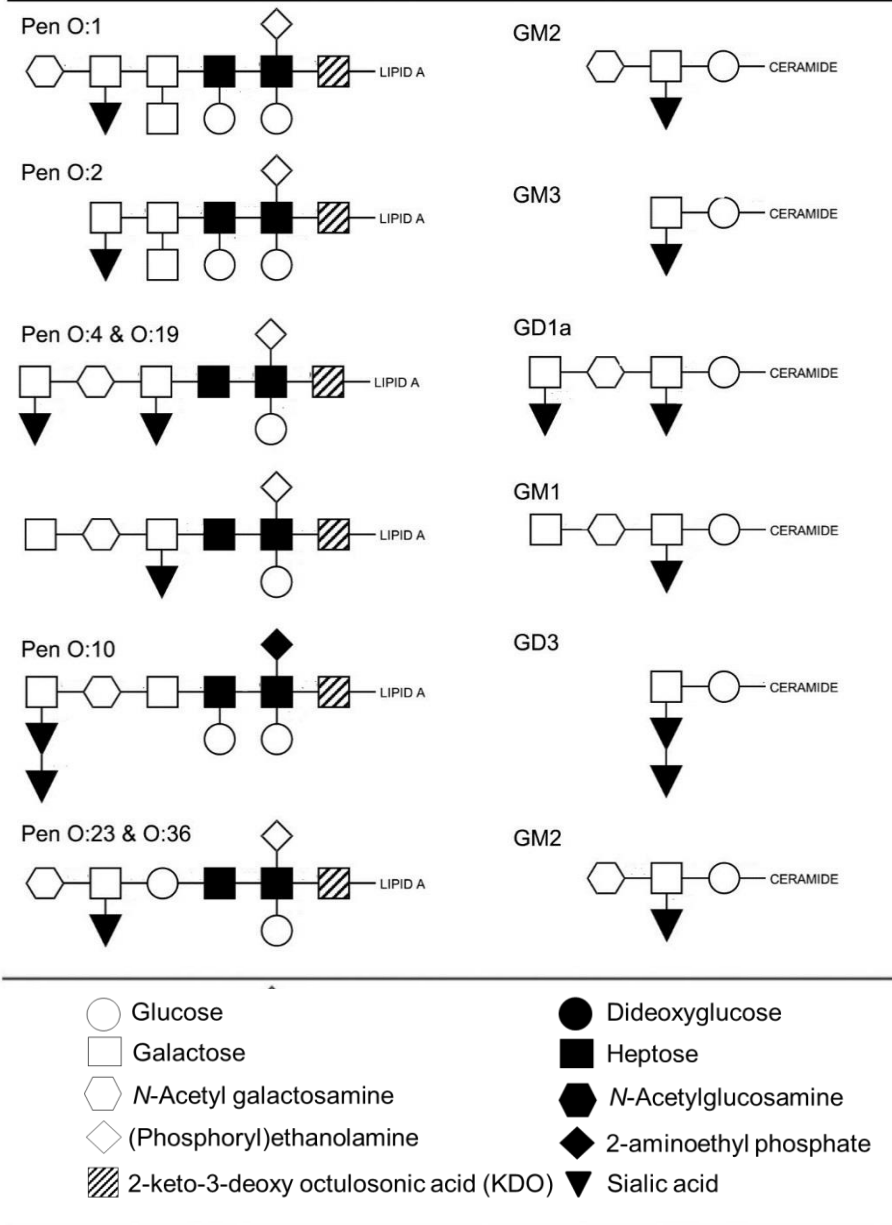
Figure 1.8: *C. jejuni* LOS structures and their corresponding human ganglioside mimics (Reproduced from Ang *et al*., 2002)

1.6. Aims and Objectives

The aim of this research project is to characterise the LOS biosynthesis gene clusters involved in cell-surface LOS biosynthesis in *C. jejuni* and *C. coli*. To achieve this primary aim, this thesis has four following aims and objectives

1) To analyse the extent of gene content variation in the *C. jejuni* LOS biosynthesis gene cluster by using a molecular typing method (Chapter 3).
   - Validate PCR as a LOS locus genotyping assay
   - Identify LOS locus type in clinical *C. jejuni* isolates by using PCR
   - Analyse LOS core of *C. jejuni* clinical isolates

2) To validate a pipeline to use for *C. jejuni* LOS locus typing, and analyse the extent of gene content variation in *C. jejuni* and *C. coli* LOS biosynthesis gene clusters *in silico* (Chapter 4).
   - Identify LOS locus type in *C. jejuni* online sequences by using Megablast and Galaxy pipeline
   - Identify LOS locus type *C. coli* online sequences by using Megablast and WG sequencing of a clinical *C. coli* strain
   - Evaluate *C. jejuni* and *C. coli* LOS loci distribution in different *Campylobacter* niches
   - Determine novel genes in *C. jejuni* and *C. coli* LOS biosynthesis loci

3) To determine the impact of LOS gene deletion on *C. coli* LOS structure and cell function (Chapter 5).
   - Validate a *rpsL*-based mutagenesis strategy
   - Construct and characterise a *C. coli* LOS biosynthesis gene region deletion mutant

4) To determine how variation of the LOS structures in *C. jejuni* and *C. coli* impacts upon infection of host cells *in vitro* (Chapter 6).
   - Measure induction of IL-1β and Caspase-1 in THP-1 cells following stimulation with extracted LOS from *Campylobacter* strains
   - Measure induction of IL-1β and Caspase-1 in THP-1 cells following infection with *Campylobacter* strains

CHAPTER 2

Materials and Methods

Materials

2.1. Bacterial Culture Media

All culture media were prepared by dissolving an appropriate amount of media in distilled water and sterilising at 121 °C for 15-20 minutes under 15 psi pressure in an autoclave (Dixons, Sussex, UK). Agar was cooled to 55 °C, poured into a petri dish (Scientific Laboratory Supplies, Nottingham, UK) under aseptic conditions, and allowed to solidify. If required, supplements were added after cooling the agar to 55 °C.

2.1.1. Mueller-Hinton agar and Mueller-Hinton broth

Mueller-Hinton agar (MHA; Oxoid Limited, Basingstoke, UK) media (38 g) containing 3 g beef dehydrate, 17.5 g casein hydrolysate, 1.5 g starch and 17 g agar was dissolved in 1 L of water. After autoclaving and cooling the media to 55 °C, 5% (v/v) defibrinated horse blood (Oxoid Limited) was added to it. Subsequently, vancomycin and trimethoprim with final concentrations (as specified in Table 2.1) were added to MHA to select *Campylobacter*. 21 g of Mueller-Hinton Broth (MHB; Oxoid Limited) containing 2 g beef dehydrate, 2 g starch and 1.5 g casein hydrolysate was dissolved in 1 L water to prepare MHB.

2.1.2. *Campylobacter* blood-free charcoal agar

Campylobacter blood-free charcoal agar base (22.75 g; Oxoid Limited) containing 4 g bacteriological charcoal, 3 g casein hydrolysate, 1 g sodium deoxycholate, 0.25 g ferrous sulphate, 0.25 g sodium pyruvate and 12 g agar was dissolved in 500 mL of distilled water. Subsequently, vancomycin and trimethoprim with final concentrations as specified in Table 2.1 were added to the media to select *Campylobacter*.

2.1.3. Luria-Bertani broth and Luria-Bertani agar

Luria-Bertani broth (LB broth; 25 g; Scientific Laboratory Supplies) was suspended in 1 L of distilled water (Bertani, 1951) to prepare LB broth. LB broth contents per L were 10 g tryptone, 5 g yeast extract and 10 g sodium chloride. To prepare Luria-Bertani agar (LB agar; Scientific Laboratory Supplies), 40 g LB agar was dissolved in 1 L of distilled water. All contents of LB agar per L were the same as LB broth with the addition of 15 g agar. After sterilisation, antibiotics were added to LB media according to the requirements (see Table 2.1). X-Gal (5-Bromo-4-chloro-3-indol-β-D-galactoside; Thermo Scientific, Loughborough, UK) dissolved in dimethylformamide (DMF) and IPTG (isopropythio-β-D-galactoside; Thermo scientific) dissolved in water, both at the final concentration of 20 µg per mL, were added into LB agar before pouring it into plates. X-gal/IPTG LB agar plates were used for blue-white screening of bacterial colonies.

2.1.4. Soft motility agar

Brain Heart Infusion broth (BHI broth; 37 g; Oxoid) containing 12.5 g brain infusion solids, 5 g beef heart infusion solids, 10 g proteose peptone, 2 g glucose, 5 g sodium chloride and 2.5 g disodium phosphate was dissolved in 1 L distilled water. To prepare the soft motility agar plates, 0.25% (w/v) bacteriological agar (Oxoid) was added into BHI broth and poured into petri plates after sterilisation. Soft motility agar plates were dried at room temperature for at least 24 hours prior to use.

2.2. Antibiotics

Stock solutions of antibiotics (Sigma-Aldrich, Dorset, UK) were prepared by dissolving in the appropriate solvents and stored at 4-20 °C in the dark. All water soluble antibiotic stock solutions were filter sterilised after preparation using 0.20 µm filters (Minisart Plus). Solvent, stock concentration and final working concentrations used are given in Table 2.1.

Table 2.1: Antibiotics used in this study

Antibiotics	Solvent	Stock concentration (mg/mL)	Final working concentration (µg/mL)
Ampicillin sodium salt	water	100	100
Vancomycin hydrochloride	50% (v/v) ethanol	10	10
Trimethoprim	50% (v/v) ethanol	5	5
Chloramphenicol	100% ethanol	20	20
Streptomycin sulphate salt	water	100	100

2.3. Buffers and Solutions

Buffers and solutions were sterilised after preparation under 15 psi pressure at 121 °C for 15-20 minutes in an autoclave, where needed.

2.3.1. TAE (Tri-Acetate-EDTA) buffer

TAE buffer (50x; ready to use) was purchased from Fisher Scientific and 1x TAE buffer was prepared by adding 20 mL of 50x TAE buffer to 980 mL distilled water for agarose gel electrophoresis.

2.3.2. Western blot transfer buffer

Sodium Dodecyl Sulfate (SDS; 1 g) was dissolved in 500 mL sterile distilled water. Then 100 mL 10 x Running buffer [0.25 M Tris (Sigma-Aldrich, UK) and 1.9 M glycine (Fisher Scientific, UK) dissolved in 1L distilled water] and 200 mL methanol were added and final volume was made up to 1 L.

2.3.3. 1X Phosphate Buffered Saline (PBS)

PBS solution was prepared by dissolving PBS tablets (Oxoid Limited) in distilled water. One tablet was used per 100 mL volume of distilled water.

2.3.4. 5M NaCl

NaCl (292.2 g; Sigma Aldrich) was dissolved in 700 mL of distilled water, total volume of solution was made up to 1 L with distilled water, and sterilised.

2.3.5. CTAB/NaCl solution

NaCl (4.1 g) was dissolved in 80 mL water to make 0.7 M NaCl solution. 10 g CTAB (hexadecyltrimethyl ammonium bromide; Sigma Aldrich) was dissolved into 0.7 M NaCl solution slowly to make CTAB/NaCl solution. The solution was stirred and heated to 60 °C to dissolve the CTAB.

2.3.6. Solutions for Tricine Polyacrylamide Gel Electrophoresis

2.3.6.1. Spacer and resolving buffer

To make the spacer buffer, 15.12 g Trizma base (Sigma-Aldrich) was dissolved in 50 mL sterile distilled water, the pH was adjusted to 6.8, and the final volume was made up to 100 mL with sterile distilled water. For the resolving buffer, 22.78 g Trizma base was dissolved in 50 mL sterile distilled water, pH 8.8, and a final volume was made up to 100 mL with sterile distilled water.

2.3.6.2. Running buffer

To make 1X running buffer, 10X running buffer (recipe given above in section 2.3.2) was diluted and 0.1% (w/v) SDS (Fisher Scientific) was added.

2.3.6.3. 10% Ammonium Persulphate (APS)

APS (0.1 g; Sigma-Aldrich) was dissolved in 1 mL of sterile distilled water.

2.3.7. Solutions for LOS gel silver staining

2.3.7.1. Gel fixing solution

Methanol (100 mL; Fisher Scientific), acetic acid (24 mL; Sigma-Aldrich) and formaldehyde (100 µL; Fisher Scientific) were added to 76 mL sterile distilled water to prepare 200 µL of fixing solution.

2.3.7.2. Wash solution (35% ethanol)

Molecular biology grade ethanol (>99.5%; 36.5 mL; Fisher Scientific) was dissolved in 63.5 mL sterile distilled water.

2.3.7.3. Sanitiser solution

Sodium thiosulfate (0.04 g; Sigma-Aldrich) was dissolved in 200 mL sterile distilled water.

2.3.7.4. Silver stain solution

Silver nitrate (0.4 g; Fisher Scientific) and formaldehyde (152 µL) were added to 200 mL sterile distilled water.

2.3.7.5. Gel developer

Sodium carbonate (24 g; Fisher Scientific), 0.02% (w/v) sodium thiosulfate (8 mL) and formaldehyde (200 µL) were added to 392 mL sterile distilled water to prepare the 400 mL of gel developer.

2.3.7.6. Stop solution

To make the 200 mL stop solution, 100 mL methanol and 24 mL glacial acetic acid were added to 76 mL sterile distilled water.

2.4. THP-1 Cell Line

The human monocytic cell line THP-1, derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia (Tsuchiya *et al*., 1980), was purchased from the European Collection of Authenticated Cell Cultures (ECACC; Catalogue no. 88081201).

Methods

2.5. Collection of Bacterial Strains

Campylobacter clinical isolates, given in Table 2.2, were collected from Northampton General Hospital (NGH), Northampton, UK, in a 12 month period from November 2015-2016. From anonymised clinical samples from NGH, bacterial isolates were collected by swabbing from already cultured Charcoal-Cefoperazone-Deoxycholate Agar (CCDA) plates. Amines and charcoal swabs (Thermo Fisher Scientific) were used for collection and transportation of *Campylobacter* isolates. Bacterial isolates were cultured again within 24 hours of collection. *Campylobacter* strains obtained from National Culture Type Collection (NCTC, Colindale) and American Type Culture Collection (ATCC) were used as reference strains throughout the project. In addition, genomic DNA (gDNA) of three *C. jejuni* strains, RM1048, RM1556 and RM1555, obtained from the United States Department of Agriculture Research Service, USA, were also used in this study.

2.6. Bacterial Cell Culture

2.6.1. Bacterial growth conditions

Campylobacter strains were grown on MHA plates at 37 °C for 24-48 hours under a microaerobic atmosphere of 5% O_2, 10% CO_2 and 85% N_2. The microaerobic environment was provided by either using the CampyGen sachets (Oxoid Limited) in 2.5 L air-tight jars or BOC gas mixture (2% H_2, 5% O_2, 10% CO_2 and 83% N_2) in a Whitley G2 workstation (Don Whitley Scientific).
E. coli strains were grown at 37 °C under aerobic conditions either on LB agar plates or in LB broth with shaking.

Table 2.2. *Campylobacter* isolates used in this study

No.	*C. jejuni* strain	No.	*C. jejuni* strain	No.	*C. coli* strain
1	CJ10[1]	35	ME112946R[1]	1	221089[1]
2	CJ4[1]	36	ME112990Z[1]	2	RM1875[4]
3	CJ12[1]	37	ME113179J[1]	3	76339[5]
4	CJ13[1]	38	ME113090Y[1]		
5	CJ18[1]	39	751[1]		
6	CJ23[1]	40	92649[1]		
7	S1[1]	41	36670[1]		
8	S2[1]	42	CJ3111[1]		
9	101[1]	43	37531[1]		
10	102[1]	44	34218[1]		
11	103[1]	45	34806[1]		
12	104[1]	46	38625[1]		
13	105[1]	47	34565[1]		
14	106[1]	48	38608[1]		
15	Moulton[6]	49	44406[1]		
16	1336[2]	50	45283[1]		
17	92740[1]	51	41999[1]		
18	92691[1]	52	40973[1]		
19	92540[1]	53	47185[1]		
20	92717[1]	54	39864[1]		
21	93133Y[1]	55	60319[1]		
22	CJ20[1]	56	60238[1]		
23	93084N[1]	57	54386[1]		
24	92661[1]	58	50702[1]		
25	112990[1]	59	59653[1]		
26	118715[1]	60	51585[1]		
27	118973[1]	61	92838[1]		
28	512[1]	62	92871[1]		
29	121097[1]	63	11168[3]		
30	118718[1]	64	81-176[3]		
31	93941P[1]	65	81116[3]		
32	11168Δ32-52[3]	66	RM1221[3]		
33	ME113262[1]	67	4031[5]		
34	ME112938s[1]				

[1] Clinical isolate
[2] University of Liverpool, UK
[3] University of Leicester, UK
[4] NCTC, Public Health England, UK
[5] University of Helsinki, Finland
[6] University of Northampton, UK

2.6.2. Bacterial strain storage

Microbanks™ (PRO-LAB Diagnostics) were inoculated with bacterial colonies from an overnight culture. Vials were inverted 4-5 times for rapid bonding of bacteria with porous bead surfaces. The cryopreservative solution was aspirated from each vial and stored at -80 °C. To prepare a fresh culture, the vial was kept cold on ice and a single bead was removed from the vial under aseptic conditions and used to directly inoculate a suitable fresh culture medium.

2.7. Mammalian Cell Tissue Culture

2.7.1. Cell counting and viability using a disposable haemocytometer

THP-1 cell suspension (100 µL) was aseptically transferred into an Eppendorf and 10 µL of it was mixed with 10 µL of 0.4% trypan blue (Gibco™) with pipetting. 10 µL of trypan blue treated cell suspension was gently injected into the counting chamber of disposable haemocytometer with pipette and examined under the inverted microscope (Nikon TMS 0.3 A). Cells were counted in 4 sets of 16 corner squares by using 10x objective of the inverted microscope. The average value of cell count from 4 sets of 16 corner squares was multiplied by 10^4 and then multiplied by 2 (dilution factor) to determine the number of live cells in the original cell suspension.

2.7.2. Cryopreservation and revival of suspension THP-1 cells

THP-1 cell suspension with >80% viable cells was transferred into a 50 mL tube and centrifuged at 100 x g for 5 minutes. The supernatant was removed and the cell pellet was dissolved in 1 mL of freezing media [95% (v/v) FBS + 5% (v/v) DMSO]. Cells in freezing media were transferred to a 1 mL cryovial. The cryovial was immediately placed in a CoolCell (Thermo Scientific™) and kept at -80 °C. After 24 hours, the cryovial was removed from the CoolCell and immediately stored at -80 °C.

For revival of the suspension of THP-1 cells, a cryovial was removed from the -80 °C freezer and snap-thawed in a 37 °C water bath for 1 minute. The cell suspension was transferred into a 50 mL tube containing 10 mL of pre-warmed cell culture media by pipetting. Subsequently, cells were centrifuged at 80 x g for 5 minutes, the supernatant was discarded and the pellet was suspended in 10 mL of cell culture media. Cell culture media containing cells was transferred into a culture flask (T25; Sarstedt). Flasks were incubated in a 37 °C humidified incubator in a vertical position for 15-20 days or until the cells reach the exponential phase of growth.

2.7.3. Tissue culture and differentiation of THP-1 cells

The THP-1 cell line was cultured in RPMI 1640 medium (Gibco®, Life Sciences) containing 10% (v/v) FBS (Gibco®, Life Sciences) at 37 °C in a 5% CO_2 humidified incubator (Eppendorf). For passaging the suspension cells, cells were counted and a volume of media required to dilute the culture to the recommended seeding density was calculated. The calculated volume of pre-warmed complete growth medium was added into the culture flask and kept at 37 °C in a 5% CO_2 humidified incubator. Cells were passaged every 3-4 days with a typical split ratio of 1:10 to minimise the accumulation of cell debris and metabolic waste by-products in suspension cell cultures.

To differentiate the THP-1 cells, cells were counted, centrifuged at 100 x g for 5 minutes and resuspended in cell culture medium supplemented with a final concentration of 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma). THP-1 cells were seeded into 12-well tissue culture plates (Eppendorf) at a density of approximately 1×10^6 cells per well and incubated for 48 hours at 37 °C in 5% CO_2 humidified incubator. After incubation, PMA-containing medium was aspirated and adherent, differentiated cells (macrophages) were washed once with culture medium. Differentiated cells were then incubated in culture medium without PMA for an additional 24 h prior to inoculation.

2.7.4. Inoculation of THP-1 cells with live bacteria, lysates or purified lipooligosaccharides

Lipooligosaccharides were extracted from *Campylobacter* cells using the hot-phenol method (specified in section 2.15.1) and whole cell lysates were used to stimulate the IL-1β secretion in THP-1 cells with procedures as described previously (Siegesmund *et al.*, 2004). To prepare *Campylobacter* whole cell lysates, *Campylobacter* cells were harvested from one overnight cultured plate, washed twice in PBS and resuspended in PBS to a final OD_{600} of 1. The suspended bacterial cells were heat killed at 65 °C for 30 minutes and lysed by using sonicator (nusonics). After sonication (six times 30 second bursts and 30 second pause), the cells were centrifuged at 15,000 x g for 20 minutes at 4 °C. The whole cell lysate was collected in a new tube, filter sterilised and stored at -20 °C. The total protein in the bacterial whole cell lysates was then quantified (section 2.16.1) and lysates equivalent to ~25 µg protein were added to THP-1 cell culture.

To infect the differentiated THP-1 cells with live bacteria, *Campylobacter* cells were harvested from an overnight cultured plate, washed once in PBS and resuspended in RPMI 1640. The optical density ($O.D_{600}$) of bacterial suspensions was adjusted to 0.8, which was almost equivalent to 2×10^9 *C. jejuni* 11168, 2×10^{10} *C. coli* RM1875 or 8×10^8 *C. coli* 76339 colony forming units (CFU). These pre-determined CFU were checked again by serially diluting and plating the bacterial suspensions. The multiplicity of infection (MOI; number of live *Campylobacter* cells required per THP-1 cell infection) was calculated by using the following formula and achieved by adding the appropriate volume of bacterial suspensions in THP-1 cells

$$\text{MOI} = \frac{\text{CFU of bacteria used for infection}}{\text{Number of THP-1 cells}}$$

The assay was carried out for 12 hours after the addition of either live bacteria, lysates or purified LOS and cell culture media from the culture plate was collected after 12 hours for further analysis. For the inhibition of K^+ channels and Caspase-1, THP-1 cells were incubated respectively with 50 µM glyburide (soluble in DMSO; Fisher Scientific, UK) and 10 µM *N*-benzyloxycarbony-Val-Ala-Asp (*O*-

methyl)-fluoromethylketone (Z-VAD-FMK; a Caspase-1 inhibitor soluble in DMSO; Invitrogen) for 3 hours prior to infecting them with live or killed bacterial lysates. THP-1 cell culture with *E. coli* O111:B4 purified Lipopolysaccharides (LPS; Sigma-Aldrich LPS25) was used as a positive control and THP-1 cell culture with PBS only was used as a negative control in all THP-1 inoculation assays.

2.8. Nucleic Acid Isolation from Bacterial Cells

The CTAB gDNA extraction method was used for large-scale DNA extraction, whereas, DNeasy Blood and Tissue kit (Qiagen) was used for small-scale DNA extraction. High quality and ultra clean DNA, required for either WG sequencing or LOS gene deletion screening PCR assays, was isolated by using the UltraClean® Microbial DNA Isolation Kit.

2.8.1. Extraction of gDNA using CTAB method

The previously described the CTAB gDNA extraction method was followed with few modifications for gDNA extraction from *Campylobacter* isolates (Dasti *et al*., 2007). Bacterial cells were collected from four cultured plates with 10 mL PBS. The mixture was centrifuged at maximum speed for 10 minutes, and 9.5 mL of PBS was added to suspend the cell pellet. To lyse the cell suspension, 2 mL of 10% (w/v) SDS (Sigma-Aldrich), 100 µL of 20 mg/mL proteinase K (Qiagen) and 20 µL of 10 mg/ml RNase A (Qiagen) were added. The solution was mixed by inverting 4-5 times and incubated for 1 hour at 37 °C. After this incubation, 1.8 mL of 5 M NaCl and 1.5 mL of CTAB/NaCl were added to the lysed cell suspension and the sample was incubated at 65 °C for 20 minutes. Chloroform:isoamyl alcohol (24:1; Sigma-Aldrich) was added to the suspension in a 1:1 ratio to isolate the DNA into the aqueous layer. The mixture was transferred to 50 mL phase lock gel tubes (Scientific Laboratory Supplies) and centrifuged in order to separate the aqueous layer and organic solvent layer at 3,020 x g at 4 °C for 20 minutes. The gDNA was extracted from the aqueous

layer with 0.7 volumes of room temperature isopropanol. The DNA was collected with a clean glass Pasteur pipette into a collection tube, washed with 70% (v/v) ethanol to precipitate the DNA, and air dried. After drying, the DNA was resuspended in 750 µL of sterile distilled water.

2.8.2. DNA extraction using DNeasy Blood and Tissue Kit

The protocol provided by the DNeasy Blood and Tissue kit (Qiagen) manufacturer was followed. Briefly, ~1 x 10^9 *Campylobacter* cells from an overnight cultured plate were harvested in 1 mL PBS and centrifuged at maximum speed for 1 minute. The cell pellet was suspended in 180 µL of ATL buffer (SDS solution) and 20 µL of supplied proteinase K (20 mg/mL), mixed and incubated at 56 °C for 3 hours. After incubation, 4 µL of RNase (100 mg/mL) was added to the cell suspension and kept at room temperature for 2 minutes. Then, 200 µL of AL buffer and 200 µL of molecular biology grade ethanol (>99.5%) were added together and the mixture was transferred to the supplied DNeasy Mini Spin column. The column was centrifuged for 1 minute and the flow-through was discarded. The column was then sequentially washed with 500 µL of Wash Buffer AW1 and 500 µL of Wash Buffer AW2 and the flow-through was discarded after every washing step. The column was re-centrifuged for 1 minute to remove the residual ethanol. The column was then placed into a clean Eppendorf, 100 µL sterile distilled water was applied to the centre of column, left for 1 minute, and finally centrifuged for 1 minute to elute the gDNA.

2.8.3. DNA extraction using UltraClean® Microbial DNA Isolation Kit

The protocol provided by the UltraClean® Microbial DNA Isolation kit (MO BIO Laboratories, Inc.) manufacturer was followed. Briefly, ~1 x 10^9 cells from an overnight cultured plate were harvested in 1 mL PBS and centrifuged at maximum speed for 1 minute. The cell pellet was resuspended in 300 µL of MicroBead solution and 50 µL of solution MD1, gently vortexed, and incubated at 70 °C for 1 hour. The cell suspension was centrifuged for 1 minute, the supernatant was collected into a new tube and mixed with 100 µL solution MD2.

The mixture was centrifuged again for 1 minute. The supernatant, collected into a fresh tube, was mixed with 100 μL of solution MD3 and transferred to spin filter. The column was centrifuged for 1 minute and the flow-through was discarded. The column was then washed with 300 μL of wash solution MD4, centrifuged for 1 minute, and then re-centrifuged for 1 minute to remove the traces of wash solution. To elute the ultraclean gDNA, the column was placed into a clean Eppendorf, 50 μL sterile distilled water or EB buffer (10 mM Tris-HCl pH 8.5) was applied to the centre of the column, left for 1 minute, and finally centrifuged for 1 minute.

2.8.4. Isolation of plasmid DNA using QIAprep Miniprep Kit

The protocol provided by the QIAprep Spin Miniprep kit (Qiagen) manufacturer was followed. Briefly, an overnight *E. coli* culture of up to 5 mL was harvested by centrifugation at 7,000 x g for 3 minutes at room temperature. The cell pellet was suspended in 250 μL of the cell suspension buffer P1 (50 μg/mL RNase A, 50 mM Tris-HCl; pH 8.0, 10 mM EDTA, and lyseblue). Then 250 μL of the lysis buffer P2 [0.2 M NaOH and 1% (w/v) SDS] was added, mixed thoroughly until the solution turned blue, and the mixture was incubated at room temperature (15–25 °C) for 5 minutes. 350 μL of the neutralization and binding buffer N3 (4 M guanidine hydrochloride and 0.5 M potassium acetate, pH 4.2) was then immediately added and mixed thoroughly until the solution became colourless. The solution was centrifuged at 18,000 x g for 10 minutes and the supernatant was transferred to the QIAprep 2.0 spin column by pipetting. The column was centrifuged for 30-60 seconds and the flow-through was discarded. The column was then washed with 0.75 mL wash buffer PE (20 mM NaCl2mM, Tris-HCl pH 7.5, and 80% ethanol) and the flow-through was discarded after centrifugation for 30-60 seconds. Each column was re-centrifuged for 1 minute to remove residual buffer PE. To elute the plasmid DNA, the column was then placed into a clean Eppendorf and 30-50 μL sterile distilled water was applied to the centre of the column, left for 1 minute, and finally centrifuged at 18,000 x g for 1 minute.

2.8.5. RNA extraction using PARIS™ Kit

The protocol provided by the manufacturer in the PARIS™ kit (Life Technologies™) was followed. Briefly, *Campylobacter* cells from an overnight cultured plate were harvested with 1 mL PBS in an RNase-free 2 mL tube and centrifuged at 7,000 x g for 3 minutes at 4 °C. Cells were washed once by resuspending in 1 mL PBS and re-pelleted via centrifugation. The supernatant was removed and 500 µL of ice-cold cell disruption buffer was added to the cell pellet to lyse the cells. Cells were completely lysed in the cell disruption buffer by vigorous pipetting. 500 µL of 2X lysis/binding solution was added to the lysed cells mixture and mixed gently by inverting the tube several times. Visible cell debris was removed by spinning at 10,000 x g for 1 minute. To purify the RNA, 500 µL of 100% (v/v) molecular biology grade ethanol was added and mixed gently by inverting the tube several times. The sample mixture (~2 mL) was transferred to a supplied filter cartridge by pipetting. The filter cartridge was then centrifuged for 60 seconds and the flow-through was discarded. The filter cartridge was sequentially washed once with 0.75 mL wash solution 1 and twice with 0.5 mL wash solution 2/3, with the flow through being discarded after each spin. The filter cartridge was re-centrifuged for 1 minute to remove the traces of wash solution 2/3. To elute the total RNA, the column was then placed into a clean RNase-free collection tube, 50 µL elution solution (preheated to 95 °C) was applied to the centre of column, left for 1 minute, and finally centrifuged for 1 minute. All samples, used for RNA extraction, were kept cold on ice between each step and each centrifugation step was carried out at 4 °C.

Following the RNA extraction, all RNA samples contaminated with gDNA were cleaned up by following the protocol provided by the DNA-free™ kit (Invitrogen) manufacturer. Briefly, the contaminated RNA sample was mixed with 5 µL 10X DNase buffer and 0.5 µL rDNase and incubated for 30 minutes at 37 °C. Then 0.5 µL rDNase was added again and sample was incubated again for 30 minutes at 37 °C. After incubation, 0.25 µL DNase inactivation reagent was added. The sample was incubated for two minutes at room temperature with occasional vortexing. The sample was centrifuged at 10,000 x g for 10 min and the

supernatant containing clean RNA was transferred to a new RNase-free collection tube.

2.9. Analysis of Nucleic acid Quality and Integrity

The concentration and quality of gDNA, plasmid DNA and RNA in nanograms (ng) per µL of sample was determined using Nanodrop 2000 spectrophotometer (Thermo Scientific).

DNA quality and integrity was also analysed by agarose gel electrophoresis. Agarose gels (1%) were prepared by dissolving agarose (1g; Sigma-Aldrich) in 100 mL of 1X TAE buffer and heating the mixture until it had dissolved completely. The agarose solution was cooled to 50 °C and 5 µL SYBR® safe stain (10,000X; Invitrogen) per 100 mL of agarose solution was added. DNA samples were then prepared by the addition of 5X loading dye (Bioline Reagents Ltd) prior to loading. DNA samples and molecular weight markers of known concentration were loaded on a gel. Hyperladder I (200bp to 10kb; Bioline Reagents Ltd) or Hyperladder IV (100bp to 1kb; Bioline Reagents Ltd) were used to determine the DNA size and concentration of unknown samples. The gel was run at 80-150 V depending upon the DNA size until the loading dye had travelled approximately 1 inch up from the bottom of gel. Loaded DNA was visualised by using G: Box (Syngene) and photographed under UV light.

2.10. Purification of DNA Fragments

2.10.1. Purification of DNA using MinElute Gel Extraction Kit

The MinElute kit (Qiagen) was used to clean DNA fragments of up to 4kb in length. Each DNA band was excised from the agarose gel with a clean, sharp scalpel and the gel slice was weighed. 1 volume of gel was mixed with 3 volumes of buffer QG (5.5 M guanidine thiocyanate and 20 mM Tris HCl, pH 6.6), for example, 100 mg of gel into 300 µL of buffer QG. Mixture was incubated at 50 °C for 10 minutes to dissolve the gel. An equal volume of 100% (v/v) isopropanol

was added to the mixture and incorporated by inversion. The mixture was transferred to the MinElute spin column and passed through by centrifugation. 500 µL of buffer QG and then 750 µL of buffer PE (10 mM Tris-HCl, pH 7.5 and 80% ethanol) were passed through the column by centrifugation, with the flow through being discarded after each spin. The column was re-centrifuged for 1 minute to remove any excessive residual ethanol from buffer PE. Finally, 10 µL of sterile distilled water was directly applied to the column membrane to elute bound DNA.

2.10.2. Purification of DNA using QIAquick PCR Purification Kit

The QIAquick PCR purification kit (Qiagen) was used to clean the DNA fragments in PCR or restriction enzyme reaction mixtures (or fragments over 4kb). One volume of reaction mixture and 5 volumes of buffer PB were mixed into a clean centrifuge tube. The mixture and 750 µL of buffer PE (10 mM Tris-HCl, pH 7.5 and 80% ethanol) were consecutively passed through the QIAquick column, with the flow through being discarded after each spin. The column was centrifuged for 1 minute to remove any excessive residual ethanol from buffer PE. Finally 30-50 µL of sterile distilled water was directly applied to the column membrane to elute bound DNA.

2.11. Enzymatic Manipulation of DNA

2.11.1. Restriction endonuclease digestion of DNA

Restriction endonucleases and buffers were purchased from New England Biolabs (NEB) or Thermo Scientific and the procedure for DNA restriction was carried out according to the manufacturers' protocols. Typically, a reaction with a maximum total volume of 50 µL including nuclease-free water, restriction enzyme buffer, 0.5-1 µg DNA, 5-10 units restriction enzyme was incubated at 37 °C for 1 hour to carry out DNA digestion. A positive control (digestion mixture with control DNA) and a negative control (digestion mixture with no enzyme) were also included.

2.11.2. DNA ligation

T4 DNA Ligase (Invitrogen) was used according to the instructions given by the manufacturer. Briefly, a reaction mixture with a maximum size of 20 µL was prepared containing 4 µL 5X ligase buffer [250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM Dithiothreitol (DTT), 25% (w/v) polyethylene glycol-8000], insert DNA and vector DNA in a Molar ratio of 3:1, distilled water, and T4 DNA ligase (2 Units). The reaction mixture was incubated overnight in PCR machine at 26 °C.

2.12. Transformation of Bacterial cells with Plasmid DNA

2.12.1. *E. coli* electrocompetent cells preparation for DNA transformation

To prepare electrocompetent *E. coli* cells (Sambrook and Russell, 2001), 100 mL of fresh LB broth was inoculated with 1 mL overnight *E. coli* culture and incubated at 37 °C with agitation until the OD_{600} nm of the culture was between 0.4 - 0.6. The cell pellet was washed three times with decreasing amounts of ice-cold, sterile, distilled water. Then, the cells were re-suspended in 10 mL of 10% (v/v) ice-cold, sterile glycerol. After washing with glycerol, harvested cells were dissolved in 1 mL of 10% glycerol and divided into 50 µL aliquots to make them ready to use either immediately or stored at -80 °C, after being snap frozen on dry ice.

2.12.2. Electroporation of plasmid DNA into *E. coli*

Plasmid DNA was electroporated into *E. coli* with a standard procedure as explained previously (Sharma and Schimke, 1996). Plasmid DNA and electrocompetent cells (2-5 µg in 50 µL of cell suspension) were carefully transferred to the bottom of an ice-cold electrocuvette (VWR International Ltd). Cells were electroporated at 2.5 kV, 5.90 ms with electroporator (Bio-RAD Micropulser) after 10 minutes incubation on ice. After electroporation, immediately cells were suspended into 1 mL of ready-to-use SOC (Super

Optimal broth with Catabolite repression; Invitrogen) media. The cuvette was inverted 2-3 times to mix the media with the cells. The mixture was then incubated with shaking for at least 1 hour at 37 °C. Cells were centrifuged and re-suspended in 200 µL of SOC media. Cells suspension was then spread on LB agar selection plates and bacterial colonies were counted to determine the transformation efficiency after 18-24 hours.

2.12.3. Preparation of Electrocompetent *Campylobacter* cells

For the preparation of electrocompetent *Campylobacter* cells (Miller *et al*., 1988), cells were harvested from two overnight cultured plates with 10 mL MHB and centrifuged at 1,500 x g for 10 minutes at 4 °C. Supernatant was removed and the cell pellet was sequentially washed four times in 50 mL, 25 mL, 10 mL and 5 mL of ice-cold CEB buffer (15% (v/v) glycerol, 272 mM sucrose). The cells were finally resuspended in 200 µL of CEB and used straight away.

2.12.4. Electroporation of plasmid DNA into electrocompetent *Campylobacter* cells

Approximately 1.5-2 µg of plasmid DNA and 40 µL of electrocompetent cells were mixed in a sterile Eppendorf (Miller *et al*., 1988). The mixture was transferred to the bottom of an ice-cold electrocuvette and incubated on ice for 10 minutes. Cells were electroporated at 2.5 kV, 5.90 ms with electroporator, suspended into 200 µL of SOC media, and immediately spread on plain MHA plate. After overnight incubation at 42 °C under microaerobic conditions, cultured cells were harvested in 2 mL MHB. An aliquot of 100 µL was cultured onto MHA supplemented with 20 µg/mL chloramphenicol and the remaining cell suspension was centrifuged for 1 minute. The cell pellet was dissolved in 100 µL of MHB and cultured onto MHA supplemented with 20 µg/mL chloramphenicol.

2.13. Polymerase Chain Reaction (PCR)

2.13.1. Standard PCR

Each Polymerase Chain Reaction (PCR) master mix was prepared after mixing the PCR reaction components in a clean, nuclease free Eppendorf, (Table 2.3 and Table 2.4). Generally, PCR master mix with MyTaq™ red DNA polymerase (Bioline Reagents Ltd) and DreamTaq Green PCR master mix (Thermo Scientific) were used for standard PCRs. The reaction mixture was transferred to the PCR tubes in the correct volume and each reaction was carried out in a thermocyler (TECHNE) using appropriate cycling conditions. A typical PCR cycling profile is given in Table 2.5.

Table 2.3: A standard PCR reaction mix with MyTaq™ red DNA polymerase

Component of PCR mix	Volume (µL)	Final Concentration
Sterile distilled water	Up to 20 µL	-
Forward primer stock solution (10 µM)	1	0.5 µM
Reverse primer stock solution (10 µM)	1	0.5 µM
MyTaq™ red DNA polymerase	0.25	~1.25 Units
gDNA	variable	50-100 ng
Plasmid DNA		10-20 ng
MyTaq™ red Reaction Buffer (includes 5 mM dNTPs, 15 mM MgCl$_2$ & enhancers)	4	5X

Table 2.4: A standard PCR reaction mix with DreamTaq polymerase

Component of PCR mix	Volume (µL)	Final Concentration
Sterile distilled water	Up to 25 µL	-
Forward primer stock solution (10 µM)	1	0.5 µM
Reverse primer stock solution (10 µM)	1	0.5 µM
Template DNA	variable	50-100 ng
DreamTaq Green Reaction Buffer (includes 1.6 mM dNTPs, 4 mM MgCl$_2$ & DreamTaq polymerase)	12.5	2X

Table 2.5: A standard PCR amplification profile.

PCR assay step	Cycle	Temperature (°C)	Duration
Initial template denaturation	1	95	5min
Template denaturation		95	30sec
Primer annealing	25-40	45-65	35sec
Primer extension		72	30s – 3 min
Final extension	1	72	5min

2.13.2. Colony PCR

Single bacterial colonies were picked with sterile loops and each colony was suspended in the same volume of sterile PCR grade water as required to make the PCR master mix (Costa *et al.*, 1994). In PCR cycling conditions, the initial denaturation temperature and time were changed to 98 °C and 10 minutes, respectively. The other conditions were kept same as for the standard PCR protocol.

2.13.3. cDNA synthesis and Real-Time PCR

To synthesize cDNA, 1-2 µg gDNA-free RNA template was mixed with 4 µL of 5X cDNA SuperMix (qScript™) in a sterile PCR tube and to a final volume of 20 µL with nuclease-free grade water. The reaction was carried out in the PCR machine under the cycling conditions as specified in Table 2.6.

Table 2.6: cDNA synthesis conditions

Step	Cycle	Temperature (°C)	Duration (minutes)
Enzyme activation	1	25	5
Enzyme reactivity	1	42	30
Enzyme inactivation	1	85	5

The master mix for real-time PCR was prepared in a clean Eppendorf after mixing the PCR reaction components, as specified in Table 2.7. The reaction components were mixed by brief centrifugation and transferred in the correct volume to a 96-well PCR plate. The PCR plate was sealed with an optical adhesive cover and centrifuged at 18,000 x g for 15 seconds to spin down the contents and eliminate any air bubbles. Each PCR reaction was carried out in a StepOnePlus (Applied Biosystems) using the cycling conditions and dissociation curve conditions as specified in Table 2.8. A no template control (NTC) reaction containing all reaction components except cDNA was also included for each primer set.

Table 2.7: Real-time PCR reaction mix

Component of PCR mix	Volume (µL)	Concentration
PowerUp™ SYBR Green master mix	10	2X
Forward primer stock solution	0.12	600nM
Reverse primer stock solution	0.12	600nM
cDNA	Variable	~ 25ng/µl
Sterile distilled water	Up to 20 µL	-

Table 2.8: Cycling and dissociation curve conditions

Cycling conditions			
PCR assay step	**Cycle**	**Temperature (°C)**	**Duration**
UDG activation	1	50	2min
Dual-Lock DNA polymerase activation	1	95	2min
Denature	40	95	15 sec
Anneal/Extend		60	1min
Dissociation curve conditions			
Step	**Ramp rate**	**Temperature**	**Duration**
1	1.6 °C/sec	95	15sec
2	1.6 °C/sec	60	1 min
3	0.15 °C/sec	95	15 sec

The amplification efficiency of *rpsL* (reference gene) and *flaA* (target gene) specific primers was checked by a standard qPCR curve where Ct (Threshold cycle) values were plotted versus the *C. coli* RM1875 gDNA dilutions (0.0001 µg, 0.001 µg, 0.01 µg, 0.1 µg, 1 µg) and it was confirmed that the correlation coefficient (R2) for the linear lines was 0.98-0.99. The $2^{-\Delta\Delta Ct}$ (Livak) method was used for the analysis of qPCR results or quantification of *flaA* expression in test sample (gDNA from *C. coli* mutant strain) relative to the calibrator sample (gDNA from *C. coli* wild-type strain). The *flaA* gene expression was normalised against the *rpsL* gene expression.

2.14. DNA Sequencing

2.14.1. Sanger sequencing using Eurofins Mix2Seq Kit

The protocol provided with the Eurofins Mix2Seq kit was followed for gene or PCR product sequencing. According to the protocol, 15 µL purified PCR product or plasmid DNA (1-15 ng/µL) was mixed with 2 µL of either forward or reverse primer stock solution (10 pmol/µL). 17 µL DNA/primer mix was pipetted into a Mix2Seq tube. The tube was tightly sealed with provided lid and sent to the Eurofins, Wolverhampton, U.K. for sequencing. Sequence data obtained online in FASTA format was analysed by Clone Manager software (Edition 9; Scientific & Educational Software).

2.14.2. DNA submission protocol for WG sequencing

The gDNA samples for WG sequencing were sent to MicrobeNG, University of Birmingham, UK. For gDNA sample submission, 2 mL screw cap tubes, each containing 50 µL of gDNA sample, were sealed, labelled with the supplied barcode labels and submitted for sequencing. Prior to sending the DNA samples, the following two steps were carried out. The results obtained were analysed by using Clone Manager as explained in section 2.19.

1) The gDNA was quantified with a Nanodrop and concentration of each sample was prepared at 100 ng/µL.

2) The integrity gDNA was verified by running ~1µg DNA sample on a 0.75% (w/v) agarose gel.

2.15. Assays for LOS Analysis

2.15.1. *Campylobacter* LOS extraction

A previously described LOS method was followed for LOS extraction from *Campylobacter* isolates (Apicella, 2008). *Campylobacter* cells were harvested from one overnight cultured plate in 1 mL sterile distilled water. Cells were washed once in 1 mL sterile water. Cells were centrifuged and resuspended in 300 µL sterile water. The cells were lysed by heating at 95 °C for 10 minutes and 1 µL of Proteinase K (20 mg/mL), DNase (Ambion Turbo DNase 2 units/µL) and RNase (20 mg/mL) was added to the lysed cell suspension. The mixture was incubated at 37 °C for 1.5 hours and 60 °C for 1.5 hours. After incubation, the cells were chilled on ice. Then 300 µL 90% aqueous phenol (pH 4.2, Fisher) was added and the mixture was incubated again at 60 °C for 10 minutes. The cells were centrifuged at 8,500 x g for 20 minutes at room temperature. The aqueous phase was carefully collected in a clean 15 mL tube. 1 mL 3M sodium acetate and 6 mL 95% ethanol were added to the tube and incubated at -20 °C overnight to precipitate the LOS. After this incubation, the mixture was centrifuged at 2,000 x g for 40 minutes at 4 °C. The supernatant was discarded and the precipitated LOS pellet was resuspended in 100 µL distilled water and 6 mL 95% (v/v) ethanol. The LOS suspension was incubated again at -20 °C overnight and centrifuged at 2,000 x g for 40 minutes at 4 °C. After this incubation, the LOS pellet was either dissolved in 100 µL of sterile distilled water or lyophilized and stored at -80 °C.

2.15.2. LOS Tricine Polyacrylamide Gel Electrophoresis

For LOS Tricine Polyacrylamide Gel Electrophoresis (PAGE), separating and stacking gels were made (Apicella, 2008) by mixing the components as specified in Table 2.9 below.

Table 2.9: Mixtures for Separating and Stacking Gels

Separating Gel Mix	Stacking Gel Mix
10.55 mL of 30% acrylamide solution	2 mL of 30% acrylamide solution
3.95 mL of resolving buffer	2 mL of spacer buffer
4.95 mL of sterile distilled water	15.6 mL of sterile distilled water
0.3 mL of 1% (w/v) APS	0.3 mL of 1% (w/v) APS
4 µL of Tetramethylethylenediamine (TEMED)	4 µL of TEMED

The gel was placed in the running buffer (1X) filled Biorad mini-gel apparatus. Samples were prepared by mixing the LOS samples with 2X Laemmli Buffer (Biorad) in equal volumes and heating at 95 °C for 5 min. The samples were cooled down on ice and loaded on to the gel. The Spectra Multicolor Low range Protein Ladder (5 µL; Thermo Scientific) was also loaded on the gel and electrophoresis was performed at 80 V for 2-3 hours.

2.15.3. Silver staining of LOS Tricine PAGE gel

A previously established method (Tsai and Frasch, 1981) with few modifications and solutions described in section 2.3.7 were used for the silver staining of SDS-PAGE LOS gels. Firstly, the gel was fixed overnight with gel fixing solution. After overnight fixation, the gel was washed 3 times with the wash solution for 20 minutes each at room temperature. After washing, the gel was immersed in the sanitizer solution for 2 minutes and washed 3 times with sterile distilled water, each for 5 minutes. The gel was stained with silver stain solution for 20 minutes and washed again 2 times with sterile distilled water for 1 minute. The gel was developed with gel developer till LOS bands become visible. The gel development was stopped by immersing the gel into the stop solution.

2.15.4. Lectin blot

The protocol for lectin blot was used as described previously (Sondej *et al*., 2009). Briefly, Polyvinylidene difluoride (PVDF) membrane (Millipore) was pre-wetted in methanol, rinsed in distilled water, and soaked in western transfer buffer for 10 minutes. The blot was set up from the bottom with

a. Paper towel stack

b. 4 x dry filter papers

c. 4 x wet filter papers (transfer buffer)

d. PVDF membrane

5 µL of LOS (~50 µg/µL) was transferred onto membrane by pipette and left to dry completely for 2 hours. 5 µL LPS extracted from *E. coli* DH5α and 5 µL sterile distilled water were also dotted on the membrane as positive and negative controls, respectively. The PVDF membrane was soaked in methanol for 2 minutes, washed in PBST (PBS + 0.1% Tween) for 2 minutes and blocked with PBST + 1% BSA for 1 hour with shaking. The blocking solution was discarded and the membrane was immersed in biotinylated peanut lectin (Sigma-Aldrich; 1:200 in the PBST-1% (w/v) BSA) for 1 hour. The membrane was washed 3 times with PBST for 10 minutes. Then the membrane was immersed in streptavidin-horseradish peroxidase (Fisher Scientific; 1:200 in PBST-1% BSA) for 1 hour and washed again 3 times for 10 minutes. Solution A and B in the chemiluminescence detection kit (Thermo Fischer Scientific) were mixed in equal volumes and used to develop the membrane.

2.16. Assays for Protein Analysis

2.16.1. Total protein quantification

To determine the total protein concentration in bacterial whole cell lysates, the protocol provided by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) manufacturer was followed. By using the 1 mL ampoule of 2 mg/mL Bovine Serum Albumin (BSA), 8 standards were prepared with final BSA concentration of 2000, 1500, 1000, 750, 500, 250, 125 and 25 µg/mL. 100 µL of

the blank, each standard and unknown samples (whole cell lysate preps) were mixed with 2 mL of working reagent [50 mL of solution A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) and 1 mL of solution B (4% cupric sulfate)]. The enzyme reaction was initiated by incubating the reaction mixtures at 37 °C for 30 minutes. After incubation, the absorbance was measured at 562 nm with a spectrophotometer. A standard curve was prepared by plotting the average measurement for each BSA standard along y-axis and standards concentration along x-axis and used to determine the total protein concentration of each unknown sample.

2.16.2. Interleukin-1β Enzyme-linked Immunosorbent Assay

The protocol provided by the Interleukin-1β (IL-1β) Enzyme-linked Immunosorbent Assay (ELISA) kit manufacturer (Thermo Fisher Scientific) was followed. Briefly, a 96-well Corning™ Costar™ 9018 ELISA plate was coated with 100 µL capture antibody diluted in 1X Coating Buffer, sealed and incubated overnight at 4 °C. Wells were aspirated and washed 3 times with 300 µL per well wash buffer (PBS + 0.05% (v/v) tween-20). Plates were soaked in the wash buffer for 1-2 minutes to increase the effectiveness of the washing solution. After each wash step, the plate was tapped several times against the paper towel to remove excess wash buffer. The wells were blocked with 200 µL of 1X ELISA diluent and incubated at room temperature for 1 hour. The wells were aspirated and washed with 300 µL per well wash buffer once. By using the provided stock solution of IL-1β, 8 standards were prepared with final IL-1β concentration of 150, 75, 37.5, 18.7, 9.4, 4.7, 2.3 and 1.2 µg/mL. 100 µL of blank (1X ELISA diluent), each standard and unknown sample (cell culture supernatant) in triplicates were added to the wells and incubated overnight at 4 °C for maximum sensitivity. The wells were aspirated and washed 3 times with wash buffer. 100 µL detection antibody diluted in 1X ELISA Diluent was added to each well, sealed, and incubated at room temperature for 1 hour. The wells were aspirated and washed again 3 times with wash buffer. 100 µL avidin-horseradish peroxidase (250X) diluted in 1X ELISA diluent was added to each well and incubated at room temperature for 30 minutes. Wells were aspirated and washed

5 times with wash buffer. Then 100 µL of 1X tetramethylbenzidine solution was added to each well and incubated at room temperature for 15 minutes. After incubation, 50 µL of stop solution was added to each well to stop the reaction and plate was read at 415 nm with plate reader (BIORAD Model 680 XR).

2.16.3. Caspase-1 ELISA

The protocol provided by the Caspase-1 ELISA Kit (R & D Systems) manufacturer was followed. In brief, a 96-well microplate precoated with Caspase-1 specific capture antibodies was incubated with 50 µL of assay diluent in each well. Wells were aspirated. By using the provided stock solution of Caspase-1 (4000 pg/mL), 7 standards were prepared with final Caspase-1 concentrations of 400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL. 100 µL of blank (calibrator diluent), each standard and unknown samples (cell culture supernatants) in duplicates were added to the wells and incubated for 1.5 hours at room temperature. Wells were aspirated and washed 3 times with 400 µL per well wash buffer. 100 µL Caspase-1 antiserum was added to each well, and the plate was incubated for 30 minutes at room temperature. After 3 washes with wash buffer, 100 µL of human Caspase-1 conjugate was added to each well and incubated at room temperature for 30 minutes. Following another wash step, 200 µL substrate solution was added to each well and incubated in dark at room temperature for 20 minutes. After incubation, 50 µL stop solution was added to each well to stop the reaction and plate was read with plate reader at 415 nm.

2.16.4. Lactate dehydrogenase (LDH) release assay

Supernatants were collected from THP-1 cell cultures after 12 hours of inoculation as specified in section 2.7.4 and LDH release assay was performed according to the protocol provided for the Cytotoxicity Detection Kit (Thermo Fisher Scientific) by the manufacturer. Briefly, 50 µL of complete cell culture media [RPMI + 10% (v/v) FBS] and 50 µL of cell culture supernatants was transferred to a 96-well flat-bottom plate in triplicate. 50 µL of reaction mixture

was added to each well and incubated in the dark at room temperature for 30 minutes. After incubation, 50 µL stop solution was added to each well and mixed by gentle tapping. The plate was centrifuged for 5 minutes to remove any bubbles and the absorbance was measured with plate reader at 415nm. The mean absorbance value of complete cell culture media was subtracted from all other absorbance values to determine the LDH activity and subsequently, relative LDH release values were calculated by using the following formula.

Relative LDH release= <u>LDH activity in unknown sample – LDH activity in negative control</u>
LDH activity in positive control – LDH activity in negative control

2.17. Assays for Phenotype Analysis of *Campylobacter* spp.

2.17.1. Scanning electron microscopy

For scanning electron microscopy (SEM), the procedure given in Yamamoto *et al.* (2013) was followed with a few modifications. In brief, agar blocks onto which *Campylobacter* colonies had grown were fixed with 3% (v/v) glutaraldehyde for 2 hours at 4 °C and 1% osmium tetraoxide overnight at 4 °C. After fixation, agar plates were kept on a shaker for 1 hour at 4 °C. The floating colonies were collected in a sterile 50 mL tube. Then cells were dehydrated with a series of graded ethanol solutions (25%, 50%, 70%, 90% and 100% ethanol) and acetone solutions (25%, 50%, 70% and 100%) for 30 minutes each at room temperature. Bacterial cells were critically dried in a mixture of isoamyl alcohol and ethyl acetate (1:1), coated with gold-palladium, and examined using a Hitachi map 3D visualisation software in Hitachi S-3000 SEM.

2.17.2. Growth assay

A single colony was recultured on the MH agar plate at 42 °C under microaerobic conditions. Bacterial cells from the cultured plate were suspended in 15 mL MHB (starter culture) and used to inoculate the MHB culture (inoculated culture) to a standard value (0.05) of $O.D_{600}$.

The inoculated cultures, containing 500 µL per well of a 12-well plate, were kept shaking at 42 °C for 48 hours under microaerobic conditions. During the incubation, samples were taken at different time points (0, 3, 6, 9, 12, 24, 36 and 48 hours), serially diluted, and plated on MHA to count CFU. The $\log_{10}$ -transformed CFU values were used to assess the growth rate of *Campylobacter* strains.

2.17.3. Motility assay

The motility assay was performed according to the procedure as explained previously (Cohn *et al.*, 2007). *Campylobacter* cells from an overnight cultured plate were harvested in 15 mL MHB (starter culture) and adjusted to O.D$_{600}$ of 0.2. 2 µL of cell suspension was stabbed with a sterile pipette tip to the centre of pre-dried soft motility agar plate. Plates were incubated for 24 hours at 42 °C under the microaerobic conditions and the average motility was estimated by measuring the diameter of growth zone.

2.18. Student's *t*-test for Statistical Analysis

Comparisons between the two experimental groups were performed by using the Student's *t*-test (Independent; equal variance *t*-test). Differences in the data mean values were considered significant with a value of alpha than 0.05 considered statistically significant.

2.19. Using Clone Manager for Designing Primers

Clone Manager Professional Suite (Version 8; Scientific & Educational Software, Morrisville, USA), was used to design the *C. jejuni* LOS class specific PCR primers and other primers (given in Appendix-I), as well as, to look for the specific primers characteristics (Primers length=18-22 bp, GC content=40-60%, GC clamp=1, Stability>1, Tm=52-58 °C, Di-nucleotide repeats<4, Runs of single base<4, Cross Dimers<5, Self 3' Dimers<2, and Hairpins=none).

2.20. Ethics

Commercially available cell lines, horse blood, and human serum were used. *Campylobacter* isolates from anonymised clinical samples were collected by the Swab method under the sterile conditions from already cultured plates. No experimentation was carried out on humans during this research and this research did not involve the use of human tissues, fluids or DNA samples. Therefore, approval from any external body (such as NHS Research Ethics Committees) was not sought for this research. Ethical principles in relation to the research work were reviewed by the University Research Ethics Committee and no ethical issues were found to apply to this research. As such there were no ethical considerations for this study, however, approval from the University of Northampton's Genetically Modified Organism Biosafety review committee and the Health & Safety Executive for *Campylobacter* mutagenesis was previously obtained.

CHAPTER 3

Analysis of the Genetic Diversity of the *C. jejuni* Lipooligosaccharide Biosynthesis Locus by Molecular Typing

3.1. Introduction

3.1.1. Genetic diversity of *C. jejuni* LOS biosynthesis locus

The WG sequencing of *C. jejuni* 11168 (1.64 MB; Parkhill *et al*., 2000), *C. jejuni* RM1221 (1.77 MB; Fouts *et al*., 2005), and *C. jejuni* 81-176 (1.62 MB; Hofreuter *et al*., 2006) has revealed that the *C. jejuni* genome is highly diverse. *C. jejuni* exhibits extensive diversity in its genome due to its natural competent property for DNA uptake (Wang and Taylor, 1989; Gilbert *et al*., 2004; Revez and Hänninen, 2012) and non-clonal population structure (Gilbert *et al*., 2004; Revez and Hänninen, 2012). In addition, low GC content (28-38%) and presence of bacteriophage-related genes in *C. jejuni* genome also facilitate the occurrence of genetic exchanges (Fouts *et al*., 2005; Parker *et al*., 2005). Interstrain genetic exchange and intragenomic genetic rearrangements in *C. jejuni* usually develop during the colonization of *Campylobacter* in chickens and humans (Prendergast *et al*., 2004; Phongsisay *et al*., 2006; Wilson *et al*., 2009). In addition to *in vivo*, genetic rearrangements also occur during *in vitro* experimental infection of chicken cells with *Campylobacter* at the rate of 1 x 10^9 – 8.3 x 10^8 (De Boer *et al*., 2002).

The *C. jejuni* LOS biosynthesis gene region is a hotspot of genetic exchange in *C. jejuni* strains, which develops variation in *C. jejuni* LOS genetically, structurally and functionally (Gilbert *et al*., 2002; Parker *et al*., 2005; Müller *et al*., 2007; Godschalk *et al*., 2007). This region has been found to be strongly diverged in several *C. jejuni* strains compared to the first sequenced strain (*C. jejuni* 11168) (Dorrell *et al*., 2001; Gilbert *et al*., 2004). The GC content of this region is only 22 to 28%, which is even lower than the GC content of the remaining genome (30%), indicating that variations within this region are most likely to involve the lengthy, AT rich DNA sequences (Parker *et al*., 2005).

The variation in the *C. jejuni* LOS biosynthesis gene region occur either due to mutations within the nucleotides of LOS biosynthesis gene sequences or recombination between LOS biosynthesis gene/gene regions, which are discussed in detail below. A single base or gene level variation within the LOS biosynthesis locus can modify the LOS genes encoded enzymes that are functional in LOS synthesis, which further leads to the development of variable LOS structures (Parkhill *et al*., 2000; Parker *et al*., 2008). Strains possessing the same LOS biosynthesis genes do not always express phenotypically similar LOS structures because the LOS structures are determined not only by the gene content of the LOS biosynthesis gene cluster, but also by the presence of several mutations within this cluster (Parker *et al*., 2005). Hence, the genetic heterogeneity in LOS synthesis region determines the ultimate structure of LOS (Godschalk *et al*., 2007).

3.1.1.1. Variation at the nucleotide level

Nucleotide level variations within the LOS biosynthesis genes may occur due to the presence of homopolymeric tracts in these genes, which are the short sequence repeats and cause phase variation (gene switching) due to slip strand mispairing within these tracts (Linton *et al*., 2000; Gilbert *et al*., 2002). The presence of phase variable homopolymeric tracts affects the length of LOS biosynthesis genes (Guerry *et al*., 2002) and variation in LOS gene length significantly increases the rate of phase variation in *C. jejuni* (Bayliss *et al*., 2012). A site directed mutagenesis of homopolymeric tract (G8 → G11) in *C. jejuni* 11168 *wlaN* or change in the *wlaN* gene length increases the rate of phase variation ~10 fold in this gene (Bayliss *et al*., 2012).

The LOS gene, *wlaN*, in *C. jejuni* 11168, *C. jejuni* 331 and *C. jejuni* 2500 with 8G homopolymeric tract produces a fully transcribed and functional gene product, β 1, 3-galactosyltransferase (Linton *et al*., 2000; Müller *et al*., 2007; Semchenko *et al*., 2012). A phase-variable 9G homopolymeric tract in *wlaN* of these strains causes the premature translational termination and a non-functional gene product, which cannot add the terminal galactose in the LOS structure and consequently, converts GM1-like LOS epitope into a GM2 mimic (Linton *et al*., 2000; Semchenko *et al*., 2012). In *C. jejuni* 224, double tracts (10A/9G; gene on

→ 10A/10G; gene off) in *cj1144-45* also lead to the development of terminal α-linked galactose lacking LOS structure (Semchenko *et al*., 2012). Another phase variable 10G tract was identified in *cgtA* in *C. jejuni* 81-176 (class B), which converts GM2 to GM3 in LOS structure (Guerry *et al*., 2002). Figure 3.1 illustrates the LOS structural modifications, which occur due to the phase variation in polyG tracts of *C. jejuni* 11168 *wlaN* and *C. jejuni* 81-176 *cgtA*. Phase variation in *C. jejuni* GC149 (class R) *cgtA* develops the expression of convertible human ganglioside (GT1a ⇔ GD3) mimics (Houliston *et al*., 2011). A 10G tract in *C. jejuni* GB27 *cst-II* and 9G tract in *C. jejuni* GB26 *cst-II* develop, respectively, a premature translation stop and a non-functional gene product (Godschalk *et al*., 2006, 2007). As a result, *cst-II* encoded sialyltransferase cannot transfer sialic acid residues to these LOS structures and strains exhibit non-sialylated LOS (Godschalk *et al*., 2007). In addition, ORF23 with 9G or 10G tracts and ORF25 with 8G or 9G tracts in *C. jejuni* 81116 (LOS locus type E) have also been observed (Parker *et al*., 2005).

Sequence variation may also occur due to the single nucleotide mutations, which can inactivate the LOS biosynthesis genes without involving the phenomenon of phase variation. For example, missing A-base at position 1234 in *lgtF* (a LOS biosynthesis gene) alters the catalytic activity of its encoded enzyme, glycosyltransferase, in four *C .jejuni* strains (ATCC 43432, ATCC 43446, OH4382, and OH4384). As a result, the produced glycosyltransferase does not have the potential to catalyse the addition of ß-1, 2-glucose to Heptose-II during the LOS synthesis. Similarly, the last base alteration in Orf5/10 (*cgtA/neuA1*) in *C .jejuni* ATCC 43430 changes the amino acid (cysteine → tyrosine) which further leads to the production of a non-functional enzyme (Gilbert *et al*., 2002). The LOS gene, *cgtA,* with missing A-base at position 71 substitutes one amino acid in the *cgtA* encoding enzyme, *N*-acetyl galactosaminyl transferase, which further leads to the inactivation of *N*-acetyl galactosaminyl transferase in *C. jejuni* OH4382 and OH4384 and truncates the LOS structure (Gilbert *et al*., 2002). Similarly, a five base deletion from the *cst-III* gene of *C. jejuni* GB1 (possess LOS class B and sialylated LOS) alters the number of amino acids (294 → 219) in sialyltransferase and eventually, produces a non-sialylated LOS (Godschalk *et al*., 2007).
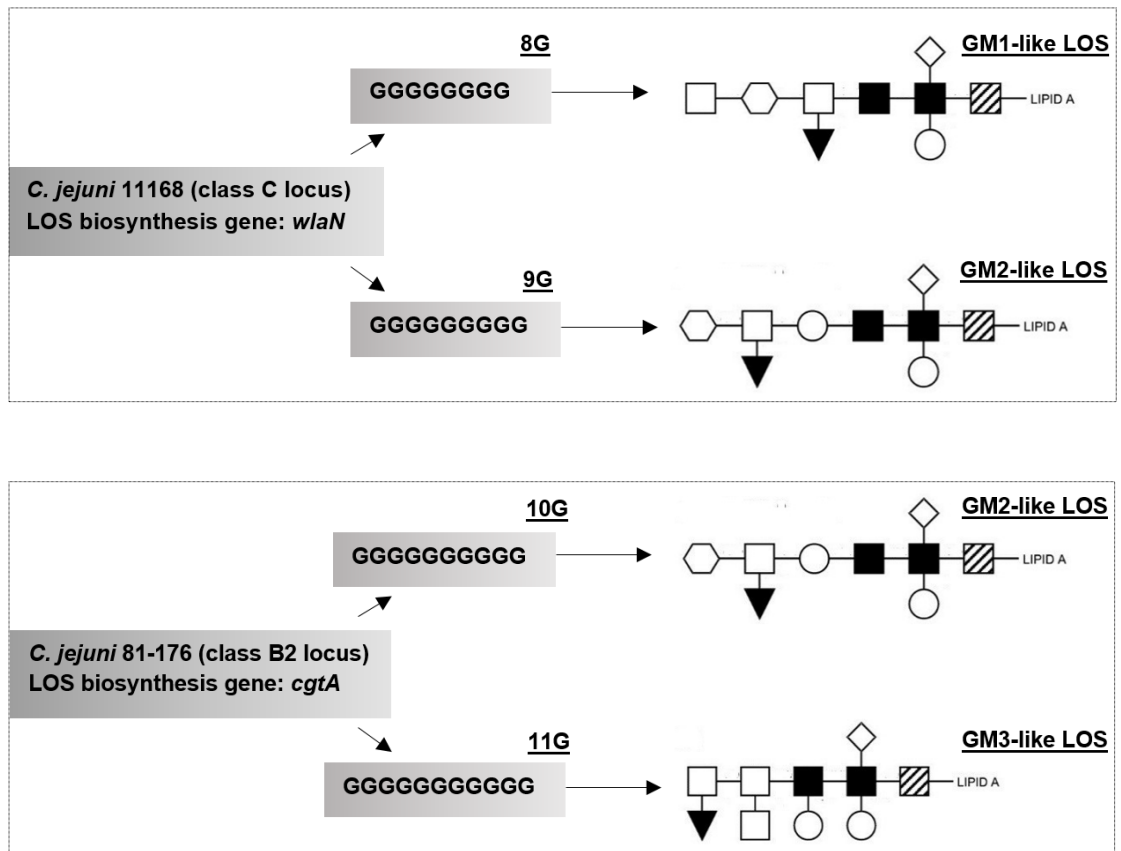
Figure 3.1. Phase variation in polyG homopolymeric tracts of *C. jejuni* 11168 *wlaN* and *C. jejuni* 81-176 *cgtA*.

The *wlaN* gene in *C. jejuni* 11168 with 8G homopolymeric tract leads to the development of GM1-like LOS structure. Phase variation of 8G into 9G removes the terminal galactose from GM1 and changes it into GM2. The *cgtA* gene in *C. jejuni* 81-176 with 10G tract develops GM2-like epitope in LOS structure and phase variation of 10G to 11G alters GM2 to GM3.
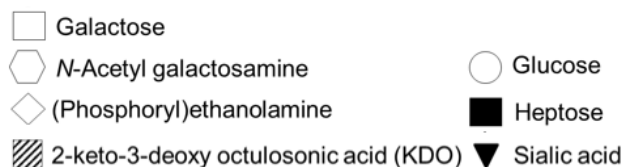
3.1.1.2. Variation at allele or gene level

An insertion or deletion of a new LOS biosynthesis gene or gene regions into LOS locus can give rise to a different locus type (Parker *et al.*, 2005, 2008). Only the portions of LOS biosynthesis genes or a deviation in gene (alleles) can also mutate to establish a new locus, such as, allele variation in *cgtA* and *wlaN* genes generate A and B subclasses including A1, A2, B1, B2 (Parker *et al.*, 2005). In addition, disruption in resident LOS biosynthesis genes can also form a new class, for instance, disruption in class E ORF26 establishes the LOS locus class P (Parker *et al.*, 2005). The developed new locus type can be variable both in gene content and gene organisation (Parker *et al.*, 2005; Revez and Hänninen, 2012).

C. jejuni acquires new genes in its LOS biosynthesis region by horizontal gene transfer. The horizontal transfer of LOS biosynthesis genes from *C. jejuni* O4 (GM1 strain) has been observed, which transformed *C. jejuni* 81116 (non-GM1 strain) into a GM1-like LOS producing strain (Phongsisay *et al.*, 2006). Similarly, a *C. jejuni* GB11 strain possessing class C locus acquired class A locus, identical to the LOS locus of *C. jejuni* ATCC 43446 (Gilbert *et al.*, 2004). *C. jejuni* 81116 and GB11, both were isolated from infected patients and horizontal gene transfer in these strains was found to have occurred during infection inside the human body (Gilbert *et al.*, 2004; Phongsisay *et al.*, 2006).

LOS biosynthesis gene alleles cause alterations in the LOS structure. For instance, *cst-II* gene alleles lead to the expression of either threonine (Thr) or asparagine (Asn) at position 51 of the translated enzyme. As a result, the enzyme retains either a monofunctional (Thr → 2, 3-sialyltransferase activity) or a bifunctional (Asn → 2, 3- and 2, 8-sialyltransferase) activity and produces LOS, respectively, with one and two sialic acids (Figure 3.2; Gilbert *et al.*, 2002).
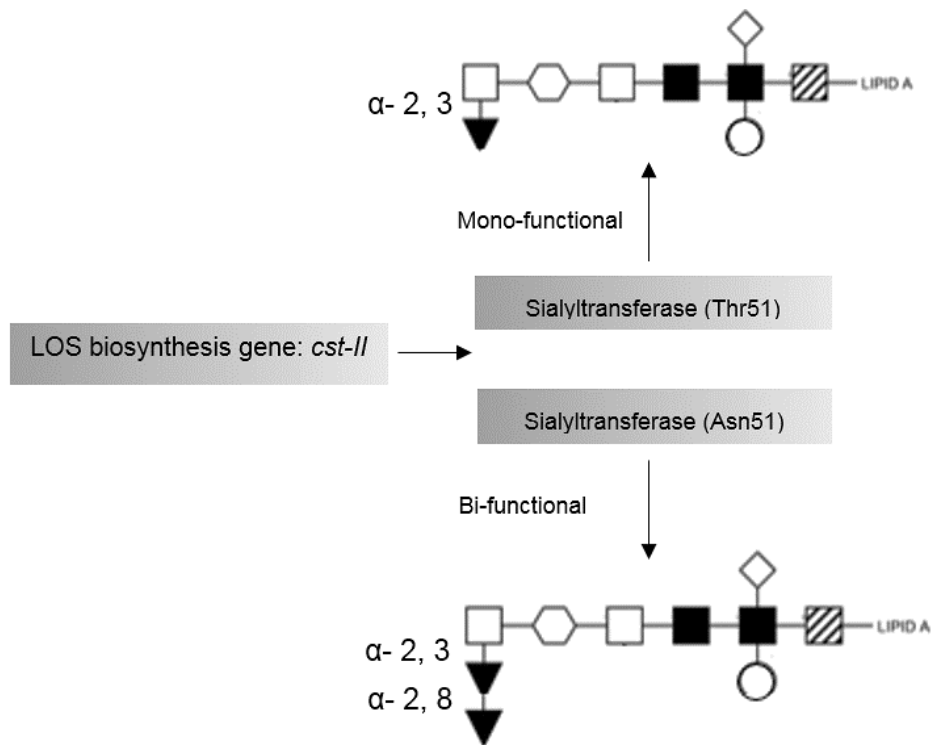
Figure 3.2: Functional variation in sialyltransferase due to allele variation in *cst-II*

Sialyltransferase with Thr at position 51 possesses mono-funtional activity and establishes α-2,3 linkage to attach one sialic acid with LOS structure. Sialyltransferase with Asn at position 51 exhibits bi-functional activity and forms α-2,3, as well as, α-2,8 linkage to join two sialic acids to LOS structure.

☐ Galactose

⬡ *N*-Acetyl galactosamine   ◯ Glucose

◇ (Phosphoryl)ethanolamine   ■ Heptose

▨ 2-keto-3-deoxy octulosonic acid (KDO) ▼ Sialic acid
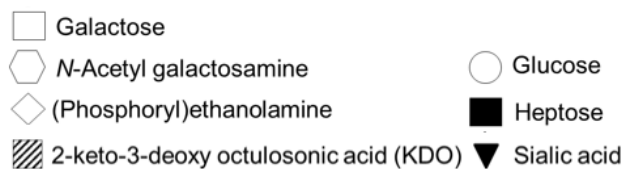
Variation in LOS locus gene content as well as in its gene organisation can also vary the cell-surface LOS structurally and functionally. It is not always the case that LOS structures belonging to the same LOS locus type present similar human ganglioside-like epitopes. *C. jejuni* 11168 and 520, both belong to class C, but *C. jejuni* 520 can produce a wider variety of human ganglioside mimics (GM1, GM2, asilo GM1, asialo GM2) than *C. jejuni* 11168 (GM1 and GM2) (Semchenko *et al*., 2012). *C. jejuni* strains containing a type A LOS locus frequently present variable human ganglioside mimics including GM1a, GM1b, GD1a, and GD1b on cell surfaces (Nachamkin *et al*., 2002; Godschalk *et al*., 2004; Mortensen *et al*., 2009). GM1a-like in *C. jejuni* 11168 (class C), GQ1b-like in *C. jejuni* 81-176 (Class B), Lewis type I-like in *C. jejuni* RM1503 (class M), and paragloboside/Pk antigens-like in *C. jejuni* RM1221 (class F) epitopes are typically presented in their LOS structures (Godschalk *et al*., 2004; Mortensen *et al*., 2009; Houliston *et al*., 2011). *C. jejuni* GC149 (class R) contains sialic acid biosynthesis genes and may present ganglioside like mimics (GT1a, GD3) as well as a hybrid form of ganglio and P-type antigens (Parker *et al*., 2008; Houliston *et al*., 2011). Other LOS classes such as D and E also possess human ganglioside-like LOS structures, but other than GM1, GD1 and GQ1b (Godschalk *et al*., 2004). Class P LOS have a lack of sialic acid and possess *N*-acetyl quinovosamine instead (Poly *et al*., 2008). The variable LOS structural epitopes or mimics presented by different *C. jejuni* LOS locus types are demonstrated in Table 3.1.

Table 3.1. Variable LOS structures synthesised by different *C. jejuni* LOS locus types

LOS locus class (Reference)	LOS structural epitopes	Mimicry
A (Nachamkin *et al.*, 2002; Godschalk *et al.*, 2004; Mortensen *et al.*, 2009)	GM1/GM1a, GM1b, GD1a, GD1b	Human Ganglioside Glycosphingolipid
B (Godschalk *et al.*, 2004; Mortensen *et al.*, 2009)	GQ1b	Human Ganglioside Glycosphingolipid
C (Linton *et al.*, 2000)	GM1, GM2	Human Ganglioside Glycosphingolipid
D (Godschalk *et al.*, 2004)	Human ganglioside-like LOS structures other than GM1, GD1 & GQ1b (Unknown)	Unknown
E (Godschalk *et al.*, 2004)	Human ganglioside-like LOS structures other than GM1, GD1 and GQ1b (Unknown)	Unknown
F (Houliston *et al.*, 2011)	Paragloboside, P1 antigen, Partial P1/GB4, Partial P1/GB3	P1 Blood Group Glycosphingolipid. Partial P1 Blood Group Glycosphingolipid. Partial Human Ganglioside Glycosphingolipid
M (Houliston *et al.*, 2011)	Sialyl- Lewis (I) Type	Lewis Type-1 Glycosphingolipid
P (Poly *et al.*, 2008)	Non-sialylated LOS with *N*-acetyl quinovosamine	No mimics
R (Houliston *et al.*, 2011)	GD3, GT1b, Partial P1/ Partial GM1	Human Ganglioside Glycosphingolipid. Partial P1 Blood Group Glycosphingolipid. Partial Human Ganglioside Glycosphingolipid

3.1.2. Correlation of LOS classes with *Campylobacter* virulence

The expression of variable cell surface LOS structures as a consequence of genes deviation in the LOS locus in *C. jejuni* is considered as an important virulence factor and may have direct connection with the progression of different neuronal disorders (Müller *et al*., 2007). For example, *C. jejuni* strains with LOS locus class A and variable human ganglioside mimics (GM1a, GM1b, GD1a, and GD1b) trigger GBS post-infection in *Campylobacter* infected patients (Nachamkin *et al*., 2002; Godschalk *et al*., 2004, 2007; Mortensen *et al*., 2009). Whereas, *C. jejuni* strains with LOS class B and corresponding GQ1b-like LOS structures are likely to develop MFS in *Campylobacter* infected patients (Godschalk *et al*., 2007; Islam *et al*., 2014). There is no doubt, genetic diversity within the LOS locus plays an important role in the *Campylobacter* virulence, as evidenced by the development of GBS or MFS post infection, however, this is not related to the development of diarrhoea or abdominal pain (Mortensen *et al*., 2009; Ellström *et al*., 2013). Neural diseases in humans do not develop only as a consequence of the *Campylobacter* infection, but other bacterial or host specific risk factors also help in stimulating the anti-gangliosides antibodies (Revez and Hänninen, 2012; Islam *et al*., 2014). In the following figure 3.3, various factors are given, which may vary among *C. jejuni* strains or infected individuals and build complexity in GBS development (Müller *et al*., 2007; Godschalk *et al*., 2007).

A strong link between the LOS biosynthesis gene variation and the invasion potential of *C. jejuni* strains *in vitro* has been identified previously. LOS class A, B and C (sialylated LOS) possessing *C. jejuni* strains show high potential of invasion into Caco-2 and INT407 cells compared to class D and E (non-sialylated) retaining *C. jejuni* strains (Guerry *et al*., 2002; Müller *et al*., 2007; Habib *et al*., 2009). Moreover, resistance of *C. jejuni* strains to antibiotics and human serum has also been considered as an attribute of the LOS locus variation. LOS locus class B possessing *C. jejuni* strains were found highly resistant to ciprofloxacin in comparison to those which had either LOS locus class A or C (Mortensen *et al*., 2009).
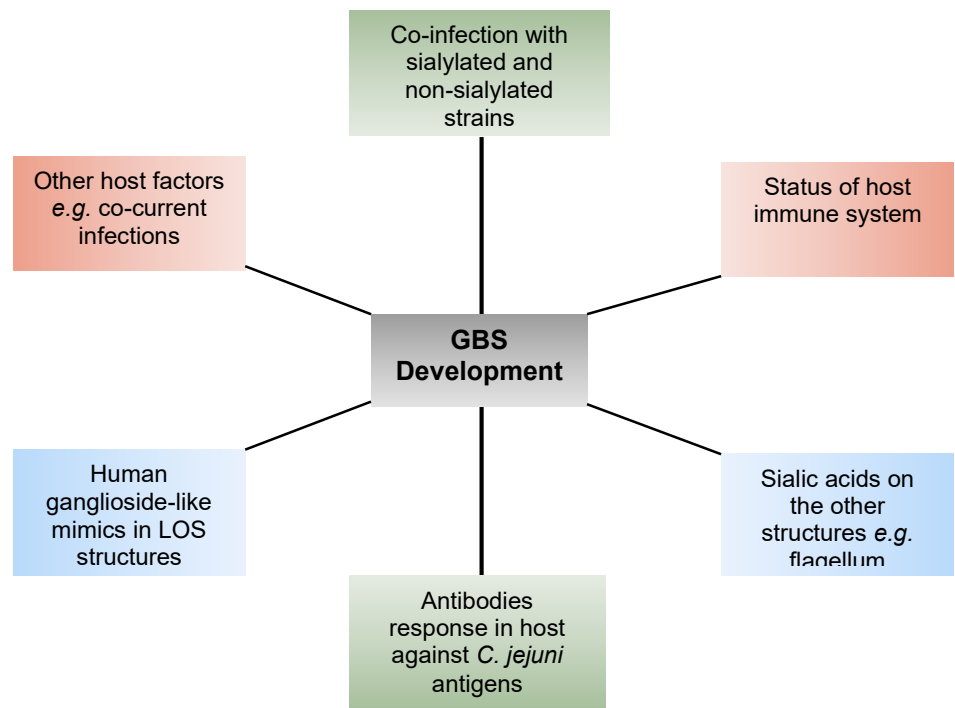
Figure 3.3: Different variable factors causing complexity in GBS development

3.2. Aims and Objectives

Based on the strong relationship between the variable LOS synthesis region and *C. jejuni* virulence, the current study aims to analyse the extent of gene variation in the *C. jejuni* LOS biosynthesis gene cluster. To achieve this aim, PCR as a LOS locus genotyping assay will be validated to use for the identification of LOS locus type in clinical *C. jejuni* isolates. The data obtained from the PCR typing of the LOS locus in clinical isolates will be used further to determine the frequency of *C. jejuni* LOS genotypes among the *C. jejuni* clinical isolates.

To examine the prevalence of various *C. jejuni* LOS locus genotypes in a clinical cohort, 122 *C. jejuni* clinical strains, isolated from faecal samples of *Campylobacter* infected patients, were collected from the Northampton General Hospital, Northampton, UK. Only 50% of the 122 *C. jejuni* isolates were able to be cultured. It is previously known that *Campylobacter* cells can undergo a viable but non-culturable state during passage in the laboratory (Cappelier *et al*., 1999; Ziprin *et al*., 2003). Therefore, it might be possible that the remaining 50% strains either had the non-cultureable state or died by the time they were cultured.

3.3. Results

3.3.1. Validation of PCR as a LOS locus genotyping assay

A PCR based screening method was used to genotype the LOS core biosynthesis gene clusters or loci of *C. jejuni* clinical isolates. This method was initially applied by Gilbert *et al*. (2002) and later, has been used in other studies (Parker *et al*., 2005; Marsden, 2007; Parker *et al*., 2008; Habib *et al*., 2009; Ellström *et al*., 2013). The PCR based LOS typing method used in this study differs from the previous established methods on the basis of the primer design strategy. This strategy involves the designing of primers that span the junction of two adjacent LOS genes and are specific to two genes rather than a single gene only, indicating the exact location of genes within the cluster.

Twenty-three LOS class specific primer pairs, as detailed in Table 1 in Appendix-I, were designed by using the WG or partial LOS biosynthesis gene region sequences of established reference *C. jejuni* strains. In addition to 23 primers pairs, two sets of control primers (1 and 2) were also designed for the amplification of two core LOS genes (*waaM* and *waaV*). These two genes are present in all *C. jejuni* LOS classes (Parker *et al*., 2005) and therefore, selected for designing the control primers. In LOS classes for which more than one reference *C. jejuni* strains were known previously, LOS gene nucleotide-level differences between the strains were considered to design the class-specific primers. Only that sequence range of a LOS gene was selected for designing primers which was present across all reference *C. jejuni* strains without any base variation.

The primer binding capacity as well as correct location of primers binding to the genomic DNA (gDNA) of reference *C. jejuni* strains were confirmed by looking at the size of PCR products. *C. jejuni* reference strains (n=9) with LOS locus types, A1, A2, B1, B2, C, E, F, G, H, O & P, were available and therefore these LOS classes specific primer pairs (A1, A2, B1, B2, C, F, 26EO, 28EP, 26HP, G) were tested (Figure 3.4). Primer pairs designed for other LOS classes could not be verified due to the unavailability of suitable reference *C. jejuni* reference strains.

Primer specificity for a single LOS class was confirmed by testing each designed primer pair against the collection of 9 *C. jejuni* reference strains belonging to different classes. Figure 3.5 represents that LOS class C specific primer pair was only specific to template DNA, which was derived from a LOS locus class C containing *C. jejuni* 11168 strain. It did not bind to gDNA of other *C. jejuni* strains including RM1048 (class A1), RM1556 (class A2), 101 (class B1), 81-176 (class B2), 81116 (class E), RM1221 (class F), RM1555 (class G) and 4031 (class P). Similarly, where possible, other primer pairs were also tested to determine their specificity for a single LOS class (data not shown). The primer pair was redesigned and retested if it showed specificity for more than one LOS class.
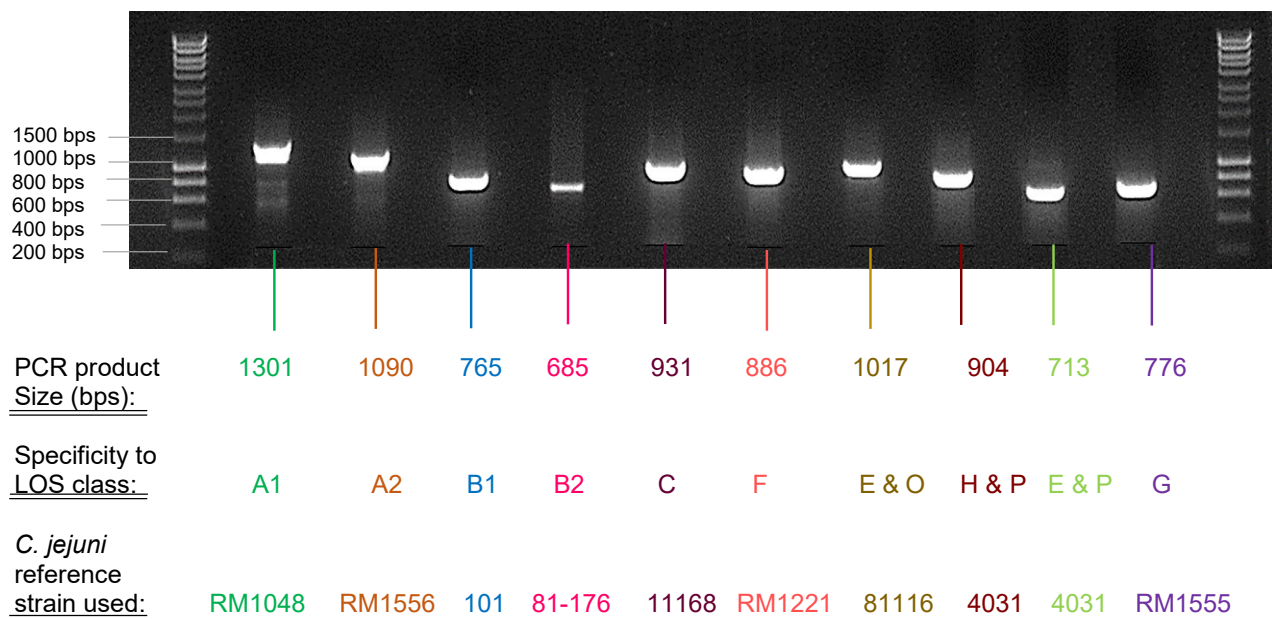
Figure 3.4: The PCR products of expected sizes amplified with *C. jejuni* LOS class specific primer pairs (A1, A2, B1, B2, C, F, 26EO, 26'HP, 28EP, and G).

The PCR product size in bp expected for LOS classes, LOS class/classes identifiable with primers, and *C. jejuni* reference strains used for PCR assay are specified. A 100 bps to 1kb hyperladder, on both sides of gel photograph, was used to estimate the size of DNA bands.
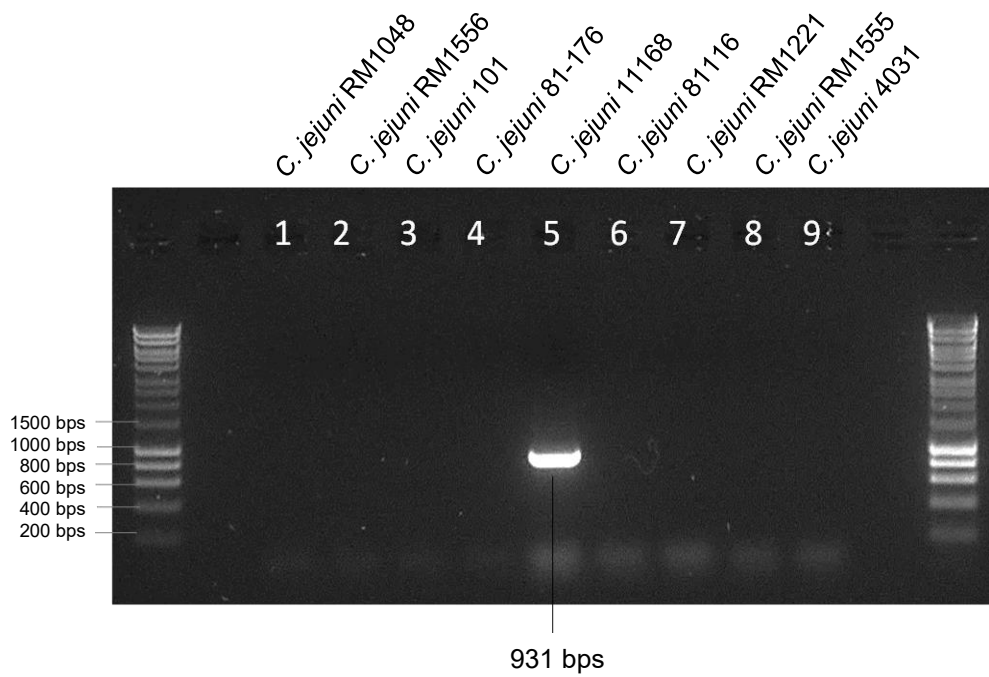
Figure 3.5: The specificity of primer pair/set C for LOS Class C related _C. jejuni_ reference strain (11168) when tested with other LOS classes associated _C. jejuni_ strains

A 100 bps to 1kb hyperladder, on both sides of gel, was used to estimate the size of DNA bands.

3.3.2. Genotyping of *C. jejuni* clinical isolates by using PCR

PCR with optimised and validated primer sets was performed to determine the LOS class of each clinical *C. jejuni* isolate. DNA from *C. jejuni* isolates was extracted and the origin of DNA only from *C. jejuni* strains was confirmed by performing PCR reactions (PCRs) with *waaM* and *waaV* LOS gene specific control primers.

The collection of 62 *C. jejuni* gDNA samples was then subjected to a number of PCRs sets; each set of PCRs contained one pair of primers to use against all gDNA samples. The results of PCRs performed with primer C and all gDNA samples have been shown in figure 3.6 as an example, where PCR products of expected size (931 bps) in reactions: 6, 12, 20, 22, 30, 33, 42, 44, 47, 50, 55 & 57, confirmed the presence of LOS locus type C respectively in *C. jejuni* CJ23, 104, 92717, CJ20, 118718, ME113262, CJ3111, 34218, 34565, 45283, 60319 and 54386. The remaining *C. jejuni* strains, negative for LOS Class C, did not show any band. A reference *C. jejuni* strain, if available, was included as a positive control in each PCR assay whereas a *C. jejuni* 11168Δ32-52 mutant strain lacking the LOS biosynthesis variable region (Marsden *et al*., 2009) was used as a negative control. A negative control for the PCR reaction was also included in each set of PCRs. In the same way, PCRs with all gDNA and other optimised primers, A1, A2, B1, B2, E, F, G, H, O, and P were performed (data not shown).

The reference strains with LOS classes D, I, J, K, L, M, N, Q, R, S, T, U, V, and W, were not available and therefore, the annealing temperature of the primer pairs specific to these classes could not be optimised. PCRs with such type of non-optimised primer pairs were carried out at annealing temperature 5 °C below the primers' theoretical melting temperatures. The presence of any specific or non-specific PCR product band was further confirmed by repeating the assay at a range of annealing temperatures (45°C to 65 °C).
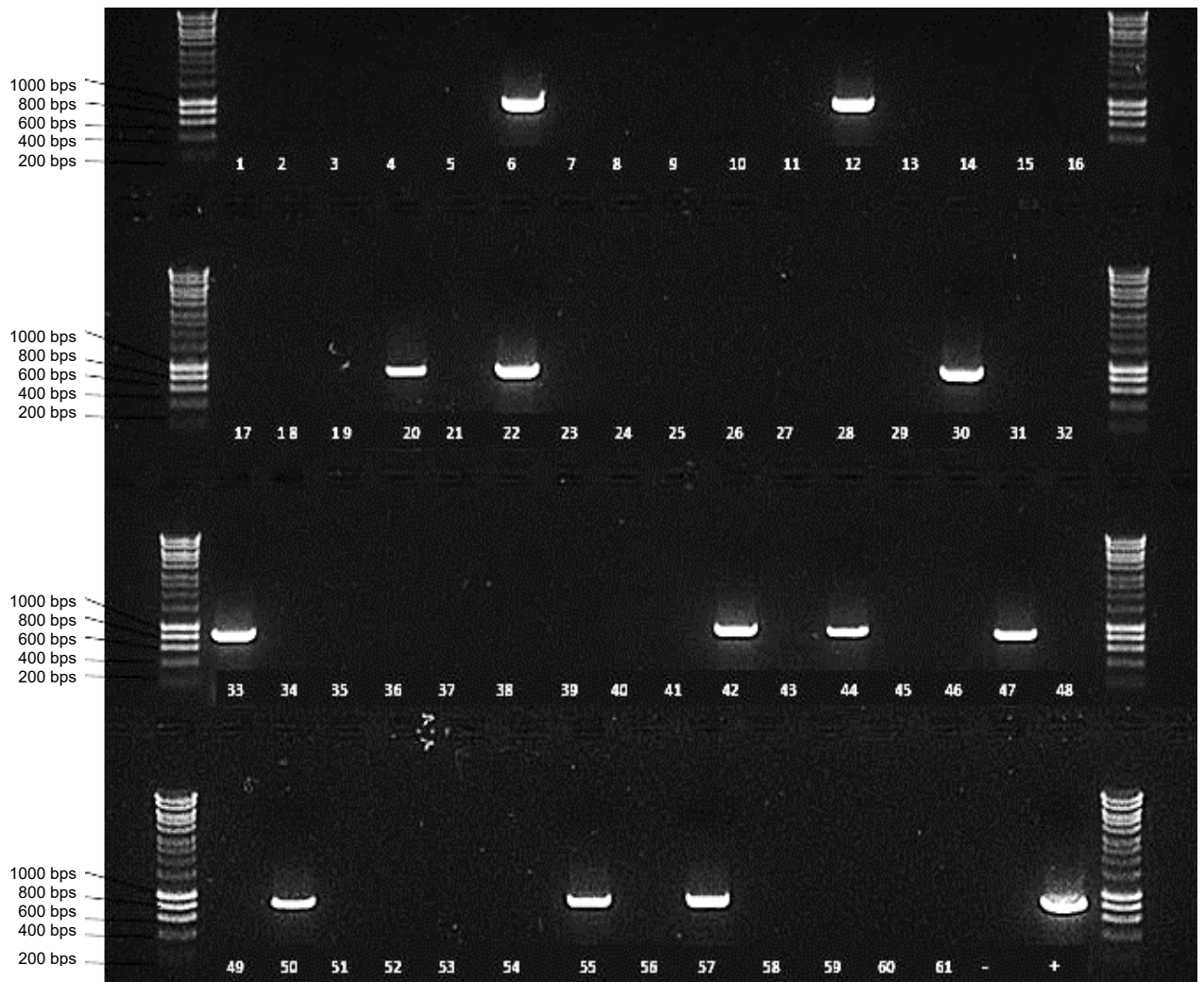
Figure 3.6: Typical results obtained by performing PCR genotyping (class C).

PCR reactions with all *C. jejuni* clinical strains' gDNA and LOS class C specific primer "C" were performed. Numbers from 1 to 61 represent individual *C. jejuni* isolates or their gDNA. *C. jejuni* 11168 strain as a positive control for PCRs (+). Negative control (a PCR reaction containing all reaction contents except gDNA) was used (-). PCR with *C. jejuni* mutant strain 11168Δ32-52 or sample 32 was an additional negative control. A 100bp to 1kb hyperladder was used to estimate the size of PCR products.

All PCRs were performed at least three times and the results obtained were also verified by Sanger sequencing of PCR products and analysis of sequenced data. The PCR and sequencing results have been summarised in Table 3.2, which were used to assign a specific LOS class to each *C. jejuni* isolate. 50 clinical *C. jejuni* isolates were LOS classified. 6 of 50 classified *C. jejuni* strains including 54386, S2, 92691, 118973, 118715 and 93133Y were positive for more than one LOS class. Typical PCR results of two *C. jejuni* strains, 54386 and 118973, positive for two LOS classes are illustrated in figure 3.7.

Table 3.2: Summary of *C. jejuni* LOS locus typing and PCR products' sequencing results

No.	Clinical *C. jejuni* strain	PCR with optimised primers										PCR with other primers	PCR product seq. identity (%)	Seq. compatible reference strain & LOS genes	Identified LOS Class
		A1	A2	B1	B2	C	26EO	F	G	26HP	28EP				
1	CJ10	+	-	-	-	-	-	-	-	-	-	-	99	RM1048 cgtA & cgtB	A1
2	CJ4	-	-	-	+	-	-	-	-	-	-	-	98	81-176 cgtA & cgtB	B2
3	CJ12	-	-	-	-	-	-	-	-	-	-	-			Unknown
4	CJ13	-	+	-	-	-	-	-	-	-	-	-	98	RM1556 cgtA & cgtB	A2
5	CJ18	-	+	-	-	-	-	-	-	-	-	-	97	RM1556 cgtA & cgtB	A2
6	CJ23	-	-	-	-	+	-	-	-	-	-	-	96	11168 neuA1 & neuC1	C
7	S1	-	-	-	-	-	-	+	-	-	-	-	95	RM1221 cgtD & waaV	F
8	S2	-	-	-	-	-	-	-	-	+	±	-			H/P
9	101	-	-	+	-	-	-	-	-	-	-	-			B1
10	102	-	-	-	-	-	-	-	-	+	+	-			P
11	103	-	-	-	-	-	+	-	-	-	-	-			O
12	104	-	-	-	-	+	-	-	-	-	-	-	98	11168 neuA1 & neuC1	C
13	105	-	-	-	-	-	-	-	-	+	-	-			H
14	106	-	-	-	-	-	-	-	-	+	+	-			P
15	Moulton	-	-	-	-	-	-	-	-	-	-	-			Unknown
16	1336	-	-	-	-	-	-	-	-	-	-	-			Unknown
17	92740	-	-	-	-	-	-	-	-	+	+	-	99	4031 Orf27 & Orf28	P
18	92691	-	+	+	-	-	-	-	-	-	-	-			A2/B1
19	92540	-	-	-	-	-	-	-	-	+	-	-			H
20	92717	-	-	-	-	+	-	-	-	-	-	-	98	11168 neuA1 & neuC1	C
21	93133Y	-	-	-	-	-	-	-	-	+	±	-			H/P
22	CJ20	-	-	-	-	+	-	-	-	-	-	-	97	11168 neuA1 & neuC1	C
23	93084N	-	-	-	+	-	-	-	-	-	-	-	99		B2
24	92661	-	-	-	-	-	-	-	-	+	+	-	98		P
25	112990	-	-	-	-	-	-	-	-	-	-	-			Unknown
26	118715	-	-	-	-	-	-	-	-	+	±	-	98	4031 Orf27 & Orf28	H/P
27	118973	-	+	-	+	-	-	-	-	-	-	-	94 99	RM1556 cgtA & cgtB 81-176 cgtA & cgtB	A2/B2
28	512	-	-	-	-	-	-	-	-	-	-	-			Unknown
29	121097	-	-	-	+	-	-	-	-	-	-	-	99	81-176 cgtA & cgtB	B2
30	118718	-	-	-	-	+	-	-	-	-	-	-	98	11168 neuA1 & neuC1	C
31	93941P	-	-	-	-	-	-	-	-	-	-	-			Unknown
32	AT large mutant	-	-	-	-	-	-	-	-	-	-	-			-ive control

#	Strain												%	Reference	Class
33	ME113262	-	-	-	-	+	-	-	-	-	-	-	99	11168 *neuA1 & neuC1*	C
34	ME112938	-	-	-	-	-	-	-	-	-	-	-			Unknown
35	ME112946	-	-	-	+	-	-	-	-	-	-	-			B2
36	ME112990	-	-	-	-	-	-	-	-	-	-	-			Unknown
37	ME113179	-	-	-	+	-	-	-	-	-	-	-	95		B2
38	ME113090	-	-	-	-	-	-	-	-	-	-	-			Unknown
39	751	-	-	-	-	-	-	-	-	-	-	-			Unknown
40	92649	-	-	-	-	-	-	-	+	+	-				P
41	36670	-	-	-	-	-	-	+	-	-	-	-	96		F
42	CJ3111	-	+	-	-	-	-	-	-	-	-	-			A2
43	37531	-	+	-	-	-	-	-	-	-	-	-			A2
44	34218	-	-	-	-	+	-	-	-	-	-	-	98	11168 *neuA1 & neuC1*	C
45	34806	-	-	-	+	-	-	-	-	-	-	-	98	81-176 *cgtA & cgtB*	B2
46	38625	-	-	-	-	-	-	-	-	+	+	-	99	4031 Orf27 & Orf28	P
47	34565	-	-	-	-	+	-	-	-	-	-	-	99	11168 *neuA1 & neuC1*	C
48	38608	-	-	-	-	-	+	-	-	-	-	-			E
49	44406	-	-	-	+	-	-	-	-	-	-	-			B2
50	45283	-	-	-	-	+	-	-	-	-	-	-	99	11168 *neuA1 & neuC1*	C
51	41999	-	-	-	+	-	-	-	-	-	-	-	99	81-176 *cgtA & cgtB*	B2
52	40973	+	-	-	-	-	-	-	-	-	-	-	98	RM1048 *cgtA & cgtB*	A1
53	47185	-	-	-	-	-	-	-	-	+	+	-			P
54	39864	-	-	-	-	-	-	-	-	+	+	-			P
55	60319	-	-	-	-	+	-	-	-	-	-	-			C
56	60238	-	-	-	-	-	-	-	-	-	-	-			Unknown
57	54386	-	-	-	+	+	-	-	-	-	-	-	98 / 98	81-176 *cgtA & cgtB*	B2/C
58	50702	-	-	-	-	-	-	+	-	-	-	-	92	RM1221 *cgtD & waaV*	F
59	59653	+	-	-	-	-	-	-	-	-	-	-	98	RM1048 *cgtA & cgtB*	A1
60	51585	-	-	-	-	-	-	-	-	+	-	-			H
61	92838	-	-	+	-	-	-	-	-	-	-	-			B1
62	92871	-	-	+	-	-	-	-	-	-	-	-			B1
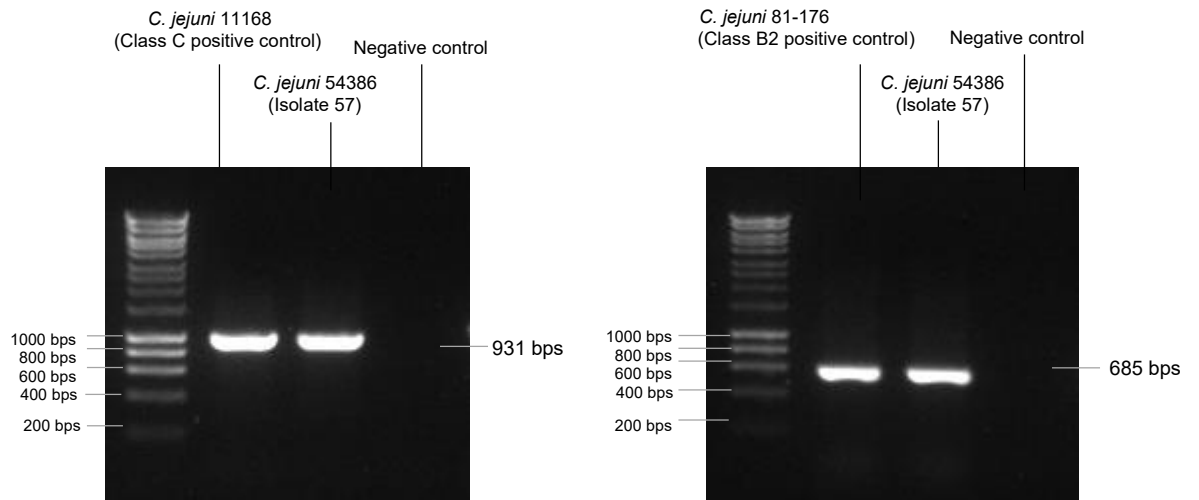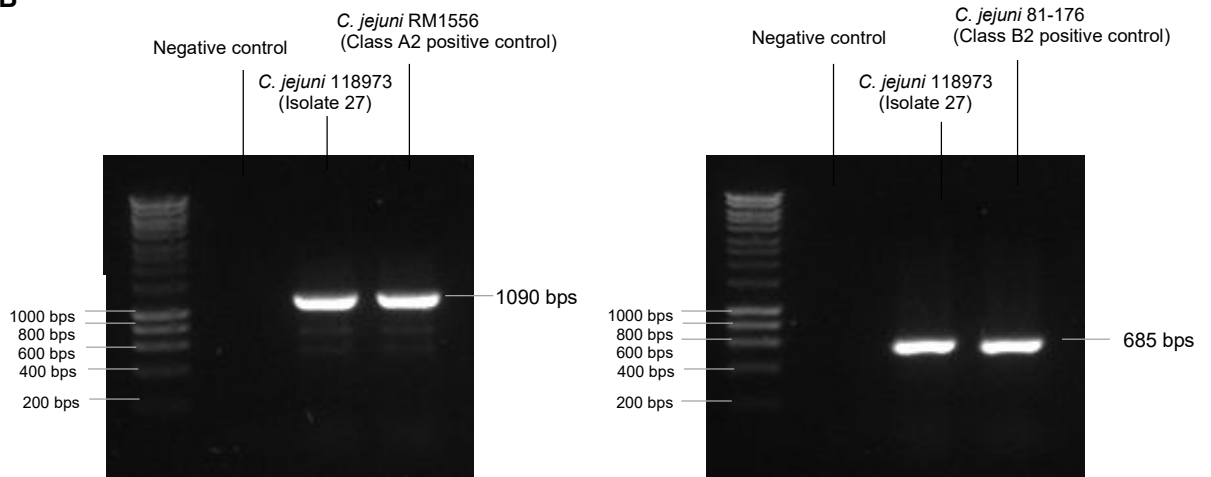
Figure 3.7: The PCR positive results for *C. jejuni* isolates, 57 and 27, which were assigned to more than one LOS class.

(A). *C. jejuni* 54386 (isolate 57) was positive for LOS classes, C and B2.
(B). *C. jejuni* 118973 (isolate 27) was positive for LOS classes, A2 and B2.

C. jejuni 11168 (class C), *C. jejuni* RM1556 (class A2) and *C. jejuni* 81-176 (class B2) were used as positive control for PCRs. PCRs containing all reaction contents except gDNA were used as negative controls. A 100bp to 1kb hyperladder was used to estimate the size of PCR products.

3.3.3. Evaluation of the *C. jejuni* LOS loci distribution at clinical level

Figure 3.8 presents the distribution of *C. jejuni* LOS locus classes (A-W), subclasses (A1, A2, B1, B2) and LOS groups (1-4) in a *C. jejuni* collection, built up from *C. jejuni* infection cases in Northampton, UK. 28 (56%) *C. jejuni* strains were identified as belonging to LOS class A1 (n=3; 6%), A2 (n=4; 8%), B1 (n=3; 6%), B2 (n=8; 16%), C (n=10; 20%) and therefore were members of LOS group 1. 16 of 50 (32%) of classified strains were positive for either class E (n=1; 2%), H (n=3; 6%), O (n=1; 2%) or P (n=8; 16%), and therefore, belonged to LOS group 2. LOS class P with 16% was the most frequently represented class within group 2. Only 3 (6%) *C. jejuni* strains were positive for LOS group 3 related class F. No *C. jejuni* strain associated with other classes of LOS group 3 (D, K, Q, N, I, S, J) was identified. In addition, *C. jejuni* strains with LOS group 4 related classes, L, G, T, & U, were also not observed among the clinical *C. jejuni* isolates. The hierarchy of LOS groups was group 1 (62%) > group 2 (32%) > group 3 (6%) > group 4, indicating the occurrence of group 1 related *C. jejuni* strains in a high proportion at clinical level. Figure 3.9 further illustrates that the hierarchy of LOS locus ABC classes within group 1 was class B (n=11) > class C (n=10) > class A (n=7), as well as, high frequency of class P (n=8) and F (n=3) respectively in group 2 and group 3.
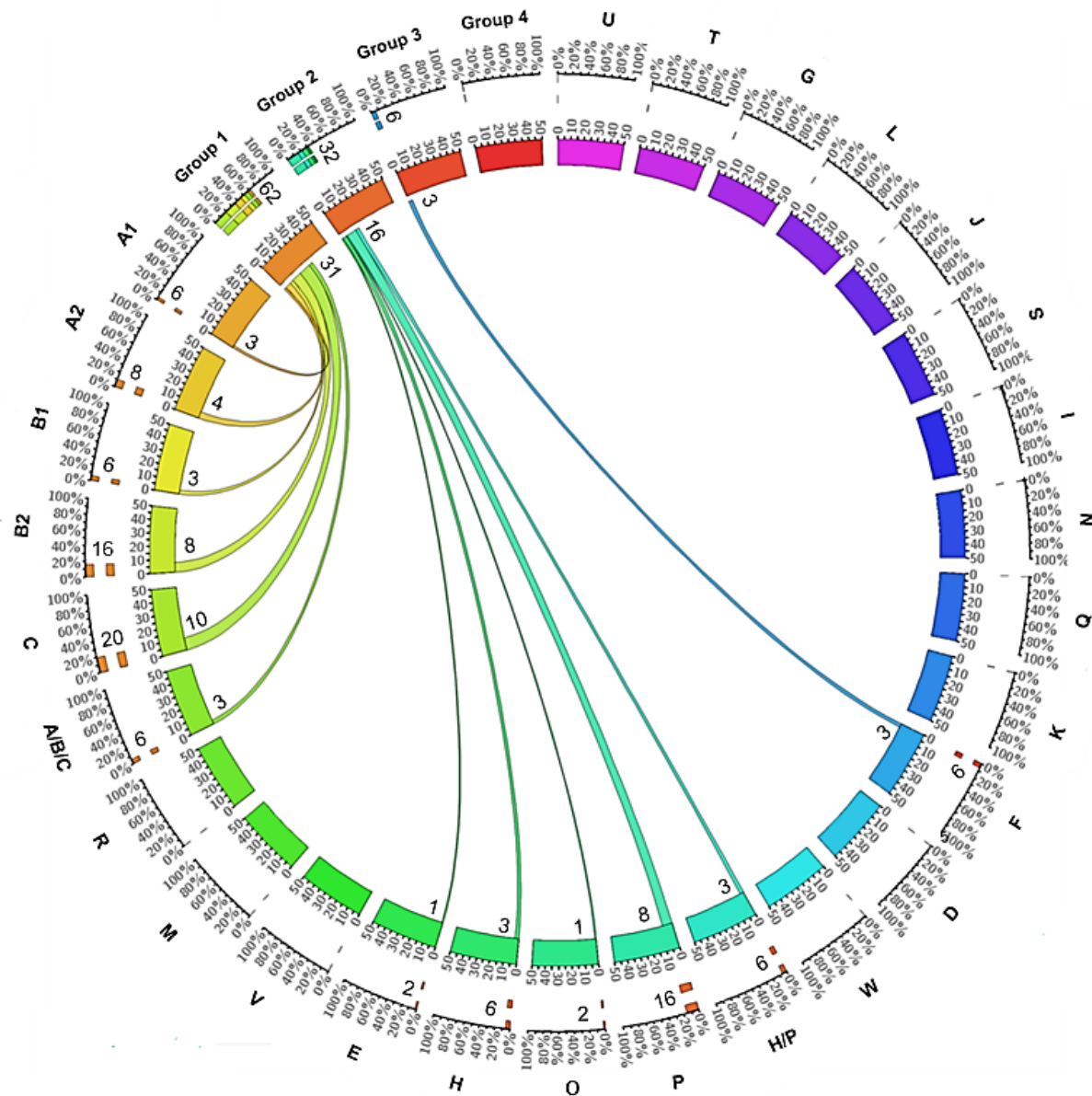
Figure 3.8: The distribution of *C. jejuni* LOS locus classes (A-W), subclasses (A1, A2, B1, B2) and LOS groups (1-4) from clinical isolates.

Each segment of inner circle specify the total number of *C. jejuni* isolates (50) used for the PCR based typing assay. The frequency of *C. jejuni* isolates classified for each particular LOS class/group is mentioned in numbers (n out of 50) on the top of each inner circle segment and presented with ribbon width. The frequency of a *C. jejuni* LOS class/group in percent is mentioned with each outer circle segment and represented by the orange or colourful blocks. Ribbon ends link each *C. jejuni* LOS class to its related LOS group.
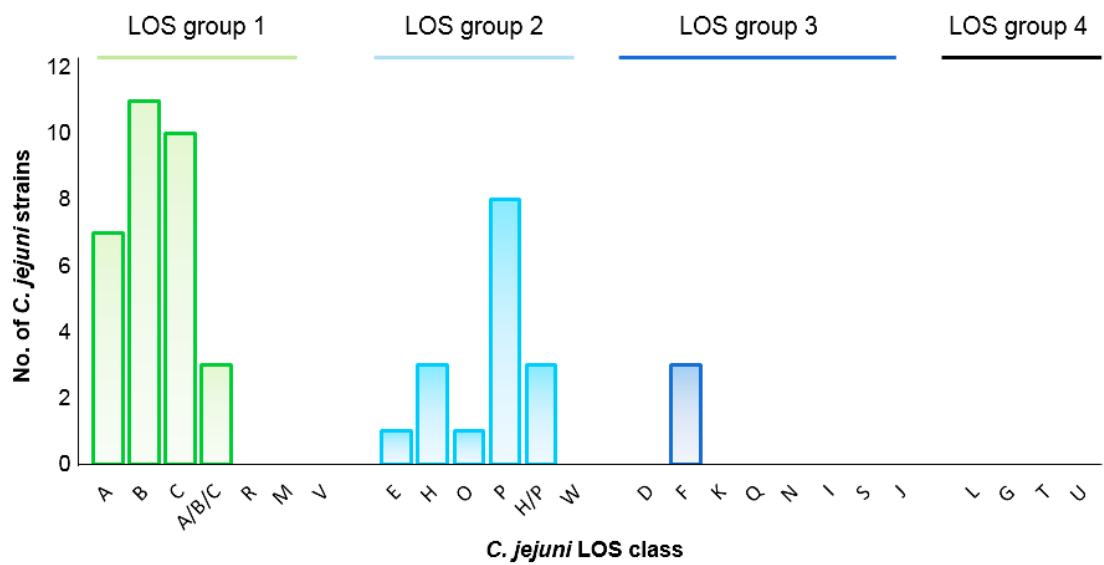
Figure 3.9: The distribution of *C. jejuni* LOS locus classes (A-W) within the LOS groups (1-4) from clinical isolates

The class B in LOS group 1 (green), class P in LOS group 2 (light Blue) and class F in LOS group 3 (dark Blue) were highly prevalent. The hierarchy of LOS classes within the Group 1 was class B > class C > class A.

3.3.4. Analysis of LOS core of *C. jejuni* clinical isolates

LOS samples extracted from *C. jejuni* clinical isolates were examined on 16% silver stained SDS-PAGE gels. Figure 3.10 (A) represents that CJ10 (class A1), CJ18 (class A2), 59653 (class A1), 37531 (class A2), and 40973 (class A1), and all *C. jejuni* strains with type A had LOS of almost same size (~12 kDa). Similarly, *C. jejuni* stains, 101 (class B1), ME113179J (class B2), 34806 (class B2), and 41999 (class B2) showed the LOS of almost same size (~13 kDa) and comparable to the LOS of class B reference strain, *C. jejuni* 81-176 (Figure 3.10 B). The LOS from class C associated strains (11168, 34565, 60319, 34218, 45283, CJ20, 92717, 104, CJ23), classes E, H, O & P linked strains (103, 51585, 92540, 47185, 38608, and 92649), and class F containing strains (RM221, 36670, 60238) are also presented respectively in figure 3.10 C, D & F. These results suggested that *C. jejuni* strains linked to the same LOS locus class or type express LOS structures of almost equal molecular weight on their cell surfaces and the size of LOS does not change even with the possession of different LOS locus subclasses.

The LOS structures were variable within different classes of a same LOS group. For instance, *C. jejuni* 92540 type H LOS and *C. jejuni* 92649 type P LOS had different sizes, although these both classes belonged to the same LOS group (Group 2; Figure 3.10 E). These results indicate that LOS size can vary between strains belonging to different LOS classes and do not significantly vary between strains of a same LOS class. The LOS structures could be variable even between the strains of a same class but these minor structural differences were unable to detect on SDS-PAGE gels.

A ~12 kDa
(LOS class A)

B ~13 kDa
(LOS class B)

C ~11-12 kDa
(LOS class C)

D ~14-15 kDa
(LOS classes E, H, O, & P)

E ~15 kDa
(LOS class H)
~14.5 kDa
(LOS class P)

F ~12 kDa
(LOS classes F)
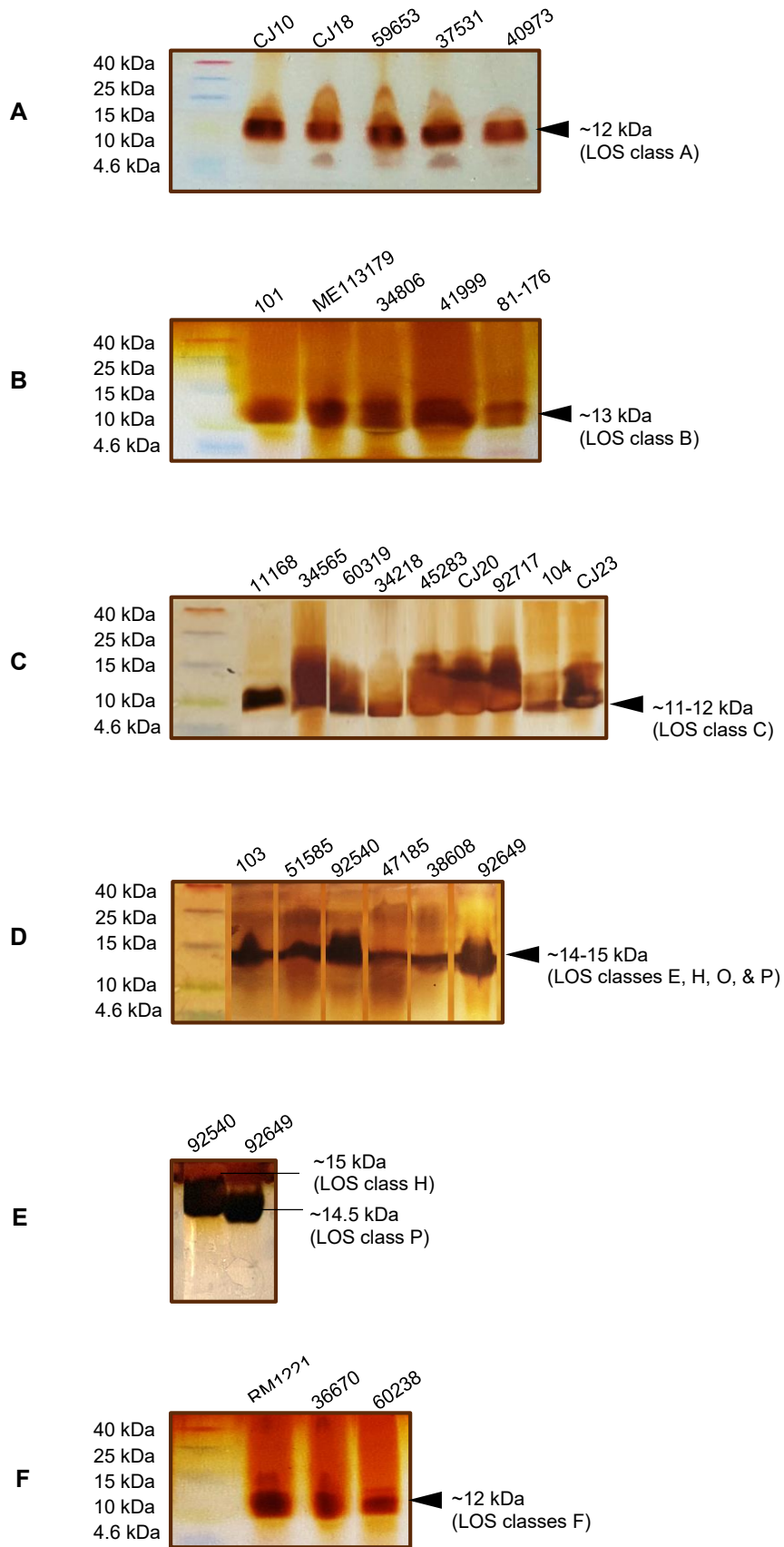
Figure 3.10: Analysis of LOS by SDS PAGE. LOS extracts from the clinical *C. jejuni* strains on silver stained 16% (v/v) SDS-PAGE gels. Multi-colour low range protein ladder (1.7- 40 kDa) was used to predict the size of LOS bands.

(A): *C. jejuni* strains, CJ10 (class A1), CJ18 (class A2), 59653 (class A1), 37531 (class A2), 40973 (class A1), showed LOS of ~12 kDa.

(B): *C. jejuni* strains, 101 (class B1), ME113179J (class B2), 34806 (class B2), 41999 (class B2), showed LOS of ~13 kDa, comparable to the LOS of class B reference strain, *C. jejuni* 81-176.

(C): *C. jejuni* strains including 34565, 60319, 34218, 45283, CJ20, 92717, 104, CJ23 showed LOS of ~11-12 kDa, comparable to the LOS of class C reference strain, *C. jejuni* 11168.

(D): *C. jejuni* strains, 103 (class O), 51585 (class H), 92540 (class H), 47185 (class P), 38608 (class E), and 92649 (class P), showed LOS of ~14-15 kDa.

(E): Difference in type H (~15 kDa; *C. jejuni* 92540) and type P (~14.5 kDa; *C. jejuni* 92649) LOS.

(F): *C. jejuni* 36670 and *C. jejuni* 60238 showed LOS of ~12 kDa, comparable to class F reference strain, *C. jejuni* RM1221.

3.4. Discussion

LOS classes from A through F were known for *C. jejuni* initially (Gilbert *et al*., 2002; Parker *et al*., 2005). Karlyshev *et al*. (2005) primarily categorised these known *C. jejuni* LOS classes into four groups and included LOS classes A, B, C in Group 1, LOS class E in Group 2, LOS class D and F in Group 3, and LOS class G in Group 4. Later, Parker *et al*. (2008) identified 11 more *C. jejuni* LOS classes including I, J, K, L, M, N, O, P, Q, R, and S. Subsequently, Richard *et al*. (2013) found *C. jejuni* strains with novel LOS loci and established 4 more LOS classes including T, U, V and W. The novel LOS loci identified in the latter two studies have never been classified into the LOS groups. To better understand the prevalence of *C. jejuni* LOS groups and groups related LOS classes, the present study has simplified the LOS classification system. Figure 3.11 is a representation of a simplified *C. jejuni* LOS locus classification system, where various already known LOS classes have been added into the pre-established LOS groups on the basis of sharing the similar LOS biosynthesis gene content. Group 1 includes all those LOS locus types, A, B, C, R, M and V, which contain the sialic acid synthesis genes whereas the other three groups have LOS loci with no sialic acid genes. Based on the similarity of LOS loci (H, O, P and W) to locus E, these four classes are added into group 2. Further, K, Q, N, I, J, and S in LOS group 3 and L, G, T, and U in LOS group 4 are assimilated.
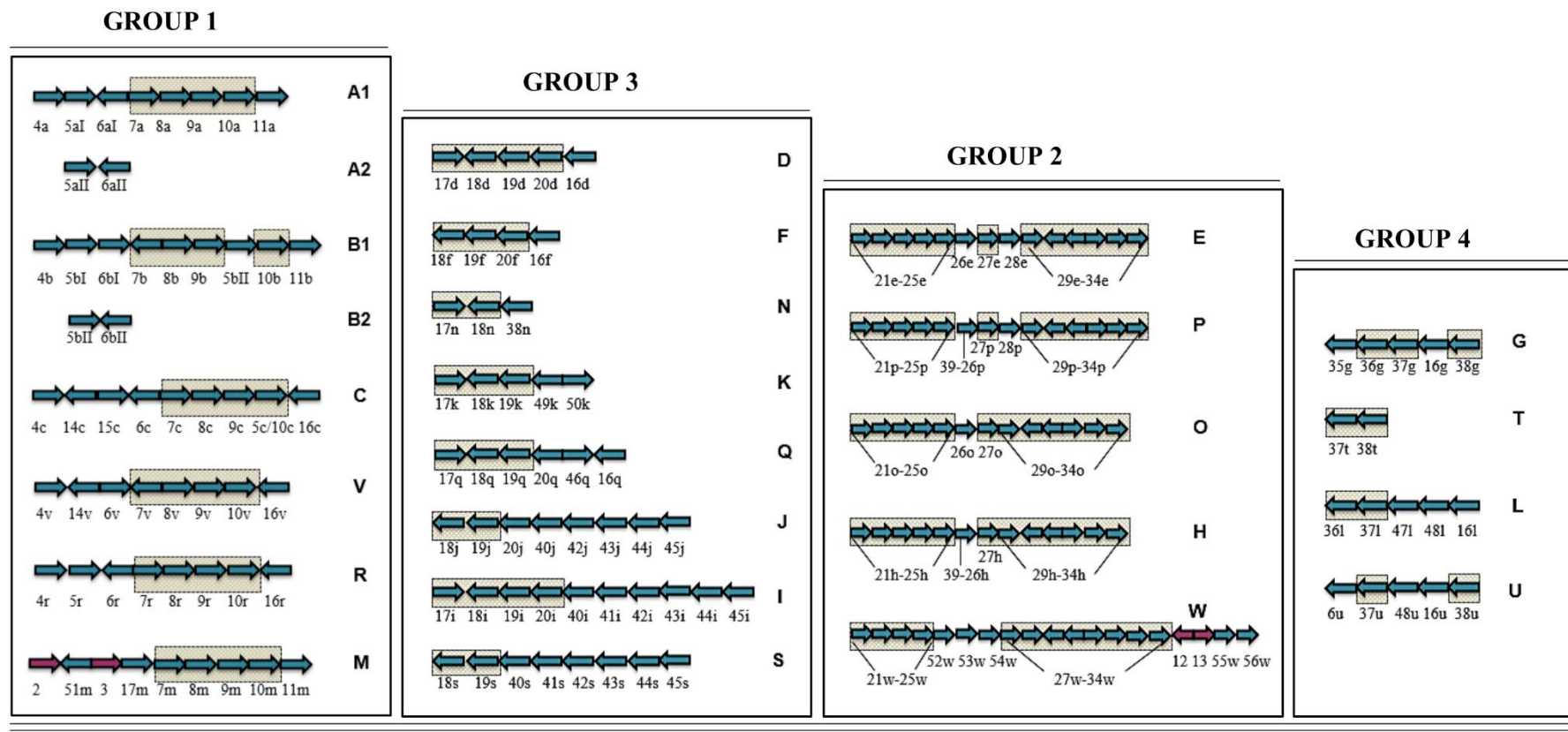
Figure 3.11: Simplified *C. jejuni* LOS locus classification system.

LOS classes, classified into already established four groups on the basis of sharing the similar LOS biosynthesis gene content (highlighted in grey coloured boxes). Genes are numbered according to the Parker *et al*. (2005) numbering system. Pink arrows: commonly present genes in all LOS classes; Blue Arrows: variable LOS genes. Arrow direction represents the direction of gene transcription (Adapted from Karlyshev *et al*., 2005).

In the present study, 6% of *C. jejuni* clinical strains possessed LOS locus subclass B1 and 16% had subclass B2. Altogether, 22% (n=11) of the analysed 50 *C. jejuni* clinical isolates were found with LOS class B, indicating the class B as the most common LOS class among these isolates. This was in line to a previous study (Islam *et al*., 2009), who observed high prevalence of *C. jejuni* LOS locus class B in enteritis patients [46% (n=18) of analysed 39 *C. jejuni* strains] and GBS patients [28% (n=2) of analysed 7 *C. jejuni* strains]. Another recent study also described the class B as the most frequent class in enteritis patients after identification of 40% (n=68) of 152 *C. jejuni* strains with LOS locus class B (Islam *et al*., 2018).

In the current study, 20% of analysed *C. jejuni* strains had LOS class C, specifying the class C as the second most abundant LOS type in the clinical cohort. The high frequency of LOS class B (22%) higher than class C (20%), observed in this study, is concordant with *C. jejuni* LOS locus class distribution data of Quinones *et al*. (2007), who LOS typed poultry *C. jejuni* strains using microarray and class-specific probes and reported class B (37%) > class C (16%). Many other studies have described the LOS class C as the major class of *C. jejuni* LOS biosynthesis locus (Marsden, 2007; Ellström *et al*., 2013, 2016). However, in comparison to the high prevalence of LOS class C (42%) in clinical isolates in Sweden (Ellström *et al*., 2016), a very small number of clinical strains (2%) in Bangladesh had association with LOS locus C (Islam *et al*., 2014). Most of the GBS-related *C. jejuni* strains in Bangladesh and China possessed the LOS locus class A rather than class C (Islam *et al*., 2009; Jiang *et al*., 2010; Islam *et al*., 2014), suggesting that *C. jejuni* LOS class distribution may vary geographically.

According to the current findings, the trend of LOS classes within group I was class B (22%) > class C (20%) > class A (14%), which was different from previously found trends of these three classes in Marsden. (2007) [class C (19%) > class B (10%) > class A (2%)] and Ellström *et al*. (2016) [class C (42%) > class B (12%) > class A (3%)]. However, a general trend of LOS groups distribution at clinical level (group 1 > group 2 > group 3 > group 4) observed in the current study was comparable to Marsden. (2007), Quinones *et al*. (2007)*,* Ellström *et*

al. (2016) and Islam *et al*. (2018). A comparison of current findings of *C. jejuni* LOS loci distribution with previous studies has been presented in figure 3.12 (A). In this comparison, it is noticeable that the hierarchy of LOS group prevalence remains similar among both human and poultry derived *C. jejuni* isolates. This also indicates that the LOS locus type A in GBS patients and B and C genotypes in clinical/enteritis patients are highly predominant. A representation of LOS ABC types' combined frequency in different populations of *C. jejuni* isolates (which may be humans or poultry), observed in current and previous studies, is also given in figure 3.12 (B). This indicates that approximately 50–65% strains in most of the clinical/enteritis associated *C. jejuni* populations and more than 80% strains in GBS linked *C. jejuni* populations, belong to LOS classes A, B or C.

Previous studies have described that a high prevalence of group 1 related ABC classes might happen due to the distinctive ability of the group 1 related strains to synthesise the sialylated LOS structures, which are beneficial for pathogenesis (Gilbert *et al*., 2002; Parker *et al*., 2005; Habib *et al*., 2009). For instance, LOS class A with the appearance of GM1-like LOS sub-structures is more likely to develop GBS whereas class B with GQ1b-like LOS sub-structures are linked with MFS development (Godschalk *et al*., 2007; Islam *et al*., 2014). According to the results of the present study, B2 and C are the most common LOS types in this population of *C. jejuni*. It might be due to this fact that both of LOS locus types exhibit high phase variations at the gene sequence level and produce heterogenous ganglioside mimics, advantageous for pathogenesis. Previously, phase variations in class B2 containing *C. jejuni* strain, 81-176 (altering $GM_2 \rightarrow GM_3 \rightarrow GD$ in LOS) and class C possessing *C. jejuni* strain, 11168 (modifying LOS $GM_1 \rightarrow GM_2$) have been observed (Guerry *et al*., 2002; St Michael *et al*., 2002). It is noticeable that the rate of GBS and MFS in *Campylobacter* infected patients is very low (Nachamkin *et al*., 2002; Mortensen *et al*., 2009) despite high predominance of GBS/MFS associated LOS classes A, B, and C, at the clinical level, supporting the fact that some other factors in addition to LOS structures contribute in the development of these neural diseases.

A

LOS Group I
- A
- A1
- A2
- B
- B1
- B2
- C

LOS Group 2
- E/O
- O
- E
- H/P
- P
- H

LOS Group 3
- D/F
- D
- F

| ENT *n*= 21 | GBS *n*= 17 | ENT *n*= 36 | GBS *n*= 15 | Chick *n*= 19 | Clinical *n*= 48 | Clinical *n*= 40 | Chick *n*= 76 | ENT *n*=163 | BB infection *n*= 73 | Chick *n*= 50 | ENT *n*= 59 | Chick *n*= 104 | ENT *n*= 152 | Clinical *n*= 50 |

Frequency of LOS Classes (%)

Godschalk *et al.* (2004)
Parker *et al.* (2005)
Quinones *et al.* (2007)
Marsden, 2007
Habib *et al.* (2009)
Ellström *et al.* (2013)
Ellström *et al.* (2014)
Islam *et al.* (2014)
Ellström *et al.* (2016)
Islam *et al.*, 2018
Current Study (2015-2017)

113

Figure 3.12: (A) A comparison of current findings of *C. jejuni* LOS loci distribution with previous studies. (B) The combined frequency of LOS types A, B, and C, present in different *C. jejuni* populations (human or poultry)

The combined frequency of classes ABC present in the range of 50–65% is represented by a green bubble. The combined frequency of classes ABC lying out of the range of 50–65% is illustrated by a grey bubble. The overlapping of green and grey bubbles shows that both bubbles belong to the same study. ENT: *C. jejuni* isolates from enteritis patients; GBS: *C. jejuni* isolates from GBS patients; Chick: *C. jejuni* isolates from chicken. BB infection: *C. jejuni* isolates from patients with Blood Borne infections.

Interestingly, other LOS classes of group 1 (R, V and M), also contain genes for the biosynthesis of sialylated LOS structures (Parker *et al*., 2008; Richard *et al*., 2013), but they were not found in the collection of *C. jejuni* clinical isolates. It might be because class R containing strains do not produce GBS related ganglioside mimics (GM1, GM2, GD1, GD2) and class M does not produce the ganglioside mimicking LOS structures at all despite having sialic acid biosynthesis genes (Houliston *et al*., 2011).

LOS group 2 was the second most predominant group with a high prevalence of LOS class P (16%). However, LOS class H has been reported previously as the most abundant class within the group 2 (Quinones *et al*., 2007, Ellström *et al*., 2016). The fact is both LOS class P and H share the almost similar gene content except for two LOS biosynthesis genes (Orf 26' and Orf28) (Parker *et al*., 2008; Jiang *et al*., 2010) and therefore, a few studies have never considered these classes as two distinct classes (Habib *et al*., 2009; Islam *et al*., 2014). To clearly differentiate between the LOS group 2 related classes, three sets of primers 26EO (for LOS class E and O), 26'HP (for class H and P) and 28EP (for class E and P) were designed in this study. Despite designing of class specific primers and testing these primers with reference strains, 81116 and 4031, a few clinical *C. jejuni* gave a mixed signal for LOS class H and P. In this case, PCR products obtained with class H and P specific primers were further sequenced and sequencing data was interpreted to assign a single class to each strain.

LOS group 2 loci related strains appeared in abundance among a small population of *C. jejuni* from the enteritis cases, although they lack the sialic acid biosynthesis genes and are likely to produce the non-sialylated LOS structures (Parker *et al*., 2008; Poly *et al*., 2008). This might be due to the presence of sialic acid biosynthesis LOS genes somewhere else in the genome of these strains rather than specifically in the LOS locus. This notion is evidenced by the presence of ganglioside-like structures other than GM1 or GQ1b in LOS locus E associated *C. jejuni* strains (Godschalk *et al*., 2004). Subsequently, only 6% of *C. jejuni* strains were identified with the group 3 related LOS class F which was relatively similar to the findings of Quinones *et al*. (2007). Six *C. jejuni* strains were positive for more than two LOS classes which can take place due to co-

infection in patients with multiple *C. jejuni* strains. The co-infection occurrence with multiple *C. jejuni* strains has been previously observed in GBS patients (Godschalk *et al.*, 2006). Another reason could be occurrence of interstrain or intrastrain LOS gene recombination during infection as it has been observed previously by Gilbert *et al*. (2004) and Phongsisay *et al*. (2006).

C. jejuni strains with the same LOS locus type expressed LOS structures of almost equal molecular weight. These results were identical to previous findings where two *C. jejuni* strains, 331 and 421, both with LOS class C had the LOS structures of similar size (Semchenko *et al.*, 2012). However, LOS were variable between different classes of the same group, such as, LOS class H and P both belonged to the same group but their LOS appeared with slightly different molecular weights. The sizes of *C. jejuni* class H related LOS (~15 kDa) and class P linked strain LOS (~14.5 kDa) were found similar to class H and P associated LOS sizes reported in a previous study (Jiang *et al.*, 2010), indicating that the size of LOS structures may vary with the change of locus type or even a single LOS biosynthesis gene of LOS locus. The work related to the LOS phenotypic characterisation was carried out by SDS-PAGE, which was not sufficient to fully explore the LOS structure and establish its link with LOS locus genotype. Another limitation of this work is that, the final LOS structure cannot be truly predicted by a particular LOS locus type as minor sequence variation in LOS biosynthesis genes can greatly impact the synthesis of LOS structure (Parker *et al*., 2005).

3.5. Conclusion

This study is the first description of the abundance of LOS subclasses (B2 > A2 > B1 > A1) in the clinical isolates of *C. jejuni*. The hierarchy of LOS group prevalence was group 1 > group 2 > group 3 > group 4 and trend of LOS classes within the group 1 was class B > class C > class A. *C. jejuni* isolates were most frequently assigned to group 1 related LOS locus types, B2 and C, which can happen due to the fact that these two loci have the potential of varying the human ganglioside-like LOS structural epitopes. Compared to the high proportion of *C. jejuni* isolates with LOS loci (A, B and C), the prevalence of strains with the other three group 1 related and sialic acid biosynthesis genes containing loci (R, V and M) was almost negligible. The reasons for this remain unclear, but it might be due to the lack of GM1-like ganglioside mimicking LOS structures in R and M loci, indicating the importance of LOS in *Campylobacter* pathogenesis. Finally, this study extends our understanding of the LOS locus classification system and represents an overview of the frequency of various LOS locus genotypes in clinical *C. jejuni* isolates.

CHAPTER 4

In Silico Analysis of the Genetic diversity of the Lipooligosaccharide Biosynthesis Locus in *C. jejuni* and *C. coli*

4.1. Introduction

4.1.1. The use of bioinformatics in the present study

Initially, the term bioinformatics was defined as "the computational methods for comparative analysis of genome data" (Hogeweg, 2011). This definition also summarises the application of bioinformatics in the current study, where computational tools were applied to compare the gene sequences and measure the similarity between them. The process of comparing the gene sequences or finding a series of similar character patterns with the same arrangement in the compared sequences is known as sequence alignment. The sequence alignment may be pairwise (performs between two sequences at a time) or multiple (performs between more than two sequences) (Pearson and Lipman, 1988).

GenBank is comprehensive and the most common database which contains nucleotide sequences from more than 260, 0000 species and is publicly available around the world at no cost over the Internet (Benson *et al*., 2007). *Campylobacter* WG sequences available in GenBank, submitted either by the individual laboratories or large scale sequencing projects, were selected for the current study.

4.1.1.1. Multiple alignment using fast Fourier transform (MAFFT)

A bioinformatic tool, MAFFT, at the MAFFT server <http://mafft.cbrc.jp/alignment/server/large.html>, was first introduced in 2002 to perform multiple sequence alignments (Katoh *et al*., 2002). The following 3 steps explain the procedure in detail which are implemented by MAFFT to perform a sequence alignment using the Needleman–Wunsch and Fast Fourier Transform (FFT) algorithms.

Step 1 - Building a guide tree: MAFFT Needleman–Wunsch algorithm initially look for the k-mers (pairs of identical 6 nucleotide long strings), determines the pairwise percent identity score, and measures the similarity between k-mers (Needleman and Wunsch, 1970). The approximate number (Tij) of k-mers shared by every pair of input sequences (sequence i and sequence j) is counted. Subsequently, k-mer numerical value for each pair is converted into a distance value (distance between two sequences in a single pair; Dij) using the following formula and mathematical calculations (Kotah *et al*., 2002).

$$Dij = 1 – [\ Tij/\min (Tii,\ Tjj)\ ]$$

An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) guide tree is constructed using the calculated distance values or a matrix of pairwise distances between all sequences (Lassmann and Sonnhammer, 2005; Katoh and Toh, 2008). The first two nodes (let's suppose A & B) of the tree are joined based on the shortest distance value and represent the two closely related or similar sequences. Further new nodes are defined and established by the MAFFT to build a guide tree. A new node (for example, node C) is defined with the smallest value of the remaining distance values. The sequence at node C will have high similarity to the sequences incorporated at node A and B. Node C is established within the tree at the specific distance from the adjacent two nodes, A and B. This specific distance is determined by the arithmetic mean value of distance values of A and B. In this manner, the process of defining and establishing the nodes eventually construct a tree. The initial tree formed in this step is known as a guide tree (Kotah *et al*., 2002; Katoh and Toh, 2007, 2008).

Step 2 - Progressive alignment based on the guide-tree branching: The guide tree dictates the order of further pairwise alignments. Once the guide tree is built, multiple alignment progressively starts building up, where multiple sequence alignment is assembled by accumulating the sequences to the alignment one by one following the branching order of guide tree. Branches and alignments, both are developed first between the most closely related sequences and then, distant ones are gradually added (Kotah *et al*., 2002; Katoh and Toh, 2008; Yamada *et al*., 2016).

Step 3 - Group-to-group alignment based on FFT and refining: MAFFT has a function of group-group alignment, similar to the profile-profile alignment of Clustal W, where a group or profile is a subset of multiple alignments. MAFFT incorporates the fast Fourier transform (FFT) algorithm in addition to dynamic programming or Needleman–Wunsch algorithm (Thompson *et al*., 1994; Katoh *et al*, 2002). Within the group-group alignment, each pair of aligned sequences is converted to a 2D wave (containing frequencies of nucleotides {A, C, G, T}) and correlation between two waves is rapidly computed using FFT. A sequence pair with longer length and maximum identity gives a high signal or peak in comparison to a less similar and short length sequence. In addition to measuring the sequences similarity, the FFT algorithm also performs an approximate distance calculation which are further used to complete a phylogenetic tree. This FFT phase is fast and takes less time to complete in comparison to the dynamic programming phase (first two steps) (Katoh *et al*., 2005; Katoh and Toh, 2008).

4.1.1.2. Using MAFFT for the classification of *C. jejuni* LOS locus

The Galaxy web based platform <http://usegalaxy.org> consists of different bioinformatics related tools to access all these simultaneously on the same platform and to produce reproducible results (Goecks *et al*., 2010; Afgan *et al*., 2018). In this study, MAFFT (version 7) in combination with other bioinformatics tools (as demonstrated in figure 4.1) was used at the Galaxy platform rather than utilising it through its own server. MAFFT alignment tree is built by this workflow as its output, where unclassified sequences belonging to a particular group are clustered together and within this cluster tree branches link each unclassified sequence to an individual, reference LOS locus type.
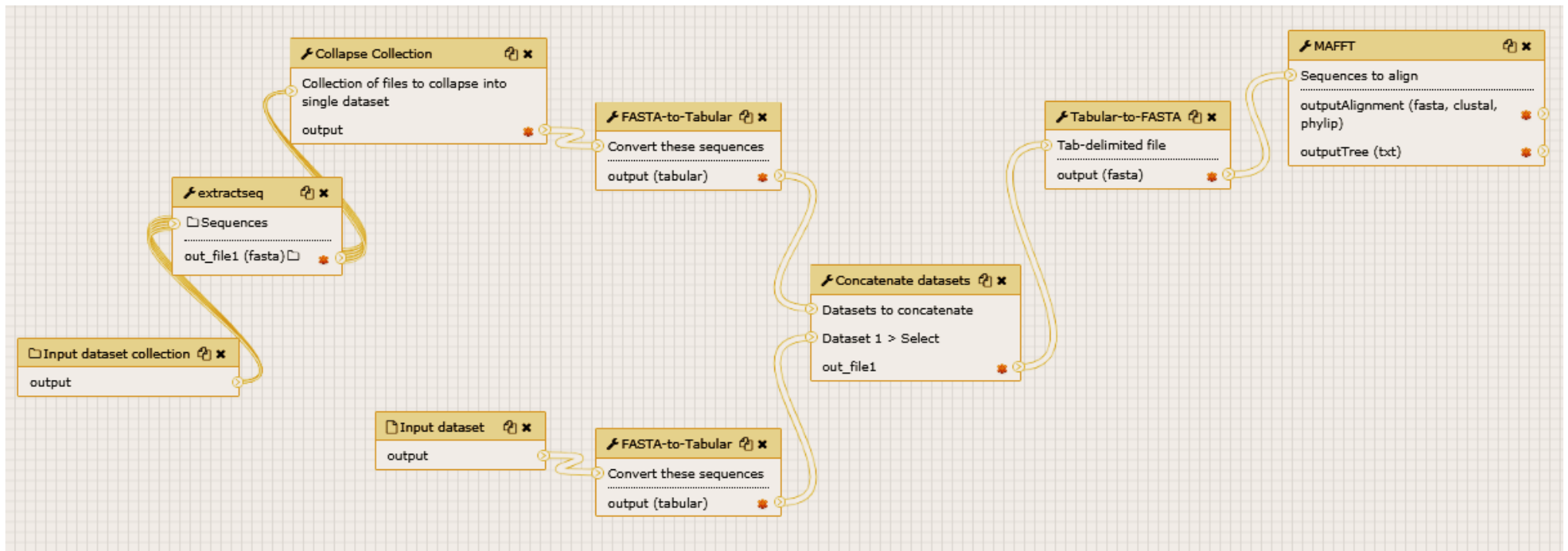
Figure 4.1: A Galaxy workflow designed for the classification of *C. jejuni* LOS locus

This workflow estimated and extracted a ~65 kb specific sequence (1064895-1130000) from every sequence in the collection of *C. jejuni* unclassified WG sequences. The extracted sequences (predicted to contain the LOS locus sequences) were collapsed. A set of collapsed sequences and a set of LOS class sequences (reference or previously known) are concatenated in tabular format and aligned in FASTA format using MAFFT.

The bioinformatics based approach has been used previously for the LOS genotyping of *Campylobacter* sequences (Culebro *et al*., 2018). In this study, *C. jejuni* and *C. coli* sequences publically available in European Nucleotide Archive were mapped against a set of reference LOS genes using the ReMatCh framework v3.2 <https://github.com/B-UMMI/ReMatCh>. This framework extracted the raw reads in fastq format, mapped the reads of the LOS locus against the reference using Bowtie237, and performed the variant calling using Samtools and ReMatCh Single Nucleotide Polymorphism call criteria. A locus was considered to be present in an unclassified sequence if 70% of the target reference sequence with ≥80% nucleotide identity aligned with the query sequence (Culebro *et al*., 2018).

4.1.1.3. Mega Basic Local Alignment Search Tool (Megablast)

Megablast searches for the conserved regions in a query sequence, where a single pair alignment process can produce many subsequence alignments in the results (local alignment) (Benson *et al*., 2007; Camacho *et al*., 2009; Chen *et al*., 2015). However, MAFFT optimises the alignment across the whole query sequence, which may include the regions of low similarity (global alignment) (Kotah *et al*., 2002; Katoh and Standley, 2013).

Megablast works by using the dynamic programming method where the Smith-Waterman algorithm is used to convert a large query sequence into small sequences (bits), pair these bits with similar bits found in the DNA databases, and mathematically assign a score to every bit pair. Subsequently, a scoring matrix is constructed based on the bits scores and high scoring optimal alignment is detected to report (Pertsemlidis and Fondon, 2001). Three steps given in figure 4.2 explain the procedure in detail which is implemented by the Megablast Smith-Waterman algorithm to perform a sequence alignment.
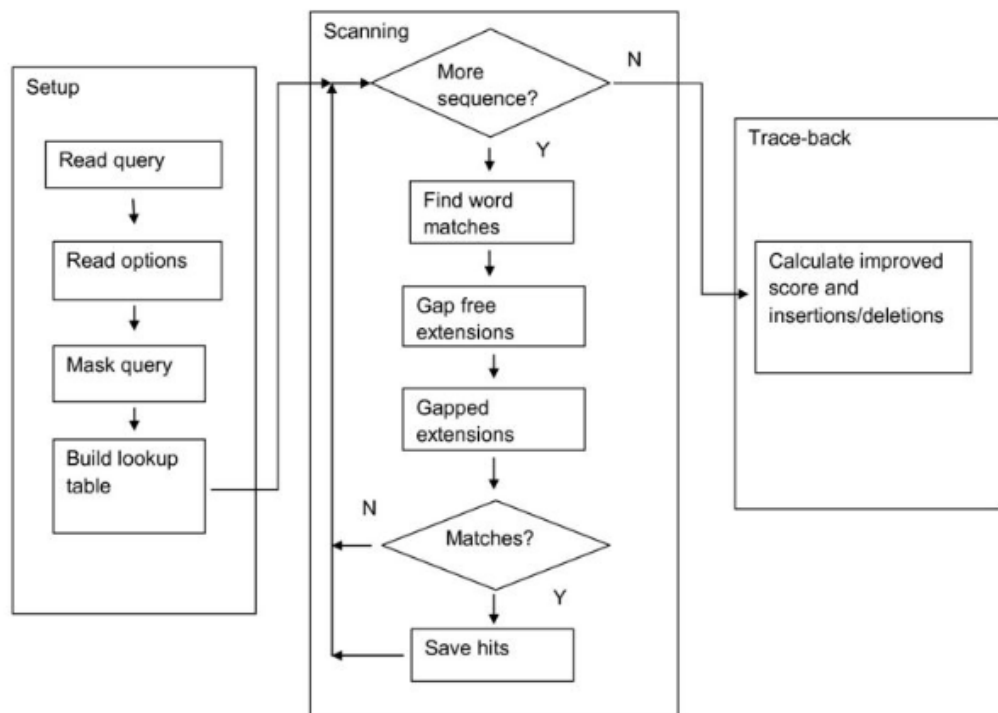
Figure 4.2. Schematic of a Blast search

(Reproduced from Camacho *et al.*, 2009).

Set-up stage: Megablast masks the short, periodic nucleotide repeats from the query sequence, scans the DNA database to find those sequences which score at least threshold (T, a Blast parameter pre-defined by the user) and builds a library of those sequences which resemble to the query sequence (lookup table).

Scanning step: Megablast seeks short word pairs (hits) typically 28 nucleotides long, which occur both in the query sequence and lookup table. Subsequent to seeding the hits, each hit or paired sequence is extended in both directions as long as its associated score continues to increase. Consequently, a segment of sequences with extended matching or alignment is produced, which is known as ungapped extensions of short word pairs or high scoring segment pair (HSP). An HSP with the highest score whose alignment cannot be further enhanced by lengthening or shortening is termed as a maximal-scoring segment pair (MSP). MSPs with the high level of similarity gains higher score than MSPs with low nucleotide similarity. MSPs possessing the highest score combine to trigger a gapped alignment ("gap" refers to the maximal uninterrupted run of spaces in a single sequence), which is then considered to report as the Blast output. The score of combined MSPs is sum up to report as a final alignment score (i.e. identity score in the Blast output).

Trace-Back stage: Megablast look up once again for the approximate matches within each MSP composition, corrects the positions of incorrect base deletions or insertions saved in the alignments during this phase.

4.1.1.4. Using Megablast for the classification of *C. jejuni* and *C. coli* LOS loci

The Blastn (Basic Local Alignment Search Tool for nucleotides) program is provided by National Center for Biotechnology Information at the Blastn server <https://blast.ncbi.nlm.nih.gov> which directly approximates the alignment between the two nucleotide sequences to measure and optimise the identity between them (Altschul *et al*., 1990). Megablast is an improvement of the existing Blastn program which is used specifically to detect the very similar nucleotide sequences between a query sequence and database sequences (subject sequences) (Benson *et al*., 2007; Camacho *et al*., 2009; Chen *et al*., 2015). In comparison to Blastn, Megablast is ten times faster, scans a large number of queries at one time, and can efficiently manipulate much longer DNA sequences originated from the same species (McGinnis and Madden, 2004; Kaur *et al*., 2008). Therefore, in the current study, alignment of an individual query sequence against all genes (present in all previously known LOS classes) was determined by performing Megablast repeatedly at its server <https://blast.ncbi.nlm.nih.gov> rather than the Blastn. A LOS gene was defined as present if ≥80% of the query sequence was effectively mapped to the reference LOS gene sequence and had ≥80% nucleotide identity to the reference LOS gene sequence. Subsequently, based on the predictions for the presence or absence of distinct LOS genes, combination of LOS genes or a LOS class was identified, and assigned to a particular *C. jejuni* or *C. coli* WG sequence.

Megablast can detect the accession number for a complete WG sequence and utilise it to find a query sequence. In contrast, Megablast is unable to incorporate the accession number of a contig-level or draft WG sequence. In this case, Megablast detects the contig unique ID number or sub accession number to find the query sequence. This means, a draft WG sequence with 100 contigs will produce 100 query sequences in the Megablast program. Thus, to reduce the burden of query sequences, contig numbers containing the LOS biosynthesis gene sequence were identified for all draft *C. jejuni* sequences. For this purpose, all sequenced contigs associated with each single strain sequence were aligned against the two reference LOS genes (*waaC* and *waaF*) sequences using Megablast, and subsequently, only identified LOS biosynthesis cluster

sequence containing contigs were used further for the LOS classification analysis. Thus, complete and annotated sequences were subjected only once and draft sequences twice to the Megablast.

In this study, Megablast was used to validate the Galaxy workflow and its classified *C. jejuni* sequences (complete and annotated), as well as, to classify those sequences of *C. jejuni* (draft) or *C. coli* (complete & draft) which could not be classified in Galaxy. Previously, 234 *Acinetobacter baumannii* genome sequences were extracted from the MLST database and their associated LOS outer core locus type was identified using Blastn (pairwise alignment between an unclassified sequence and one of the reference OCL1, OCL2 and OCL3 sequences). 9 Novel LOS outer core biosynthesis locus types (OCL4 - OCL12) of *Acinetobacter baumannii* were identified and OCL1 type was found to be most abundant in the MLST database of *Acinetobacter baumannii* using Blastn *(*Kenyon *et al*., 2014).

4.2. Aims and Objectives

This study aims to validate the use of the designed *in silico* Galaxy workflow for the classification of the *C. jejuni* LOS biosynthesis locus. For this purpose, complete online sequences of *C. jejuni* (n=125) classified by Galaxy workflow were compared with those 125 sequences which were classified using the Megablast. The designed Galaxy workflow was unable to classify the draft *C. jejuni* sequences, therefore, this study also aims to classify the draft *C. jejuni* sequences (n=578) using Megablast. Further, this study also aims to compare the distribution of *C. jejuni* LOS genotypes present within the GenBank database of *C. jejuni* sequences [complete (n=125) and draft (n=578)] with the frequency of *C. jejuni* LOS genotypes in a collection of 50 *C. jejuni* clinical Northampton General Hospital (NGH) isolates (previously identified in Chapter 3).

C. coli LOS locus class II contains *cst-IV* (a homologues of sialic acid biosynthesis gene, *cst-II*) and class III consists of a pseudogenized *cst-IV*, which are responsible for the transfer of sialic acid residues to the outer core of LOS structure (Culebro *et al.*, 2018; Kolehmainen *et al.*, 2019). This might be a reason of occurrence of *C. coli* strains in GBS patients (Wulffen *et al.*, 1994; van Belkum *et al.*, 2009). The relation between different *C. coli* LOS locus structures and virulence is not fully established yet (van Belkum *et al.*, 2009). The reasons behind it is the presence of a wide variety in *C. coli* LOS biosynthesis locus type (I-VIII)/LOS structures and availability of limited knowledge of *C. coli* LOS structures (Richard *et al.*, 2013; Kolehmainen *et al.*, 2019). Therefore, to focus future research at the specific, predominant *C. coli* LOS structures only, it is very important to identify those LOS locus types, which have been dominantly evolved and are frequently present in *C. coli* as the result of natural selection. Thus, this study also aims to determine the frequency of *C. coli* LOS types present in the worldwide GenBank database of *C. coli* sequences [complete (n=22) and draft (n=542)].

4.3. Results

4.3.1. LOS locus typing of *C. jejuni* GenBank sequences using Megablast and Galaxy MAFFT alignment tools

Complete *C. jejuni* sequences (n=125) and contig-level *C. jejuni* sequences (n=578) of high quality (with >20x coverage) available in GenBank were subjected to Megablast and the LOS class associated with each sequence was determined. The accession numbers of both complete and draft sequences as well as identified contig numbers of all draft sequences containing LOS biosynthesis region are reported (Table 1 in Appendix-II). Megablast was performed repeatedly (taking a manual approach) to determine the alignment of *C. jejuni* unknown or query sequences against all reference LOS gene sequences. The identity score (similarity between the two sequences in the form of percentage; cut-off value 80%) and query cover (percent of query sequence identical to the subject sequence) confirmed the presence of a particular LOS gene sequence in the given query sequence. On the basis of the presence or absence of LOS gene or LOS genes' combination in a query sequence, an individual LOS class from extant *C. jejuni* LOS classes was identified and assigned to the query sequence. Using this manual Megablast pairwise alignment approach, sequences of 400 *C. jejuni* strains were assigned to LOS group 1 related classes, 214 to LOS group 2 related classes, 73 to LOS group 3 related classes, and only 16 to LOS group 4 related classes, were associated (Table 1; Appendix-II).

The LOS class identification process for 125 complete *C. jejuni* sequences was also carried out using the Galaxy multiple alignment workflow. Altogether 81 of 125 sequences were linked to LOS groups (leaving 44 of 125 unclassified) and 39 of 81 were further associated with LOS classes using Galaxy MAFFT alignment trees (Figure 4.3). The same LOS group or class was also determined for each of these 81 sequences using Megablast, validating the use of Galaxy MAFFT alignment tool for the classification of *C. jejuni* LOS biosynthesis locus. Table 4.1 summarises the total number of *C. jejuni* sequences of 125, which were classified using Galaxy and Megablast.

(B)

1_NZ_CP028911_1_Campylobacter_jejuni_strain_NCTC_12661◄
11_NZ_CP020045_1_Campylobacter_jejuni_subsp__jejuni_strain_ATCC_35925◄
12_NZ_CP019965_1_Campylobacter_jejuni_strain_NCTC12662◄
85_NZ_CP010906_1_Campylobacter_jejuni_subsp__jejuni_strain_35925◄
96_NC_017281_1_Campylobacter_jejuni_subsp__jejuni_S3◄
101_Class_F
100_Class_D
102_Class_I
107_Class_S
103_Class_J
13_NZ_CP007190_1_Campylobacter_jejuni_strain_HF5-7-1◄
14_NZ_CP007189_1_Campylobacter_jejuni_strain_HF5-5-1◄
104_Class_K
89_NZ_CP006851_1_Campylobacter_jejuni_subsp__jejuni_F38011◄

19_NZ_CP017863_1_Campylobacter_jejuni_strain_IF1100
48_NZ_CP010508_1_Campylobacter_jejuni_strain_CJ677CC523
61_NZ_CP010496_1_Campylobacter_jejuni_strain_CJ677CC032
64_NZ_CP010492_1_Campylobacter_jejuni_strain_CJ677CC531
54_NZ_CP010502_1_Campylobacter_jejuni_strain_CJ677CC014
79_NZ_CP010472_1_Campylobacter_jejuni_strain_CJ677CC002
82_NZ_CP012149_1_Campylobacter_jejuni_strain_CJM1cam
34_NZ_CP010467_1_Campylobacter_jejuni_strain_CJ677CC024
74_NZ_CP010477_1_Campylobacter_jejuni_strain_CJ677CC526
56_NZ_CP010495_1_Campylobacter_jejuni_strain_CJ677CC538
76_NZ_CP010475_1_Campylobacter_jejuni_strain_CJ677CC073
97_NZ_CP005388_1_Campylobacter_jejuni_subsp__jejuni_CG8421
55_NZ_CP010501_1_Campylobacter_jejuni_strain_CJ677CC520
73_NZ_CP010479_1_Campylobacter_jejuni_strain_CJ677CC036
67_NZ_CP010489_1_Campylobacter_jejuni_strain_CJ677CC530
2_NZ_CP028910_1_Campylobacter_jejuni_strain_NCTC_12660
86_NZ_CP010301_1_Campylobacter_jejuni_subsp__jejuni_strain_00-0949
92_NC_022362_2_Campylobacter_jejuni_subsp__jejuni_00-2425
93_NC_022353_2_Campylobacter_jejuni_subsp__jejuni_00-2544
94_NC_022351_2_Campylobacter_jejuni_subsp__jejuni_00-2538
26_NZ_CP014744_1_Campylobacter_jejuni_strain_OD267
83_NZ_CP012690_1_Campylobacter_jejuni_subsp__jejuni_strain_RM3196
84_NZ_CP012689_1_Campylobacter_jejuni_subsp__jejuni_strain_RM3197
90_NZ_CP008787_1_Campylobacter_jejuni_subsp__jejuni_strain_MTVDSCj20
23_NZ_CP017032_1_Campylobacter_jejuni_subsp__jejuni_strain_MTVDSCj13
98_CP010058_1_Campylobacter_jejuni_subsp__jejuni_strain_YH001
7_NZ_CP022470_1_Campylobacter_jejuni_strain_RM1246-ERRC
72_NZ_CP010480_1_Campylobacter_jejuni_strain_CJ677CC524
77_NZ_CP010474_1_Campylobacter_jejuni_strain_CJ677CC536
91_NC_022529_1_Campylobacter_jejuni_4031
8_NZ_CP022080_1_Campylobacter_jejuni_subsp__jejuni_strain_FDAARGOS_266
105_Class_N
27_NZ_CP010487_1_Campylobacter_jejuni_strain_CJ677CC012
69_NZ_CP010483_1_Campylobacter_jejuni_strain_CJ677CC535
28_NZ_CP010486_1_Campylobacter_jejuni_strain_CJ677CC095
36_NZ_CP010465_1_Campylobacter_jejuni_strain_CJ677CC008
49_NZ_CP010507_1_Campylobacter_jejuni_strain_CJ677CC078
65_NZ_CP010491_1_Campylobacter_jejuni_strain_CJ677CC529
41_NZ_CP010460_1_Campylobacter_jejuni_strain_CJ677CC058
45_NZ_CP010511_1_Campylobacter_jejuni_strain_CJ677CC061
31_NZ_CP010470_1_Campylobacter_jejuni_strain_CJ677CC026
71_NZ_CP010481_1_Campylobacter_jejuni_strain_CJ677CC016
42_NZ_CP010459_1_Campylobacter_jejuni_strain_CJ677CC047
53_NZ_CP010503_1_Campylobacter_jejuni_strain_CJ677CC039
52_NZ_CP010504_1_Campylobacter_jejuni_strain_CJ677CC085
70_NZ_CP010482_1_Campylobacter_jejuni_strain_CJ677CC041
43_NZ_CP010458_1_Campylobacter_jejuni_strain_CJ677CC533
59_NZ_CP010498_1_Campylobacter_jejuni_strain_CJ677CC537
68_NZ_CP010488_1_Campylobacter_jejuni_strain_CJ677CC092
44_NZ_CP010457_1_Campylobacter_jejuni_strain_CJ677CC539
32_NZ_CP010469_1_Campylobacter_jejuni_strain_CJ677CC525
50_NZ_CP010506_1_Campylobacter_jejuni_strain_CJ677CC527
80_NZ_CP010471_1_Campylobacter_jejuni_strain_CJ677CC519
63_NZ_CP010493_1_Campylobacter_jejuni_strain_CJ677CC062
66_NZ_CP010490_1_Campylobacter_jejuni_strain_CJ677CC532
33_NZ_CP010468_1_Campylobacter_jejuni_strain_CJ677CC064
37_NZ_CP010464_1_Campylobacter_jejuni_strain_CJ677CC094
38_NZ_CP010463_1_Campylobacter_jejuni_strain_CJ677CC522
40_NZ_CP010461_1_Campylobacter_jejuni_strain_CJ677CC013
46_NZ_CP010510_1_Campylobacter_jejuni_strain_CJ677CC040
106_Class_Q
10_NZ_CP022076_1_Campylobacter_jejuni_subsp__jejuni_strain_FDAARGOS_262
21_NZ_CP017862_1_Campylobacter_jejuni_strain_FJ3124
57_NZ_CP010500_1_Campylobacter_jejuni_strain_CJ677CC528
25_NZ_CP014742_1_Campylobacter_jejuni_strain_WP2202
15_NZ_CP007188_1_Campylobacter_jejuni_strain_HF5-4A-4
16_NZ_CP017856_1_Campylobacter_jejuni_strain_ZP3204
20_NZ_CP017860_1_Campylobacter_jejuni_strain_TS1218
17_NZ_CP017859_1_Campylobacter_jejuni_strain_YQ2210
87_NZ_CP010072_1_Campylobacter_jejuni_subsp__jejuni_strain_01-1512
18_NZ_CP007749_1_Campylobacter_jejuni_subsp__jejuni_M129
24_NZ_CP017029_1_Campylobacter_jejuni_subsp__jejuni_strain_14980A
88_NZ_CP010306_1_Campylobacter_jejuni_subsp__jejuni_strain_00-1597
5_R_NZ_CP023867_1_Campylobacter_jejuni_strain_FDAARGOS_422
29_NZ_CP010485_1_Campylobacter_jejuni_strain_CJ677CC086
39_NZ_CP010462_1_Campylobacter_jejuni_strain_CJ677CC100
30_NZ_CP010484_1_Campylobacter_jejuni_strain_CJ677CC034
75_NZ_CP010476_1_Campylobacter_jejuni_strain_CJ677CC521
47_NZ_CP010509_1_Campylobacter_jejuni_strain_CJ677CC540
78_NZ_CP010473_1_Campylobacter_jejuni_strain_CJ677CC534
4_R_NZ_CP023866_1_Campylobacter_jejuni_strain_FDAARGOS_421
35_NZ_CP010466_1_Campylobacter_jejuni_strain_CJ677CC541
51_NZ_CP010505_1_Campylobacter_jejuni_strain_CJ677CC052
62_NZ_CP010494_1_Campylobacter_jejuni_strain_CJ677CC059
58_NZ_CP010499_1_Campylobacter_jejuni_strain_CJ677CC542
60_NZ_CP010497_1_Campylobacter_jejuni_strain_CJ677CC033
9_NZ_CP022077_1_Campylobacter_jejuni_subsp__jejuni_strain_FDAARGOS_263
3_CP005081_1_Campylobacter_jejuni_subsp__jejuni_R14
81_NZ_LN831025_1_Campylobacter_jejuni_genome_assembly_NCTC11351
6_NZ_CP023543_1_Campylobacter_jejuni_strain_CFSAN032806
95_R_NC_021834_1_Campylobacter_jejuni_32488
22_NZ_CP017033_1_Campylobacter_jejuni_subsp__jejuni_strain_MTVDSCj16
99_NZ_KK365768_1_Campylobacter_jejuni_Cj3

1_NZ_CP028910_1_Campylobacter_jejuni_strain_NCTC_12660
80_NZ_CP010301_1_Campylobacter_jejuni_subsp__jejuni_strain_00-0949
85_NC_022362_2_Campylobacter_jejuni_subsp__jejuni_00-2425
86_NC_022353_2_Campylobacter_jejuni_subsp__jejuni_00-2544
87_NC_022351_2_Campylobacter_jejuni_subsp__jejuni_00-2538
21_NZ_CP014744_1_Campylobacter_jejuni_strain_OD267
18_NZ_CP017032_1_Campylobacter_jejuni_subsp__jejuni_strain_MTVDSCj13
90_CP010058_1_Campylobacter_jejuni_subsp__jejuni_strain_YH001
78_NZ_CP012690_1_Campylobacter_jejuni_subsp__jejuni_strain_RM3196
79_NZ_CP012689_1_Campylobacter_jejuni_subsp__jejuni_strain_RM3197
11_NZ_CP017856_1_Campylobacter_jejuni_strain_ZP3204
15_NZ_CP017860_1_Campylobacter_jejuni_strain_TS1218
12_NZ_CP017859_1_Campylobacter_jejuni_strain_YQ2210
81_NZ_CP010072_1_Campylobacter_jejuni_subsp__jejuni_strain_01-1512
13_NZ_CP007749_1_Campylobacter_jejuni_subsp__jejuni_M129
19_NZ_CP017029_1_Campylobacter_jejuni_subsp__jejuni_strain_14980A
82_NZ_CP010306_1_Campylobacter_jejuni_subsp__jejuni_strain_00-1597
6_NZ_CP022470_1_Campylobacter_jejuni_strain_RM1246-ERRC ◄
14_NZ_CP017863_1_Campylobacter_jejuni_strain_IF1100 ◄
95_Class_H
89_NZ_CP005388_1_Campylobacter_jejuni_subsp__jejuni_CG8421 ◄
84_NC_022529_1_Campylobacter_jejuni_4031 ◄
94_Class_P
93_Class_E
67_NZ_CP010480_1_Campylobacter_jejuni_strain_CJ677CC524 ◄
72_NZ_CP010474_1_Campylobacter_jejuni_strain_CJ677CC536 ◄
83_NZ_CP008787_1_Campylobacter_jejuni_subsp__jejuni_strain_MTVDSCj20 ◄
92_Class_O
43_NZ_CP010508_1_Campylobacter_jejuni_strain_CJ677CC523 ◄
56_NZ_CP010496_1_Campylobacter_jejuni_strain_CJ677CC032 ◄
59_NZ_CP010492_1_Campylobacter_jejuni_strain_CJ677CC531 ◄
49_NZ_CP010502_1_Campylobacter_jejuni_strain_CJ677CC014 ◄
74_NZ_CP010472_1_Campylobacter_jejuni_strain_CJ677CC002 ◄
29_NZ_CP010467_1_Campylobacter_jejuni_strain_CJ677CC024 ◄
69_NZ_CP010477_1_Campylobacter_jejuni_strain_CJ677CC526 ◄
51_NZ_CP010495_1_Campylobacter_jejuni_strain_CJ677CC538 ◄
71_NZ_CP010475_1_Campylobacter_jejuni_strain_CJ677CC073 ◄
50_NZ_CP010501_1_Campylobacter_jejuni_strain_CJ677CC520 ◄
68_NZ_CP010479_1_Campylobacter_jejuni_strain_CJ677CC036 ◄
62_NZ_CP010489_1_Campylobacter_jejuni_strain_CJ677CC530 ◄
77_NZ_CP012149_1_Campylobacter_jejuni_strain_CJM1cam ◄
96_Class_W
22_NZ_CP010487_1_Campylobacter_jejuni_strain_CJ677CC012 ◄
64_NZ_CP010483_1_Campylobacter_jejuni_strain_CJ677CC535 ◄
23_NZ_CP010486_1_Campylobacter_jejuni_strain_CJ677CC095 ◄
31_NZ_CP010465_1_Campylobacter_jejuni_strain_CJ677CC008 ◄
44_NZ_CP010507_1_Campylobacter_jejuni_strain_CJ677CC078 ◄
60_NZ_CP010491_1_Campylobacter_jejuni_strain_CJ677CC529 ◄
36_NZ_CP010460_1_Campylobacter_jejuni_strain_CJ677CC058 ◄
40_NZ_CP010511_1_Campylobacter_jejuni_strain_CJ677CC061 ◄
26_NZ_CP010470_1_Campylobacter_jejuni_strain_CJ677CC026 ◄
66_NZ_CP010481_1_Campylobacter_jejuni_strain_CJ677CC016 ◄
37_NZ_CP010459_1_Campylobacter_jejuni_strain_CJ677CC047 ◄
48_NZ_CP010503_1_Campylobacter_jejuni_strain_CJ677CC039 ◄
47_NZ_CP010504_1_Campylobacter_jejuni_strain_CJ677CC085 ◄
65_NZ_CP010482_1_Campylobacter_jejuni_strain_CJ677CC041 ◄
38_NZ_CP010458_1_Campylobacter_jejuni_strain_CJ677CC533 ◄
54_NZ_CP010498_1_Campylobacter_jejuni_strain_CJ677CC537 ◄
63_NZ_CP010488_1_Campylobacter_jejuni_strain_CJ677CC092 ◄
39_NZ_CP010457_1_Campylobacter_jejuni_strain_CJ677CC539 ◄
27_NZ_CP010469_1_Campylobacter_jejuni_strain_CJ677CC525 ◄
45_NZ_CP010506_1_Campylobacter_jejuni_strain_CJ677CC527 ◄
75_NZ_CP010471_1_Campylobacter_jejuni_strain_CJ677CC519 ◄
58_NZ_CP010493_1_Campylobacter_jejuni_strain_CJ677CC062 ◄
61_NZ_CP010490_1_Campylobacter_jejuni_strain_CJ677CC532 ◄
28_NZ_CP010468_1_Campylobacter_jejuni_strain_CJ677CC064 ◄
32_NZ_CP010464_1_Campylobacter_jejuni_strain_CJ677CC094 ◄
33_NZ_CP010463_1_Campylobacter_jejuni_strain_CJ677CC522 ◄
35_NZ_CP010461_1_Campylobacter_jejuni_strain_CJ677CC013 ◄
41_NZ_CP010510_1_Campylobacter_jejuni_strain_CJ677CC040 ◄
4__R_NZ_CP023867_1_Campylobacter_jejuni_strain_FDAARGOS_422
24_NZ_CP010485_1_Campylobacter_jejuni_strain_CJ677CC086
34_NZ_CP010462_1_Campylobacter_jejuni_strain_CJ677CC100
25_NZ_CP010484_1_Campylobacter_jejuni_strain_CJ677CC034
70_NZ_CP010476_1_Campylobacter_jejuni_strain_CJ677CC521
42_NZ_CP010509_1_Campylobacter_jejuni_strain_CJ677CC540
73_NZ_CP010473_1_Campylobacter_jejuni_strain_CJ677CC534
9_NZ_CP022076_1_Campylobacter_jejuni_subsp__jejuni_strain_FDAARGOS_262
16_NZ_CP017862_1_Campylobacter_jejuni_strain_FJ3124
52_NZ_CP010500_1_Campylobacter_jejuni_strain_CJ677CC528
30_NZ_CP010466_1_Campylobacter_jejuni_strain_CJ677CC541
46_NZ_CP010505_1_Campylobacter_jejuni_strain_CJ677CC052
57_NZ_CP010494_1_Campylobacter_jejuni_strain_CJ677CC059
2_CP005081_1_Campylobacter_jejuni_subsp__jejuni_R14
76_NZ_LN831025_1_Campylobacter_jejuni_genome_assembly_NCTC11351
53_NZ_CP010499_1_Campylobacter_jejuni_strain_CJ677CC542
55_NZ_CP010497_1_Campylobacter_jejuni_strain_CJ677CC033
88__R_NC_021834_1_Campylobacter_jejuni_32488
3__R_NZ_CP023866_1_Campylobacter_jejuni_strain_FDAARGOS_421
7_NZ_CP022080_1_Campylobacter_jejuni_subsp__jejuni_strain_FDAARGOS_266
5_NZ_CP023543_1_Campylobacter_jejuni_strain_CFSAN032806
8_NZ_CP022077_1_Campylobacter_jejuni_subsp__jejuni_strain_FDAARGOS_263
17_NZ_CP017033_1_Campylobacter_jejuni_subsp__jejuni_strain_MTVDSCj16
10_NZ_CP007188_1_Campylobacter_jejuni_strain_HF5-4A-4
20_NZ_CP014742_1_Campylobacter_jejuni_strain_WP2202
91_NZ_KK365768_1_Campylobacter_jejuni_Cj3

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Figure 4.3. Illustration of trees, obtained using the Galaxy-MAFFT workflow and used to predict the LOS locus types in *C. jejuni* **WG sequences**

(A). The clustering of 19 *C. jejuni* strains sequences (12664, 12567, 11168/*lacY,* 00-2426, IA3902, NS4-9-1, NS4-5-1, NS4-1-1, MTVDSCj07, RM1285, 11168-GSv, 11168-Kf1, 11168-K12E5, 11168-BN148, 11168-mfK12E5, PT14, D42a, 11168H/*araE*, YH0002) around the reference class C, 2 sequences (81-176_G1_B0, RM3194) around the class B and 2 sequences (00-6200, RM3420) around the class A are represented. All these sequences belonged to LOS group 1.

(B). The clustering of 5 sequences (12661, ATCC35925, 12662, 35925, S3) around the LOS class F and 3 sequences (HF5-7-1, HF5-5-1, F38011) around the reference LOS class K are depicted. All these sequences belonged to LOS group 3.

(C). The clustering of RM1246-ERRC & IF1100 with class H, CG8421 & 4031 with class P, CJM1cam with class W, and MTVDSCj20, CJ677CC524 & CJ677CC536 around the class O are presented. All these sequences belonged to LOS group 2.

Each blue coloured arrow represents that *C. jejuni* sequence for which the same LOS class was identified using both Megablast and Galaxy MAFFT alignment tools. Purple coloured arrows represent those sequences which could not be LOS classified correctly using Galaxy MAFFT, but correctly LOS grouped.

Table 4.1: Comparison of Galaxy and Megablast classified *C. jejuni* sequences (n=125)

C. jejuni LOS locus class (Group)	No. of LOS classified *C. jejuni* sequences by Galaxy	No. of LOS grouped *C. jejuni* sequences by Galaxy	No. of LOS classified *C. jejuni* sequences by Megablast	No. of LOS grouped *C. jejuni* sequences by Megablast
A (1)	2		11	
B	2		10	
C	19		30	
V	-		1	
Total	23	25	52	52
E (2)	-		1	
H	2		2	
O	3		56	
P	2		2	
W	1		1	
Total	8	48	62	62
D (3)	-		1	
F	5		7	
K	3		3	
Total	8	8	11	11
Sum of all totals	39	81	125	125

4.3.2. *C. jejuni* LOS loci distribution in GenBank database and its comparison to *C. jejuni* LOS loci distribution in NGH clinical isolates

The distribution of *C. jejuni* LOS locus classes and LOS groups in a collection of 703 *C. jejuni* sequences available from GenBank is presented in figure 4.4. 400 of 703 (58%) *C. jejuni* sequences belonged to the LOS group 1 related classes. The LOS classes A, B, and C with trend [B1 (n=125) > C (n=109) > A1 (n=68) > A2 (n=44) > B2 (n=36)] were the most common classes and the other three classes including R (n=10), M (n=7), and V (n=1) were rare. 214 (30%) classified sequences were positive for either class E (n=16), class H (n=72), class O (n=63), class P (n=44) or class W (n=13) and therefore, belonged to LOS group 2. 73 (10%) *C. jejuni* sequences were positive for LOS group 3 related classes including D (n=2), F (n=36), K (n=16), Q (n=1), N (n=1), I (n=3), S (n=6), J (n=4). Only 16 (2%) strains belonged to group 4 related classes, where 10 were associated to class G and only 6 were linked to class L.

A few *C. jejuni* sequences contained some LOS genes which had identity to the reference LOS genes below the cut-off value and therefore, identity scores for these genes were not considered to include in the Megablast score results (Table 1 in Appendix-II). For this reason, these sequences were not linked to a particular class, and categorised into a mix class category within a group, such as, six to H/P (Group 2) and four to I/J/S (Group 3).

A comparison of *C. jejuni* LOS class and group frequencies identified in both collections of clinical *C. jejuni* isolates and online *C. jejuni* sequences has been presented in Figure 4.5. The frequency of LOS classes within the LOS group 1 was almost similar in both collections of *C. jejuni* clinical isolates [class B (22%) > class C (20%) > class A (14%)] and *C. jejuni* online sequences [class B (23%) > class C (16%) $\geq$ class A (16%)]. Other group 1 related classes (R, V and M) were less frequent (~ 2.5 %) in the online *C. jejuni* sequence database, whereas, these three classes were absent in the clinical collection. 16% of LOS typed *C. jejuni* clinical strains had the LOS group 2 related class P and 10% of analysed *C. jejuni* sequences belonged to the LOS group 2 related class H, marking the class P in local clinical *C. jejuni* collection and class H in the online *C. jejuni* sequence database as the most predominant LOS group 2 classes. LOS class

F was the most common class of LOS group 3 among both local (6%) and global (5%) *C. jejuni* collections. A very small proportion of online *C. jejuni* sequences belonged to LOS group 2 related class W (2%), LOS group 3 related classes D (0.3%), N (0.1%), Q (0.1%), I (0.5%), J (0.5%), S (1%) and LOS group 4 related classes G (1.4%) and L (1%). These classes were all absent in the clinical collection of *C. jejuni* isolates. Finally, the trend of prevalence of LOS groups, group 1 > group 2 > group 3 > group 4, was similar in both types of collection of *C. jejuni* strains.
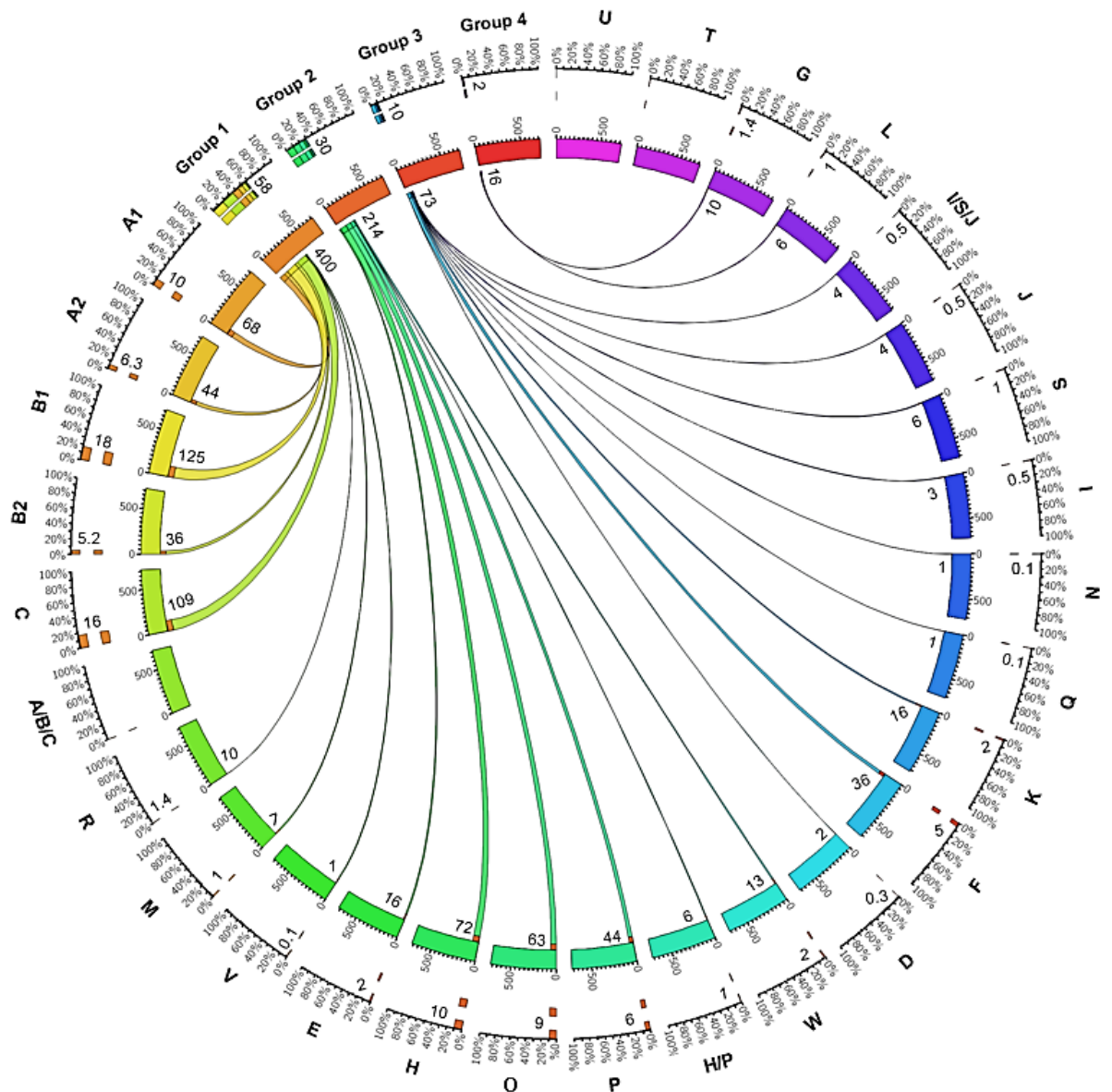
Figure 4.4: A Circos plot showing the distribution of *C. jejuni* LOS locus classes (A-W), subclasses (A1, A2, B1, B2) and LOS groups (1-4) in the online *C. jejuni* sequence database.

Each segment of inner circle specify the total number of *C. jejuni* strains sequences (703) extracted for the LOS classification. The frequency of *C. jejuni* isolates classified for each particular LOS class/group is mentioned in numbers (n out of 703) on the top of each inner circle segment and presented with ribbon width. The frequency of a *C. jejuni* LOS class/group in percent is mentioned with each outer circle segment and represented by the orange or colourful blocks. Ribbon ends link each *C. jejuni* LOS class to its related LOS group.
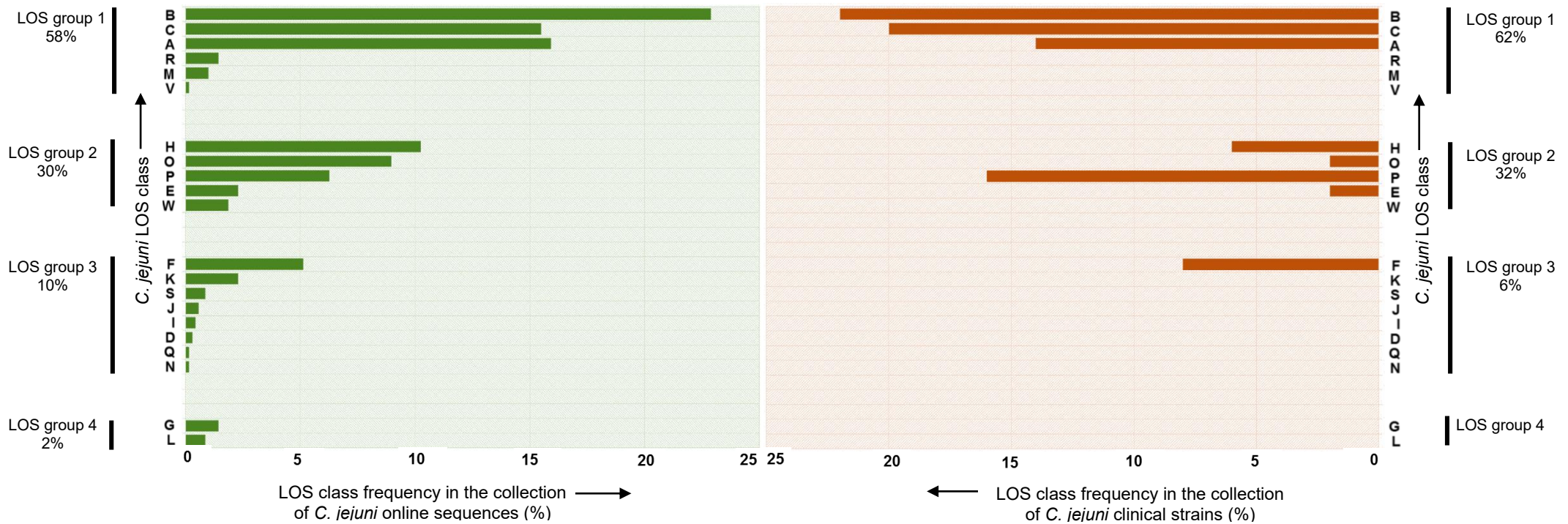
Figure 4.5: A comparison of *C. jejuni* LOS biosynthesis locus class and group frequencies found in the collections of GenBank *C. jejuni* sequences (n=703) and *C. jejuni* clinical strains (n=50)

4.3.3. LOS locus typing of *C. coli* GenBank sequences and WG shotgun sequencing of a clinical *C. coli* strain

The WG sequence data of 564 *C. coli* strains was obtained from GenBank, available either in the complete, annotated version or in the form of discrete contigs. 22 complete and 542 contig-level *C. coli* sequences were subjected to Megablast and a LOS class for each sequence was identified. The accession numbers for both complete and draft *C. coli* sequences as well as identified LOS containing contig numbers associated to draft sequences have been reported in Table 2 in Appendix-II. The identity score and query cover, both with cut-off values of 80%, confirmed the presence of an individual LOS gene sequence in a specified query sequence. On the basis of presence or absence of LOS gene or LOS gene combinations in a query sequence, an individual LOS class from already known *C. coli* LOS classes was identified, and assigned to the query sequence. Using this sequence pairwise alignment approach, sequences of 564 *C. coli* strains were LOS classified (Table 2; Appendix-II).

In the previous chapter, 50 *C. jejuni* clinical isolates were collected from NGH and the LOS type for every strain was identified using a PCR based typing method. *C. coli* strains in equal number to the *C. jejuni* isolates could not be obtained from NGH, indicating a reduced prevalence of *C. coli* clinically than *C. jejuni*. Due to the lack of availability of *C. coli* strains, a PCR typing method for *C. coli* could not be established or validated. Only one *C. coli* strain (*C. coli* 221089) was obtained from NGH, which was WG sequenced to analyse its LOS biosynthesis cluster sequence. The WG sequence of *C. coli* 221089 was submitted in GenBank under the accession number, RJLP00000000. Figure 4.6 was obtained using the Artemis comparison tool (ACT; Carver *et al*., 2005), where, *C. coli* 221089 and *C. coli* RM2228 (reference strain for *C. coli* LOS class III) LOS biosynthesis region sequences were aligned pairwise. This figure is an illustration of high similarity (>99%) between the LOS cluster sequences of *C. coli* 221089 and *C. coli* RM2228, confirming the presence of *C. coli* LOS type III in *C. coli* 221089 strain.
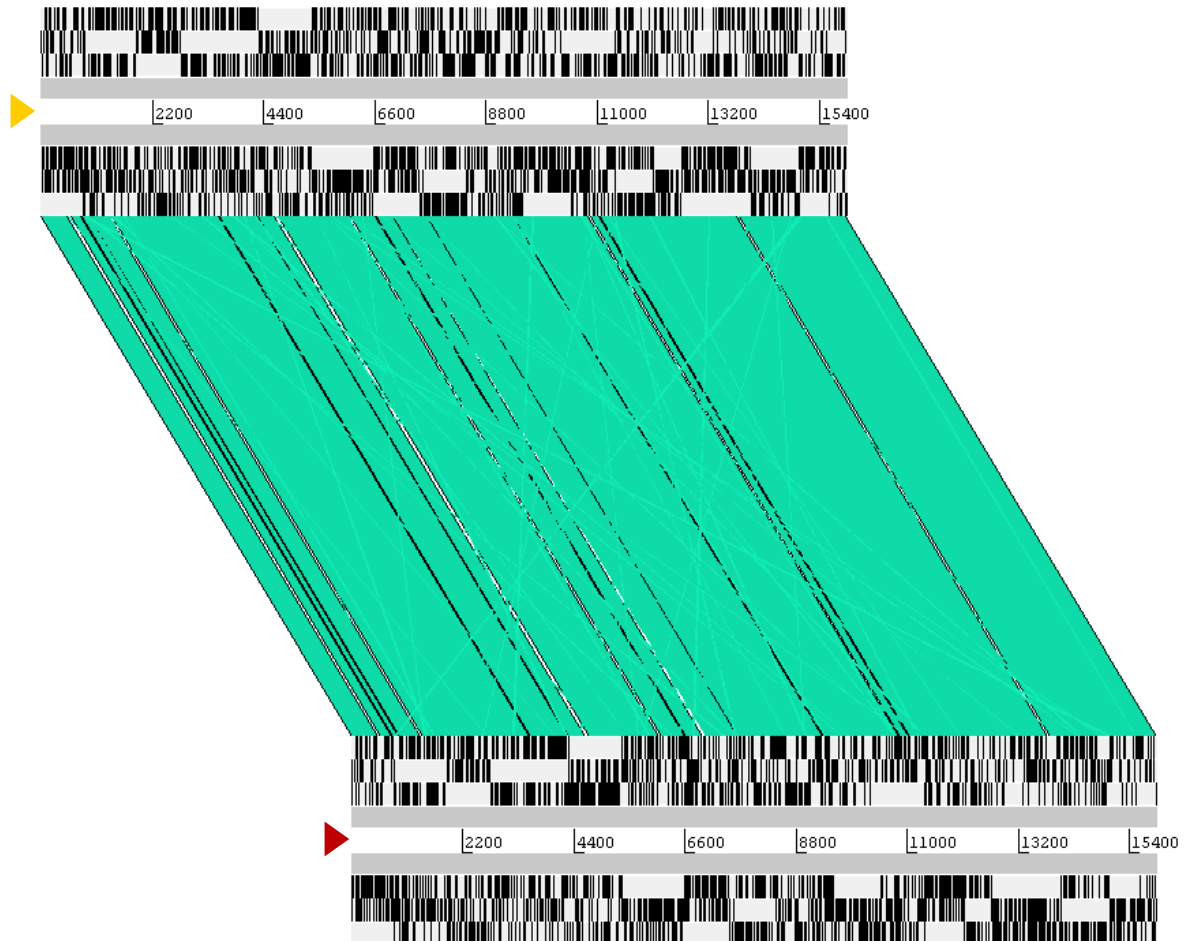
Figure 4.6: Pairwise comparison of *C. coli* RM2228 (reference strain, yellow arrow) and *C. coli* 221089 (clinical strain; red arrow) LOS cluster sequences, obtained via ACT. It shows the extent of similarity (green coloured area) between aligned LOS sequences (each containing 15,400 bases).

Black lines link sequences present in the same orientation. Light green lines link sequences present in the reverse orientation.

4.3.4. *C. coli* LOS loci distribution in GenBank database

The frequency of *C. coli* LOS locus classes in a collection of 564 GenBank *C. coli* sequences has been represented in figure 4.7. *C. coli* LOS class III (41%; n=229) was the most abundant class followed by class VIII (18%; n=103) > I (13%; n=70) > II (8%; n=47) > VII (7%; n=42) > IV (5%; n=27) > VI (4%; n=25) > V (4%; n=21) within the collection of 564 *C. coli* GenBank sequences.
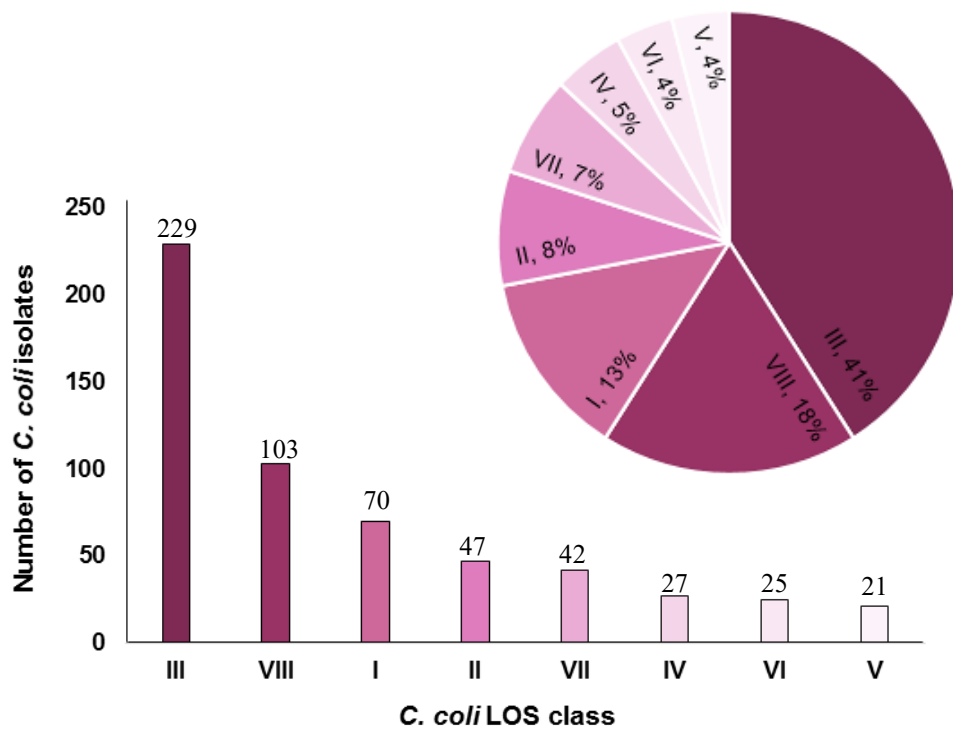
Figure 4.7: Frequency of *C. coli* LOS locus classes within the online *C. coli* sequences GenBank database.

The number of *C. coli* strains associated with each *C. coli* LOS class in column chart and corresponding percentages of *C. coli* strains in Pie chart, represent the frequency of *C. coli* LOS locus classes within the collection of *C. coli* online sequences.

4.3.5. *C. jejuni* and *C. coli* LOS loci distribution in different *Campylobacter* niches

A source of microbe isolation is usually reported with each sequence of GenBank database. The prevalence of *C. jejuni* and *C. coli* LOS genotypes in different *Campylobacter* niches was estimated (Figure 4.8) by looking at the online published sources from which these *C. jejuni* and *C. coli* strains were isolated (Table 1 & 2, Appendix-II). *C. jejuni* and *C. coli* strains, isolated from faeces, were not included in this analysis as the actual source of faeces was unknown. Chicken and animal farm environment (water and soil) were found as the most common reservoir respectively for *C. jejuni* and *C. coli*.

C. jejuni LOS group 1 associated *C. jejuni* strains were found mostly in humans [A (n=24; 6%), B (n=33; 8%), C (n=29; 7%)] and chickens [A (n=14; 3%), B (n=23; 5%), C (n=9; 2%)]. The LOS class P (the most predominant LOS group 2 class among clinical isolates) associated strains (n=8 of 23) were majorly isolated from humans and a large proportion (n=22 of 55) of LOS class H (the most common LOS group 2 class in GenBank) related *C. jejuni* strains was obtained from milk. For the common LOS group 3 related class F, cows were the main source of isolation. Nine LOS class C linked *C. jejuni* strains were isolated from goats, one LOS class A related strain was isolated from a monkey, and five LOS class L related strains were from American crows. Other reservoirs of *C. jejuni* included turkey and pigeon.

The most predominant LOS class III related *C. coli* strains were isolated in a high number from the farm environment (n=165; 37%) and humans (n=20, 5%), indicating that these are both the most common niches for type III LOS locus containing *C. coli* strains. The second most prevalent LOS class VIII was also frequent in the environment (n=31, 7%) and at similar levels to humans (n=26, 6%) and chickens (n=25, 6%). Only one LOS class I associated *C. coli* strain was found in monkey and one LOS class II related *C. coli* strain was recovered from a dog. Rarer reservoirs of *C. coli* were turkey, cow, duck, pig and milk.
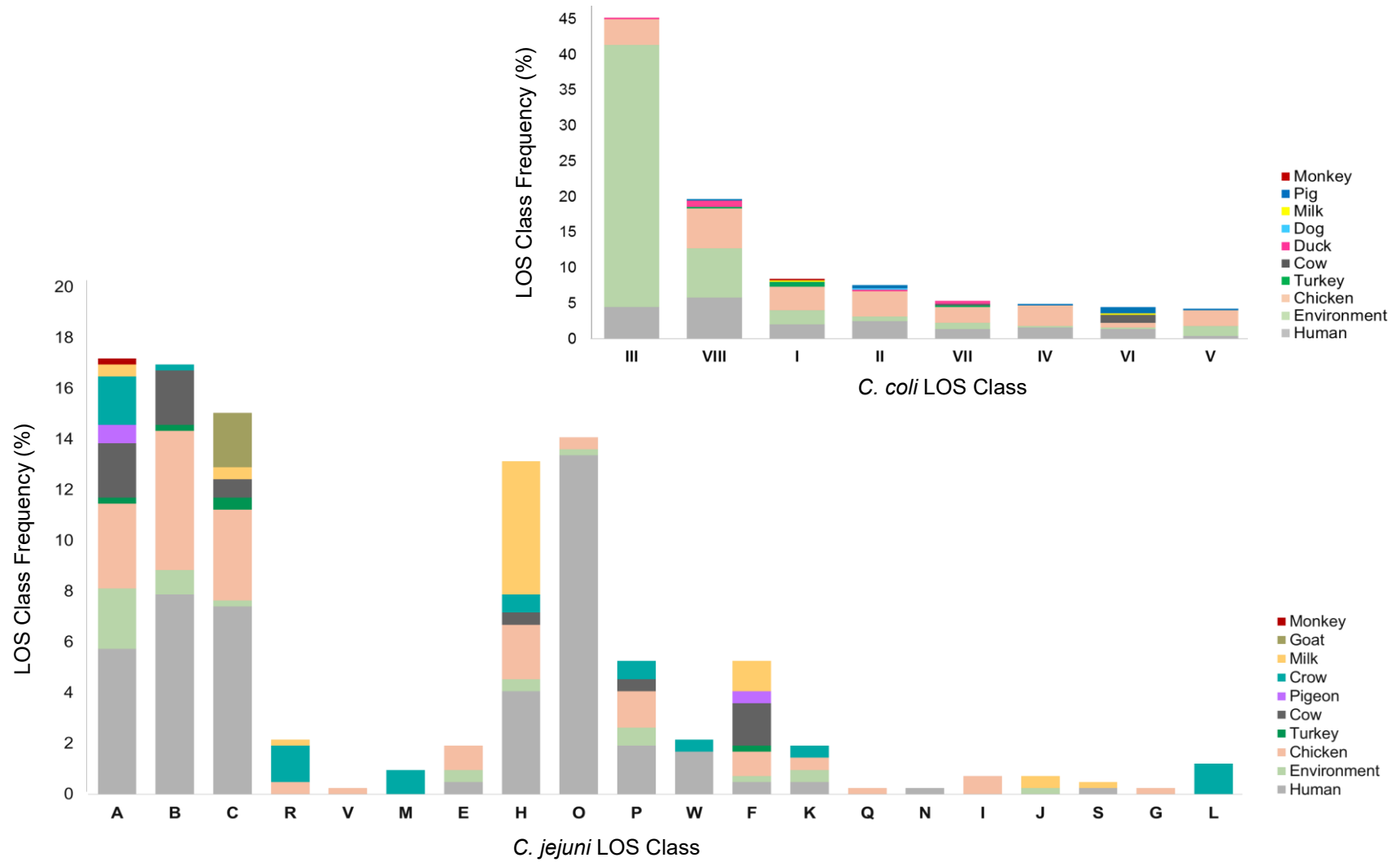
Figure 4.8: Frequency of *C. jejuni* and *C. coli* LOS locus classes in different *Campylobacter* sources

4.3.6. Identification of novel genes in *Campylobacter* LOS biosynthesis loci

By *in silico* analysis, LOS biosynthesis genes clusters of two *C. jejuni* strains, *C. jejuni* 414 (Accession no: ADGM01000014.1) and *C. jejuni* 1336 (Accession no: CM000854.1), were found with novel LOS gene content. The variable regions of *C. jejuni* 414 and 1336 LOS biosynthesis locus had 5 and 13 novel LOS genes of unknown functions, respectively (Figure 4.9). Further, the origin of these novel genes was predicted by blasting each gene against all sequences available in the GenBank database. Four novel LOS genes in *C. jejuni* 1336 locus had >99% similarity with the capsular polysaccharide (CPS) biosynthesis genes of other *C. jejuni* strains, suggesting a possible gene transfer from other *C. jejuni* strains CPS loci to *C. jejuni* 1336 LOS biosynthesis locus. Six novel LOS genes of *C. jejuni* 1336 and one gene of *C. jejuni* 414 did not have any identity with the previously known *C. jejuni* LOS genes. Instead, they had >99% similarity with various LOS biosynthesis genes of *C. coli*, suggesting the interspecies gene recombination. Another *C. jejuni* strain, CFSAN054107 (Accession no: CP028185.1), was also found with 5 previously unreported LOS genes (Figure 4.9). One of them encodes a methlytransferase and the remaining four encode glycosyltranferases (data from GenBank database). In addition to looking at the complete novel *C. jejuni* LOS loci with major modifications, two *C. jejuni* strains' (PT14 & 34288) LOS loci with a few minor gene modifications were also observed. *C. jejuni* PT14 (Accession no: CP003871.3) had a type C LOS locus with two modifications; deletion of ORF14 (class C related gene; *cj1137c*) and the addition of ORF48 (generally belong to LOS class L). Similarly, *C. jejuni* 34288 (Accession no: CP006006.1) had a type A2 LOS locus, but with the duplication of a sialic acid biosynthesis gene (*neuA*).

Genes present in the middle region of LOS biosynthesis cluster usually undergo recombination compared to those LOS genes which are present at the proximal and distal ends of the cluster. The cluster ends' genes often have nucleotide level variations, but are less likely to vary in gene order or content (Parker *et al*., 2008; Richard *et al*., 2013). In the current study, previously unreported LOS genes were identified, which were found localised at the same position in the distal end of five *C. coli* LOS biosynthesis locus types (I, II, III, V and VIII; Figure 4.10). These were found to be extant in all those *in silico* typed *C. coli* strains

sequences which had the LOS type I (n=63), II (n=43), III (n=229), V (n=3), and VIII (n=98) (Grey column in Table 2 in Appendix-II). These LOS genes were present at the same position in all five types of *C. coli* LOS locus, but altered in size and nucleotide composition from LOS class to class. The class I gene had a maximum size of ~1.4 kb and was distantly related to those genes which occurred at the same position in other *C. coli* LOS locus types (II, III, V & VIII). The class II gene had similarity (~91%) to genes present in other classes (III, V and VIII). The type III and VIII genes were identical (100%) to each other and the class V gene was partially (~51%) similar to both of these identical genes. Class I gene encoded the β-Kdo transferase, class II gene encoded the phosphoheptose isomerase and genes in other classes produced the glycosyltranferases for LOS synthesis (Data extracted from GenBank; Table 4.2).
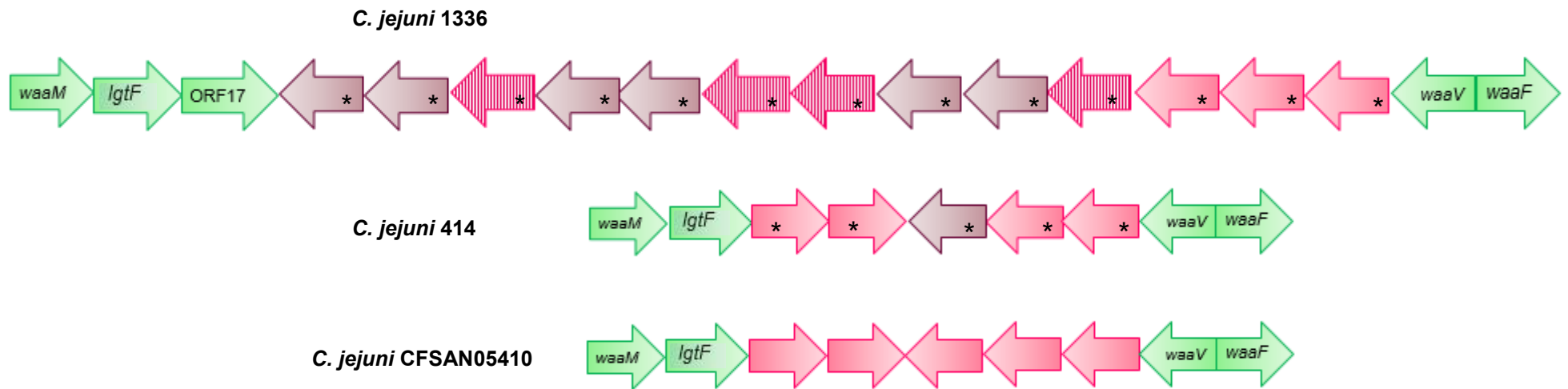
Figure 4.9: The illustrations of variable regions of *C. jejuni* 1336 (containing 13 novel LOS genes), *C. jejuni* 414 (containing 5 novel LOS genes), and *C. jejuni* CFSAN05410 (containing 5 previously unknown LOS genes) between the common LOS genes (*lgtF*, ORF17 and *waaV*).

Green arrows: LOS genes common in all *C. jejuni* strains; Pink arrows: LOS genes originated from the LOS loci of other *C. jejuni* strains; Pink arrows with vertical lines: LOS genes originated from CPS loci of other *C. jejuni* strains; Purple arrows: LOS genes originated from the LOS biosynthesis region of *C. coli* strains. The direction of arrow represents the direction of gene transcription. Black star: Gene with an unknown function.
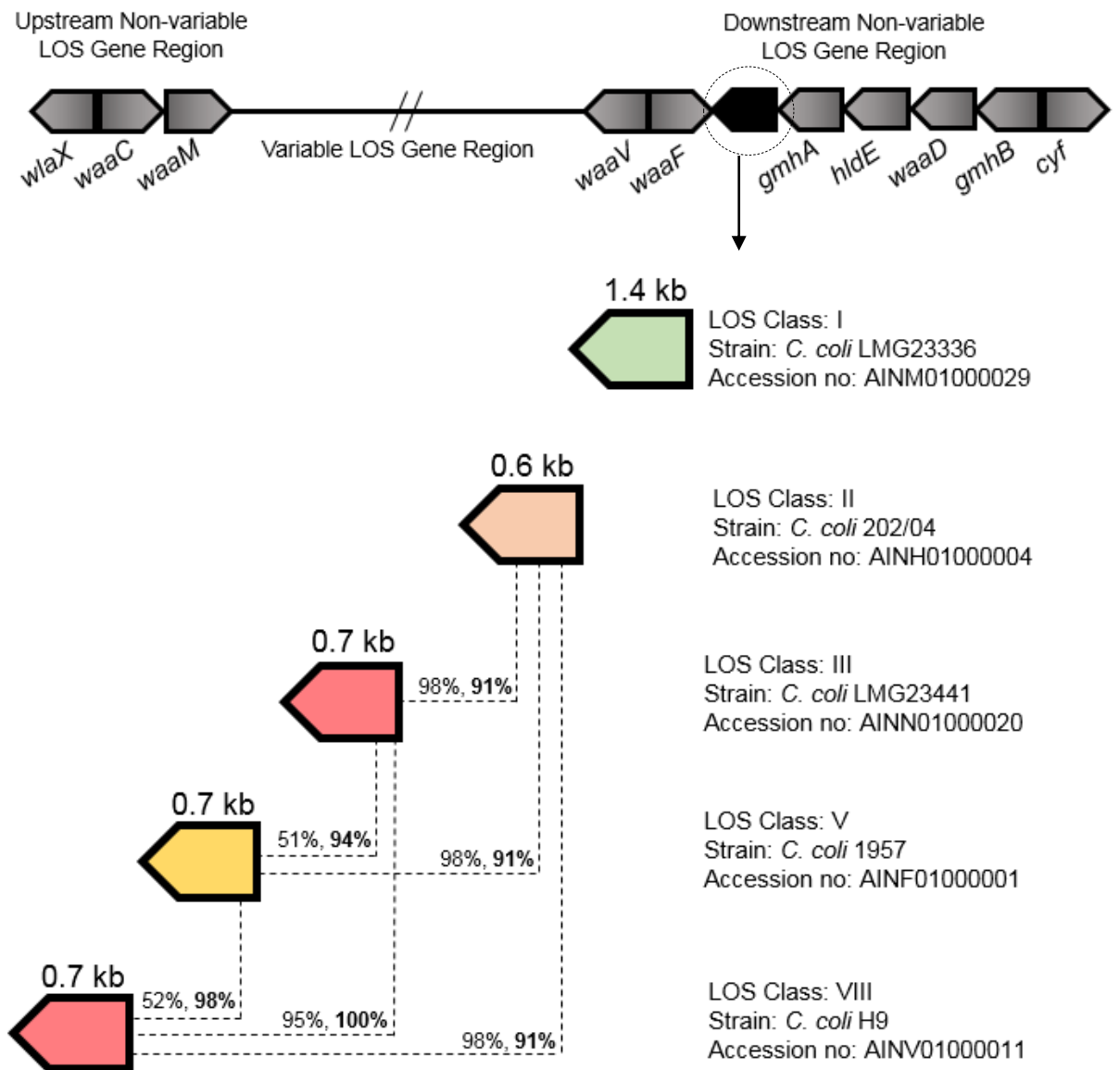
Figure 4.10: Illustration of previously unreported *C. coli* LOS core genes

These genes occur in *C. coli* LOS types (I, II, III, V and VIII) at the same location (between *waaF* and *gmhA*) and vary in size and nucleotide composition. The sizes of these genes, obtained from the GenBank database, are given in kb. Each dotted line links two genes to represent the similarity between them in terms of query cover score (non-bold words) and Megablast identity score (bold words).

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Table 4.2: Proteins encoded by LOS genes in *C. coli* LOS type I, II, III, V and VIII (Data extracted from the GenBank)

Gene Location (Strain; Accession No)	LOS Class	Protein Accession No.	Encoded Protein
45451-46905 (*C. coli* LMG2336; AINM01000029)	I	EIA95603.1	Beta-Kdo transferase
24584-25144 (*C. coli* 202/04; AINH01000004)	II	EIA86515.1	Phosphoheptose isomerase
42860- 43621 (*C. coli* LMG23341; AINN01000020)	III	EIA96492.1	Glycosyltransferase
35579-36334 (*C. coli* 1957; AINF01000001)	V	EIA83727.1	Glycosyltransferase
25177-25926 (*C. coli* H9; AINV01000011)	VIII	EIB11470.1	Glycosyltransferase

4.4. Discussion

4.4.1. Application of bioinformatics for the analysis of *C. jejuni* LOS biosynthesis cluster

81 of 125 *C. jejuni* sequences obtained from GenBank were LOS grouped using the Galaxy built-in MAFFT multiple alignment tool, indicating that the designed Galaxy workflow can successfully classify the complete and annotated *Campylobacter* sequences. Similar results for 81 classified *C. jejuni* sequences were obtained by using Megablast, which validated the Galaxy workflow for the *C. jejuni* LOS locus genotyping. This Galaxy workflow extracts the approximate coordinates (65 kb; predicted to contain the LOS biosynthesis locus sequence) from the unclassified sequences. Ideally, the desire was to make it able to extract the sequence of variable LOS biosynthesis genes from a complete *C. jejuni* WG sequence flanking the conserved LOS biosynthesis genes. Currently, it is unable to detect the exact LOS biosynthesis locus sequence and therefore, it can sometimes extract the partial LOS sequence. This was the reason that only ~50% sequences (39 of 81) were linked to a particular LOS class. This also reflects the fact that Galaxy workflow was more efficient in identifying a LOS group rather than the LOS class. Another limitation of the Galaxy workflow is that it is not applicable to draft (scaffold or contig level) sequences.

The use of each *in silico* method, applied in the current study, has its own pros and cons. Megablast and MAFFT, both can scan a large number of sequence queries (McGinnis and Madden, 2004; Katoh and Toh, 2007). Gene-by-gene searching in a WG sequence and performing a Megablast operation repeatedly is a very time consuming process, however, running a Galaxy-MAFFT workflow once to predict the LOS types consumes less time in comparison to Megablast. The MAFFT progressive approach becomes less reliable when it deals with the aligned sequences of only 25-30% identity (Thompson *et al*., 1994). Megablast hit searching is a biased approach for *Campylobacter* LOS genotyping in contrast to MAFFT alignment in Galaxy. It is important to be stated here that complete or draft sequences deposited in GenBank are also not 100% correct due to occurrence of errors at the experimental or sequenced data analysis stages (Pertsemlidis and Fondon, 2001), which may give false positive results.

In this study, variation in LOS biosynthesis locus was analysed at the nucleotide sequence level in order to determine the presence of clustered whole LOS genes rather than finding the modifications within the LOS gene nucleotides. The identification of several LOS genes within a single sequence excludes the possibility of false-positive results, but not 100%.

4.4.2. Gene content diversity in *C. jejuni* LOS locus

By analysing the distribution of *C. jejuni* LOS locus types within the *C. jejuni* GenBank database, LOS class B (23%) was found to be most common class, whereas, classes A and C (each with 16%) were the second most frequent classes. These findings were concordant with the *C. jejuni* LOS locus class prevalence within the clinical *C. jejuni* isolates, described in the previous chapter, where LOS class B was the most predominant followed by the LOS classes A and C. Class B as the most common LOS class in humans and poultry *C. jejuni* isolates has been reported previously in other studies (Quinones *et al*., 2007; Islam *et al*., 2018). It was discussed in the previous chapter that 50 – 64% strains belong to LOS group 1 related classes (A, B and C) in almost every type of *C. jejuni* population which may be human or poultry. The combined frequency of these three classes, identified within the online *C. jejuni* database (~55%), also falls within the same range. A very high rate of these three classes might occur due to distinctive ability of these classes to synthesise the sialylated LOS structures, which may be advantageous for pathogenesis. In contrast, the other three LOS group 1 related classes (R, V, & M) were absent at the clinical level and infrequent (~2.5%) within the *C. jejuni* worldwide database. Despite having association with the synthesis of the sialylated LOS structures (Houliston *et al*., 2011), these classes were less distributed. The classes, R and M, with only single gene modifications are the variants of LOS type A (Parker *et al*., 2008) and class V with the absence of only one gene (*cj1137c*) is the variant of LOS class C (Richard *et al*., 2013). These loci can undergo gene selection and recombination process and re-arrange into class A, B or C, which might explain the reduced prevalence of these three sialylated LOS producing types.

The LOS group 2 was the second most predominant group in both sorts of *C. jejuni* LOS genotypes distribution analyses. Class P within the clinical local *C. jejuni* collection, whereas, class H within the global *C. jejuni* collection, were found as the most common group 2 linked classes. These contrasting results might be explained because both LOS class P and H share almost similar gene content except for two LOS biosynthesis genes (Orf 26' and Orf28) (Jiang *et al.*, 2010) and therefore few studies have ever considered these classes as two separate classes (Habib *et al.*, 2009; Islam *et al.*, 2014). The LOS class H has been reported previously as the most abundant class in group 2 (Quinones *et al.*, 2007; Ellström *et al.*, 2016).

Within the LOS group 3, class F was the most prevalent class from clinical isolates (6%) and more globally (5%), similar to the findings of Marsden (2007) and Quinones *et al*. (2007). Class K (2%) was the second most common class of group 3, whereas, other LOS classes (I, S, J, D, Q, and N) were less frequent (≤1%). *C. jejuni* sequences in a very small number (< 3%) belonged to group 4 related class G (1.4%) and class L (1%). In contrast, no group 3 related classes (K, I, S, J, D, Q and N) as well as group 4 related classes (G and L) were identified from *C. jejuni* clinical isolates, which may be because of the relatively small size of the collection. A general trend of *C. jejuni* LOS groups distribution (group 1 > group 2 > group 3 > group 4) was similar both from clinical and Genbank derived sequences, which agrees with the previous studies (Marsden, 2007; Quinones *et al*., 2007; Ellström *et al*., 2016; Islam *et al*., 2018).

4.4.3. Gene content diversity in *C. coli* LOS locus

In this study, LOS locus type III was found abundant (41%; n=229 of 564) in the online GenBank database of *C. coli* sequences. Culebro *et al*. (2018) also reported the high frequency of classes III (28%; n=72 of 261) within the agriculture associated *C. coli* strains. The class III was also found prevalent among those 33 *C. coli* strains which were collected by Richard *et al*. (2013) from different sources including human, turkey, chicken, swine and bovine. Within this collection of *C. coli* strains, LOS classes III, II and IV (21% each), I (12%), VI (9%), V & VIII (6% each), and VII (3%) were identified.

C. coli strain 76339 contains the sialic acid biosynthesis genes (*cst*, *neuA*, *neuB*, & *neuC*) as well as sialic acids in the LOS structure (Skarp-de Haan *et al*., 2014; Kolehmainen *et al*., 2019). In the current study, all *C. coli* sequences available in the GenBank database were screened for the presence of these sialic acid biosynthesis genes, but no other *C. coli* strain was found with these genes except the *C. coli* RM4661 which had three sialic acid synthesis genes (*cst*, *neuB* & *neuC*) in its LOS locus. Sialic acid synthesis related genes in *C. coli* strains (maximum one or two in each strain) are mostly located elsewhere in the genome rather than specifically in the LOS and CPS biosynthesis loci (Richard *et al*., 2013). Despite having genes (sialyltransferases) for the synthesis of sialylated LOS structures, a few *C. coli* strains (*e.g. C. coli* 664H2004) are unable to initiate the molecular mimicry or GBS development in humans (Funakoshi *et al*., 2006; van Belkum *et al*., 2009; Culebro *et al*., 2018; Kolehmainen *et al*., 2019).

4.4.4. Association of *C. jejuni* and *C. coli* LOS loci distribution to *Campylobacter* sources

The frequency of LOS genotypes within the pool of human *C. jejuni* isolates from online sequences was comparable to the frequency of LOS genotypes, identified within the collection of *C. jejuni* NGH isolates (Chapter 3). For example, group 3 related class F possessing strains were common in NGH clinical isolates and similarly, class F belonging GenBank *C. jejuni* strains were more frequently isolated from humans (or *Campylobacter* infected patients). Moreover, the frequency trend in LOS group 1 related classes was also similar in NGH *C. jejuni* strains [B (22%), C (20%), A (14%)] versus GenBank *C. jejuni* sequences [B (8%), C (7%), A (6%)]. Humans, chickens, and the animal farm environment were the common isolation sources for *C. jejuni* and almost every *C. jejuni* LOS class was associated with at least one of these sources. Workman *et al*. (2005) screened the faecal samples from 596 animals for the presence of *Campylobacter* species and found that *C. jejuni* is mostly prevalent in humans (63.6%) and chickens (86.6%) and can also be recovered from monkeys (17.1%) and sheep (4.2%).

The most common *C. coli* LOS class III and the second most common class VIII linked *C. coli* strains were mostly isolated from humans which was concordant to a previous study, where half (57%) of the clinical isolates belonged to class III, VIII and II (Culebro *et al*., 2016). All *C. coli* classes were associated with farm environment, suggesting that animal farm water and soil are also important niches for *C. coli*, in addition to chickens. This was in accordance to the previous studies in which agriculture associated *C. coli* was reported as an emerging human pathogen (Sheppard *et al*., 2010, 2013).

4.4.5. Novel genes in *C. jejuni* and *C. coli* LOS biosynthesis clusters

The presence of six *C. coli* LOS genes in *C. jejuni* 1336 and one *C. coli* LOS gene in *C. jejuni* 414 indicated the occurrence of interspecies gene recombination events. *C. jejuni* does not only harbour the genes from *C. coli*, but *C. coli* can also uptake and accumulate *C. jejuni* DNA, especially when they are present in the same niche (Sheppard *et al*., 2013). *C. jejuni* and *C. coli* share 71% of LOS biosynthesis genes and 65% of CPS biosynthesis genes as a consequence of recombination events (Richard *et al*., 2013). Subsequently, transfer of 4 genes in *C. jejuni* 1336 LOS locus from the CPS locus of other *C. jejuni* strains, insertion of *C. jejuni* class L related ORF48 to class C linked *C. jejuni* PT14, and duplication of sialic acid synthesis gene, *neuA*, in *C. jejuni* 34288, indicated that intraspecies gene recombination events have occurred. The duplication phenomenon in *neuA* and *cgtA* LOS genes has been observed previously in *C. jejuni* strains (Richard *et al*., 2013). A few *C. jejuni* genes and their encoded enzymes are functional in both LOS and CPS biosynthesis clusters, for example, *gmhB* (*cj1152c*; phosphatase) gene, and therefore, they are subject to the horizontal transfer between LOS and CPS loci (Karlyshev *et al*., 2005).

In this study, previously unreported LOS genes present in *C. coli* classes III and VIII, were found to be identical structurally (100% similar nucleotide content) and functionally (both encode the glycosyltransferase). The presence of these LOS core biosynthesis genes in *C. coli* classes, III and VIII, might be linked to the high

predominance of *C. coli* LOS type III and type VIII within the online database of *C. coli* sequences. This prediction requires verification with further research.

4.5. Conclusion

A Galaxy pipeline employing the alignment software MAFFT can predict the LOS type associated with a *C. jejuni* strain sequence. It can cluster the sequence with a particular *C. jejuni* reference LOS group where it is unable to assign an individual LOS class. This study validates the use of Galaxy workflow <available @https://usegalaxy.org/u/amberimran/w/workflow-for-*Campylobacter-jejuni-*lipooliogosaccharide-biosynthesis-locus-typing> for the typing of the *C. jejuni* LOS locus. The sequence alignment tools including Megablast and MAFFT, used for the current *in silico* analysis of *C. jejuni* and *C. coli* LOS biosynthesis loci, are not 100% reliable and may incorporate errors in the alignment results. *C. jejuni* strains containing group I related LOS loci continue to be the most prevalent group in GenBank and amongst our clinical isolates. This may be due to the distinctive ability of these group I strains to synthesise sialylated LOS structures, which may be advantageous for pathogenesis. The abundance of *C. jejuni* LOS class B and trend of *C. jejuni* LOS group prevalence (group 1 > group 2 > group 3 > group 4) were similar in both types of *C. jejuni* collections. *C. coli* strains possessing LOS class III are the most common in a worldwide database of *C. coli* and this study represents the first exploration of LOS locus genotype distribution in *C. coli* GenBank database via *in silico* analysis. The novel *C. jejuni* LOS types identified may have arisen due to interspecies (between *C. jejuni* and *C. coli*) and intraspecies LOS genes recombination. The novel *C. jejuni* and *C. coli* LOS genes identified in the current study need to be further characterised in order to understand their roles in the LOS biosynthesis. In addition to poultry, animal farm soil and water are also important sources of *C. jejuni* and *C. coli* transmission to humans.

CHAPTER 5

Validation of a Mutagenesis Strategy to Construct a *Campylobacter coli* RM1875 Mutant

5.1. Introduction

5.1.1. Mutagenesis strategies used previously to construct *Campylobacter* mutants

Several mutagenesis methods have been used previously to mutate the LOS biosynthesis genes in *Campylobacter* species. The most common mutagenesis strategy involves the use of inverse PCR (Wren *et al.*, 1994). In this method, a gene of interest is amplified using standard PCR and the produced amplicon is cloned into a vector. This vector is further used as a template for inverse PCR in order to delete the centre of the gene of interest as well as inserting one or two unique restriction sites. An antibiotic resistance cassette is inserted between the engineered, unique restriction sites and subsequently, the constructed vector is used to introduce a double-crossover event within the bacterial genome. This double-crossover event disrupts the gene of interest with the antibiotic cassette, resulting in defined gene mutagenesis. A *cj0256* mutant in *C. jejuni* 81-176, *galE* mutant in *C. jejuni* 81116, *cj1136* mutant in *C. jejuni* 11168, and *waaF* mutant in *C. jejuni* 84-25 and *C. jejuni* 84-19 strains have been previously constructed using this method to determine the functions of LOS biosynthesis genes (Fry *et al.*, 2000; Cullen *et al.*, 2010; Keo *et al.*, 2011; Javed *et al.*, 2012). For example, the role of, *cj0256* encoded a lipid A phosphoethanolamine (pEtN) transferase (adds pEtN to LOS lipid A), *galE* encoded UDP-glucose 4-epimerase (interconverts UDP-galactose and UDP-glucose), *cj1136* encoded galactosyltransferase (transfers a galactose), and *waaF* encoded heptosyltransferase-II (adds an heptose residue), in the biosynthesis of LOS structure was identified (Fry *et al.*, 2000; Cullen *et al.*, 2010; Keo *et al.*, 2011; Javed *et al.*, 2012).

Another mutagenesis strategy implements the use of the overlapping extension PCR. In this method, upstream and downstream regions of a target gene as well as antibiotic cassette are amplified separately using standard PCR and these

three amplified DNA fragments are then joined using overlapping extension PCR (Horton *et al*., 1990). The overlapping extension PCR product is cloned into a vector, which is further used to introduce a double-crossover and mutagenesis of the gene of interest. Various LOS biosynthesis genes, *gmhA*, *hldE*, *hldD*, *waaC*, *waaF*, *lgtF*, *cj1136* and *cj1138*, *cj1152*, and *cj1165*, have been mutated in *C. jejuni* 11168 and 81-176 by using this method (Iwata *et al*., 2013).

Tn5 (Transposase) based *in vitro* transposition (Goryshin and Reznikoff, 1998) has also been used to construct LOS mutants. With this mutagenesis strategy, a plasmid containing the antibiotic cassette is used as a donor DNA and another plasmid comprising the cloned gene of interest is used as a target DNA. An antibiotic resistance cassette is transposed to the target DNA from the donor DNA using the Tn5 based *in vitro* transposition system. The resulting plasmid containing the antibiotic cassette is then used to introduce a double-crossover and mutate the gene of interest. The mutants of various LOS genes, *cgtA*, *lgtF*, *waaC*, and *neuC1*, in *C. jejuni* 81-176 have been generated using this Tn5 based mutagenesis strategy (Guerry *et al*., 2002; Kanipes *et al*., 2006, 2008). Mutation of *waaC* and *neuC1* genes in *C. jejuni* 81-176, respectively, excised the terminal sugars and sialic acid residues from its LOS structure (Guerry *et al*., 2002; Kanipes *et al*., 2006). Accordingly, the role of *waaC* gene encoded heptosyltransferase-I in the addition of first heptose to Kdo-lipid A and *neuC1* function in the synthesis of sialic acids were explored. Similarly, *cgtA* encoded *N*-acetyl galactosaminyltransferase was examined for its role in the catalysis of *N*-acetyl galactosamine and the *lgtF* gene encoded glucosyltransferase was identified as having dual function in the transfer of a β-1,4-glucose on heptose-I and a β-1,2-glucose on heptose-II (Guerry *et al*., 2002; Kanipes *et al*., 2008). Briefly, LOS gene mutants in various *C. jejuni* and *C. coli* strains have been produced using different mutagenesis methods to investigate the contribution of *Campylobacter* LOS genes in the synthesis of cell-surface LOS structures.

5.1.2. Mutagenesis strategy used in the current study to construct a *C. coli* LOS mutant

This strategy involves a gene, *rpsL*, which encodes a S12 protein - a component of the 30S subunit of prokaryotic ribosome (Figure 5.1) (Calidas *et al*., 2014). The nucleotide composition of the *rpsL* gene defines the structure of the S12 protein, regulates the binding of streptomycin to ribosomes, and determines the streptomycin sensitive (strep^S) or streptomycin resistant (strep^R) phenotype in bacteria (Torii *et al*., 2003; Miller *et al*., 2016). The S12 protein amino acids encoded by codons 43-88 of the strep^S *rpsL* gene produce a site in the S12 protein for streptomycin binding (Llano-Sotelo *et al*., 2009). Streptomycin binds to the 12S protein or 16S rRNA present within the 30S subunit of the ribosome, stabilises the binding of aminoacyl tRNA at the 30S-ribosomal A site, reduces the translocation of these aminoacyl tRNA from the A to P site and consequently, causes mismatching between the mRNA-codons and tRNA-anticodons (Llano-Sotelo *et al*., 2009; Dale *et al*., 2009). The misreading of codons leads to the synthesis of an unstable ribosome-mRNA complex and defective proteins with missense errors, which is detrimental to bacterial growth and causes cell death (Ruusala *et al*., 1984).

It was identified that the strep^S *rpsL* gene becomes dominant over the strep^R gene and bacterial cells maintain the strep^S phenotype when strep^S and strep^R *rpsL* genes are present together in the chromosome of prokaryotes (Lederberg, 1951). This hypothesis was tested in strep^R *E. coli* which became phenotypically strep^S following the transformation with strep^S gene carrying plasmids (Dean, 1981). This principle of strep^S dominance over strep^R was used first time by Skorupski and Taylor (1995) for positive selection and construction of deletion mutants. For this study, a plasmid containing a strep^S gene (*E. coli rpsL* with strep^S phenotype) was introduced into strep^R *Vibrio cholerae* via conjugation of *E. coli* and *Vibrio cholerae* strains. The occurrence of the first DNA exchange event between plasmid and chromosomal DNA produced the strep^S phenotype upon a strep^R background in *Vibrio cholerae* transconjugates. The second DNA exchange event caused the plasmid excision for the loss of strep^S and gain of strep^R as well as the desired mutation in *Vibrio cholerae* transconjugates.

P-site
tRNA

E-site
tRNA

50S

S12

30S

Decoding
center

mRNA

Ribosomal
protein S12
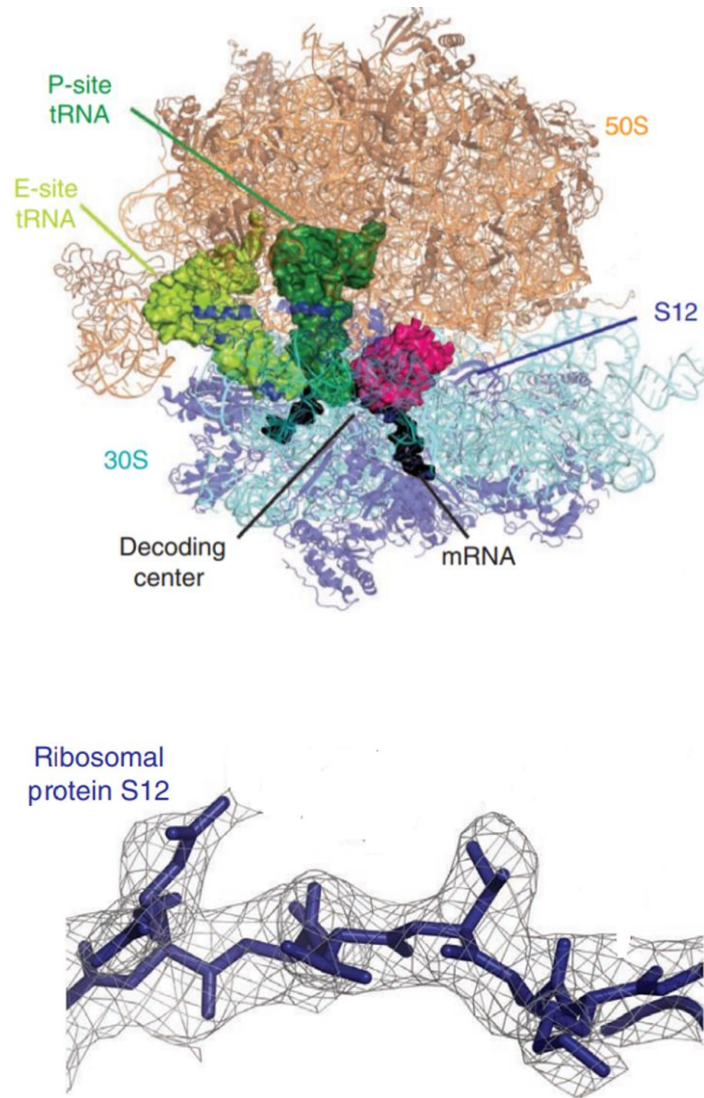
Figure 5.1 (A): Location of the S12 protein in the 30S ribosomal subunit (B): Structure of ribosomal S12 protein (Reproduced from Ng *et al*., 2010).

The method introduced by Skorupski and Taylor (1995) was then applied with few modifications by Hendrixson *et al*. (2001) to construct motility gene (*fliA*, *rpoN*, *cj1189c* and *cj1190c*) deletion mutants in the *C. jejuni* 81-176 strain. In Hendrixson *et al*. (2001), two plasmids were introduced into the bacterial cells by electroporation step-by step rather than by conjugation. In the first step, strep[R] *C. jejuni* 81-176 mutant was developed by replacing its genomic *rpsL* with *C. jejuni* 11168 *rpsL* gene and a vector or suicide plasmid A (pUC19 acts as suicide vector in *Campylobacter*) containing the Cat-*rpsL* (conferring resistance against chloramphenicol and sensitivity against streptomycin) cassette flanked by homologous regions upstream and downstream of gene of interest was electroporated into strep[R] *C. jejuni* 81-176 mutant. The occurrence of homologous recombination excised the gene of interest and incorporated the Cat-*rpsL* cassette at the targeted gene location. The transformed *C. jejuni* cells (intermediate mutants) were selected for the chloramphenicol resistance and electroporated with another plasmid (suicide plasmid B) containing the homologous regions upstream and downstream of genes of interest. The occurrence of a second homologous recombination in the intermediate mutants excised the Cat-*rpsL* cassette and subsequently, final *C. jejuni* 81-176 deletion mutants were selected for the gain of strep[R]. Marsden *et al*. (2009) created intermediate deletion mutants of *C. jejuni* 11168 (Δ*cj1138c–cj1144c*) by using the mutagenesis strategy of Hendrixson *et al*. (2001), where a Cat-*rpsL* cassette replaced the 7 LOS biosynthesis genes (*cj1138c–cj1144c*). In Hendrixson *et al*. (2001), Cat-*rpsL* cassette was subsequently removed from the intermediate mutants to create the final, defined deletion mutants. In comparison, Marsden *et al*., 2009 replaced the Cat-*rpsL* cassette in an intermediate *C. jejuni* 11168 Δ*cj1138c–cj1144c* mutant with a kanamycin cassette to construct a final, large deletion mutant (Δ*cj1132c-cj1152c*).

The approach of creating the smaller and then larger LOS cluster deletion mutants established by Marsden *et al*. (2009) and cassette eliminating *rpsL*-based mutagenesis strategy developed by Hendrixson *et al*. (2001) are aimed to be used in combination in the current study to construct a LOS deletion mutant of *C. coli* RM1875 strain. Figure 5.2 explains this mutagenesis procedure, which will be used to generate two smaller deletion mutants (with and without Cat-*rpsL*

cassette) and two larger deletion mutants (with and without Cat-*rpsL* cassette) in *C. coli*. For smaller deletion, two plasmids (pAH1, pAH3) and for larger deletion, two plasmids (pAH2, pAH4), will be constructed. Hendrixson *et al*. (2001) cloned the *C. jejuni* 11168 *rpsL* gene with its own promoter into pUC19. In the current study, *Helicobacter pylori rpsL* gene and *C. jejuni* 11168 *rpsL* gene promoter, cloned into pUC19, were used. *C. jejuni* 11168 *rpsL* in the suicide plasmid was replaced with *Helicobacter pylori rpsL* gene for reducing the occurrence of false gene conversions during the site-directed mutagenesis in *Campylobacter* (Haigh, unpublished data).
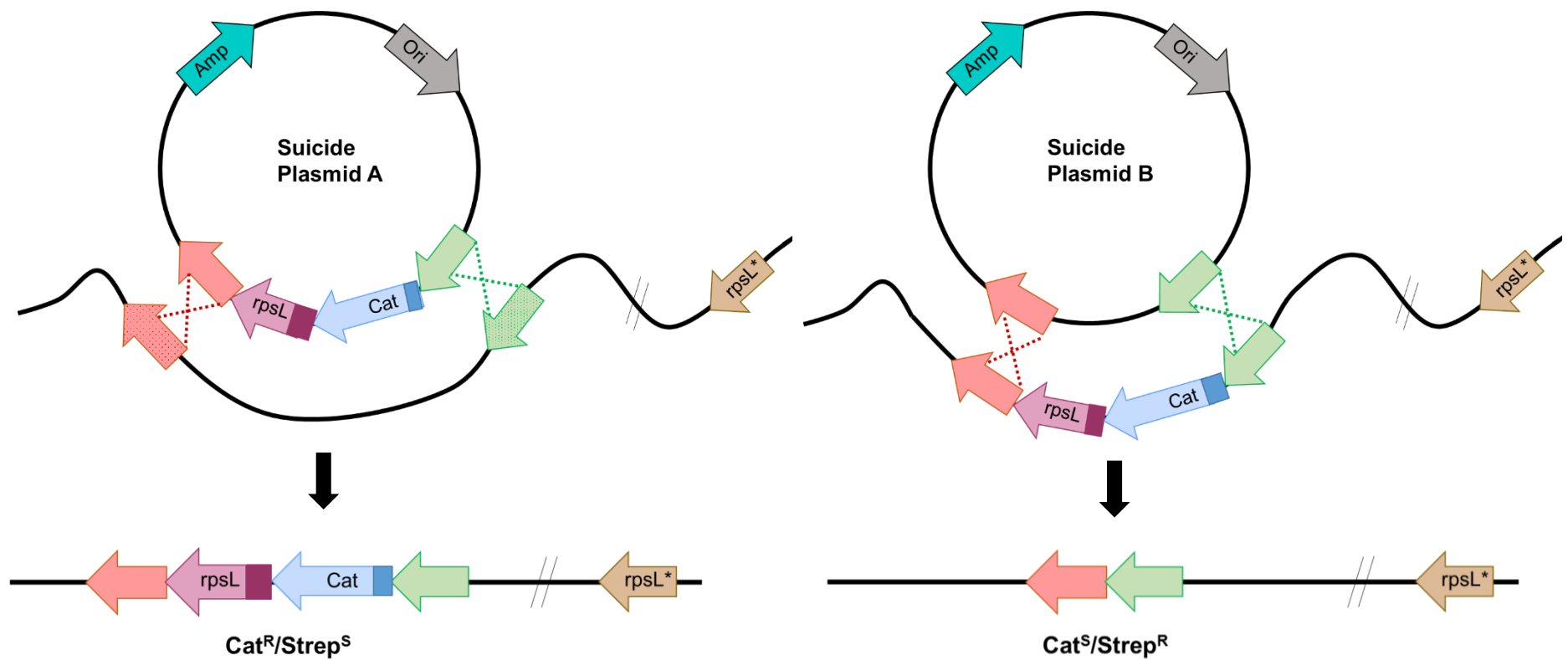
Figure 5.2: Strategy for *rpsL*-based positive selection mutagenesis (Adapted from Hendrixson *et al*., 2001).

Green and pink arrows represent the cloned genes or gene sequences in suicide plasmid A & B which are homologous to the mutation site flanking gDNA sequences and introduce the double homologous recombination (pink and green x marks). The first double homologous recombination events between suicide plasmid A and bacterial DNA produce the mutation of interest and replace it with Cat-*rpsL* cassette. The intermediate mutant with strep^S (*rpsL* domination on *rpsL** gives streptomycin sensitivity) and chloramphenicol resistance is selected. Subsequently, the second double homologous recombination between suicide plasmid B and intermediate mutant gDNA excises the Cat-*rpsL* cassette from its gDNA and the final mutant with strep^R phenotype (the presence of only *rpsL** gene gives streptomycin resistance) is selected. Amp: Ampicillin resistance gene; Ori: Origin of replication (replicates in *E. coli* but do not replicate in *Campylobacter*).

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5.2. Aims and Objectives

A large deletion mutant (Δ*cj1132c-cj1152c*) of the *C. jejuni* 11168 strain was produced previously by deletion of LOS biosynthesis gene region from *cj1132c* to *cj1152c* from a small deletion mutant of *C. jejuni* 11168 (Δ*cj1138c-cj1144c*) (Marsden *et al*., 2009). The phenotypic characterisation of the *C. jejuni* Δ*cj1132c-cj1152c*, lacking the almost entire LOS biosynthesis gene cluster, revealed that cell surface LOS structures hugely impact the *C. jejuni* potential of invasion into host cells, natural transformation, and resistance against antibiotics. Similarly, this study aims to determine the impact of LOS gene deletion on the *C. coli* cell phenotype by validating the *rpsL*-based mutagenesis strategy, using this strategy to construct a *C. coli* LOS biosynthesis gene region deletion mutant, and characterising the constructed *C. coli* LOS mutants. To generate a smaller LOS deletion (ΔORF15-ORF4) and larger deletion (Δ*waaD-waaC*) in the LOS biosynthesis gene region, *C. coli* RM1875 strain (Accession number: CP007183.1) will be used.

5.3. Results

5.3.1. Mutagenesis of *C. coli* RM1875

5.3.1.1. Identification of a SNP in *C. coli* RM1875 *rpsL* gene

The first step of the mutagenesis procedure was to introduce a spontaneous single-base mutation within the *rpsL* gene of *C. coli* RM1875 to establish the streptomycin resistance in this wild-type (WT) *C. coli* RM1875 strain. However, *C. coli* RM1875 strain was already resistant to streptomycin in its wild type state when grown on MHA with various streptomycin concentrations (100-1000 µg/ml), higher than its Minimum Inhibitory Concentration (MIC; 50 µg/ml). To further confirm that *C. coli* RM1875 was already resistant to streptomycin and there was no need to produce a spontaneous mutation in the *rpsL* gene, a 250 bp *C. coli* RM1875 *rpsL* amplicon was generated using PCR and Sanger sequenced. The *rpsL* gene specific primer and all other primers used in this chapter are given in Table 2 in Appendix-I. A single nucleotide polymorphism (SNP; A to G) in codon 43 of *C. coli rpsL* gene was present. This change replaces a lysine with an arginine in the S12 protein resulting in streptomycin resistance, (Figure 5.3). In subsequent steps of the mutagenesis procedure, *C. coli* RM1875 on a strep[R] *rpsL* background was used.

A

GTG CCT ACC ATA AAT CAA TTG GTT AGA AAA GAG CGC AAA AAA GTT TTA GAA AAA TCT AAA TCT
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
GTG CCT ACC ATA AAT CAA TTG GTT AGA AAA GAG CGC AAA AAA GTT TTA GAA AAA TCT AAA TCT

CCA GCG CTT AAA AAT TGT CCA CAA AGA AGG GGA GTT TGC ACT AGG GTT TAT ACT ACA ACA CCT
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
CCA GCG CTT AAA AAT <u>TG**C**</u> CCA CAA AGA AGG GGA GTT TGC ACT AGG GTT TAT <u>AC**A**</u> ACA <u>AC**T**</u> CCT
                        27   39   41

<u>AAA</u> AAA CCA AAC TCA GCG TTA AGA AAA GTT GCC AAA GTA AGA CTT ACT AGT GGC TTT GAA GTG
 ||| ||| ||| ||| || ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| || ||| ||| ||| ||| |||
<u>A**G**A</u> AAA CCA AAC TCA <u>GC**A**</u> TTA AGA AAA GTT GCC AAA GTA AGA CTT ACT <u>AG**C**</u> GGC TTT GAA GTG
 43   48   59

ATC AGC TAT ATC GGC GGT GAA GGT CAT AAC TTG CAA GAA CAC AGC ATT GTT TTA GTG CGT GGT
||| ||| ||| ||| ||| ||| ||| ||||||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
<u>AT**T**</u> AGC TAT ATC GGC GGT GAA GGT <u>CA**C**</u> AAC <u>C**TA**</u> CAA GAA CAC AGC ATT GTT TTA GTG CGT <u>GG**G**</u>
64   72   74   84

GGT AGG GTA AAA GAC TTA CCA GGG GTT AAA TAT CAC ATC GTT CGT GGT GCT CTT GAT ACA GCA
||| ||| ||| || ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
GGT AGG GTA AAA GAC TTA CCA <u>GG**T**</u> <u>GT**G**</u> AAA TAT CAC ATC <u>GT**G**</u> CGT GGT <u>GC**G**</u> CTT GAT <u>AC**T**</u> <u>GC**G**</u>
                             92   93   98   101   104 105

GGT GTT GCA AAA AGA ACA GTT TCT CGT TCT AAA TAT GGT GCT AAA CGT CCT AAA
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
GGT GTT GCA AAA AGA ACA GTT TCT CGT TCT AAA TAT GGT <u>GC**A**</u> AAA <u>CG**C**</u> CCT AAA
                                                    119   121

B
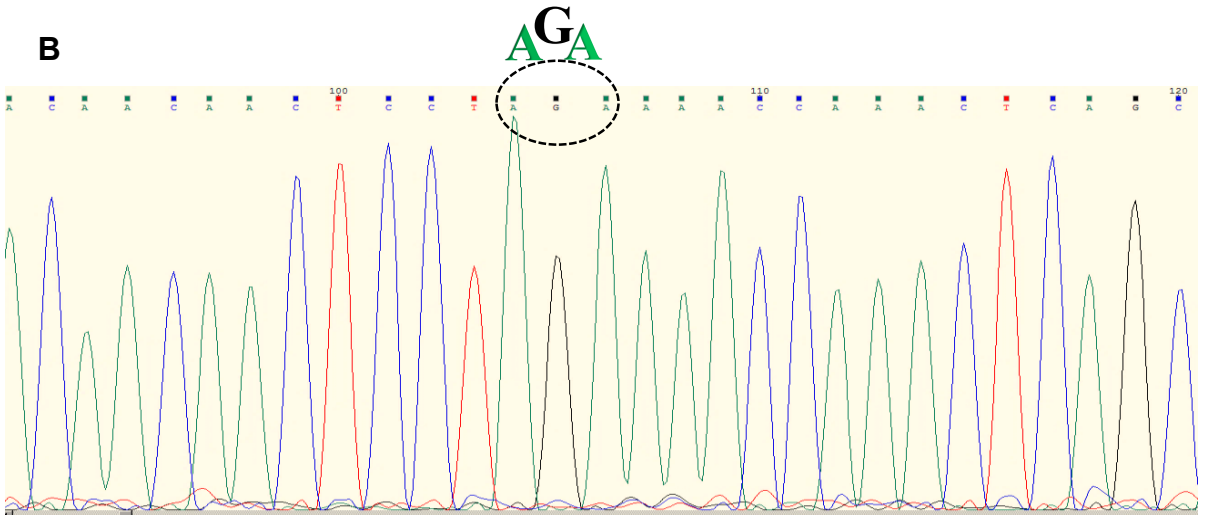
C

VPTINQLVRKERKK**+**LEKSKSPALKNCPQRRGVCTRVYTTTP**K**KPNSALRKVAKVRLTSGFE
|||||||||||||||| ||||||||||||||||||||||||||||| ||||||||||||||||||||||
MPTINQLVRKERKK**V**LEKSKSPALKNCPQRRGVCTRVYTTTP**R**KPNSALRKVAKVRLTSGFE

VISYIGGEGHNLQEHSIVLVRGGRVKDLPGVKYHIVRGALDTAGVAKRTVSRSKYGAKRPKA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VISYIGGEGHNLQEHSIVLVRGGRVKDLPGVKYHIVRGALDTAGVAKRTVSRSKYGAKRPKA

Figure 5.3 (A): Alignment between *C. jejuni* 11168 *rpsL* sequence (pink) and *C. coli* RM1875 *rpsL* sequence (green) for the identification of a SNP (A to G) in codon 43 of *C. coli* RM1875 *rpsL* gene; (B): SNP confirmation by Sanger sequencing of PCR amplified *rpsL* gene; (C): Pairwise alignment between 11168 and RM1875 S12 protein sequences; where the replacement of K (lysine) with R (arginine) in the amino acid sequence of S12 protein is indicated by a blue arrow.

5.3.1.2. Construction of plasmids, pAH1 and pAH2

The plasmid pAH1 was constructed by cloning those regions of LOS biosynthesis genes, ORF4 and ORF15, in pUC19 which flanked the LOS gene region of interest to be deleted (~9 kb). The constructed pAH1 plasmid was used to introduce the double homologous recombination events in gDNA of *C. coli* RM1875 and generate a smaller deletion mutant (ΔORF15-ORF4) in *C. coli*. Figure 5.4 illustrates that gene sequences of the *C. coli* RM1875 LOS biosynthesis ORF15 (795 bps: 642827-643620) and ORF4 (758 bps: 652735-653481), flanking the mutation of interest, were PCR amplified. Two restriction sites specific for *Sac*I and *Xho*I enzymes in the amplified PCR product of ORF15 were added correspondingly with two primers, S1-F and S1-R. Similarly, two sites for *Xho*I and *Kpn*I restriction in the amplified PCR product of ORF4 were incorporated using S2-F and S2-R primers. The amplified ORF15, restricted with *Sac*I and *Xho*I, and amplified ORF4, restricted with *Xho*I and *Kpn*I, were ligated with common *Xho*I restricted, sticky ends. Simultaneously, the ligated ORF15 and ORF4 were cloned into *Sac*I and *Kpn*I restricted pUC19 to construct a plasmid, pAH1.

Figure 5.5 (A) represents a map of 4233 bp pAH1 and indicates the annealing positions of different primers (pUC-F, pUC-R, S1-F, S1-R, S2-F, S2-R), used for the confirmation of cloning of ORF15 and ORF4 into pUC19. PCR with pUC-F & pUC-R primers amplified the cloned ORF15 and ORF4 gene sequences together and gave a 1755 bps PCR product (Figure 5.5 B), indicating the presence of these two ORFs in pAH1. To further confirm the presence of these *C. coli* LOS biosynthesis gene regions into pAH1, a 1636 bps PCR product was obtained with pUC-F and S2-R primers and a 1684 bps PCR product was amplified using a set of S1-F & pUC-R primers. These PCR positive results, sequence analysis of PCR products, and restriction digestion of pAH1 with *Sac*I, *Xho*I, and *Kpn*I (Figure 5.6), confirmed the successful cloning of ORF15 and ORF4 into pUC19.

A

YSQ_03240 AHK72997.1
YSQ_03265 AHK73002.1
YSQ_03285 AHK73006.1
YSQ_03260 AHK73001.1
YSQ_03275 AHK73004.1
YSQ_03290 AHK73007.1
YSQ_03235 AHK72996.1
YSQ_03255 AHK73000.1
YSQ_03270 AHK73003.1
YSQ_03250 AHK72999.1
YSQ_03280 AHK73005.1
YSQ_03245 IK72998.1

PCR amplified ORF15 (642827-643620)

PCR amplified ORF4 (652735-653481)
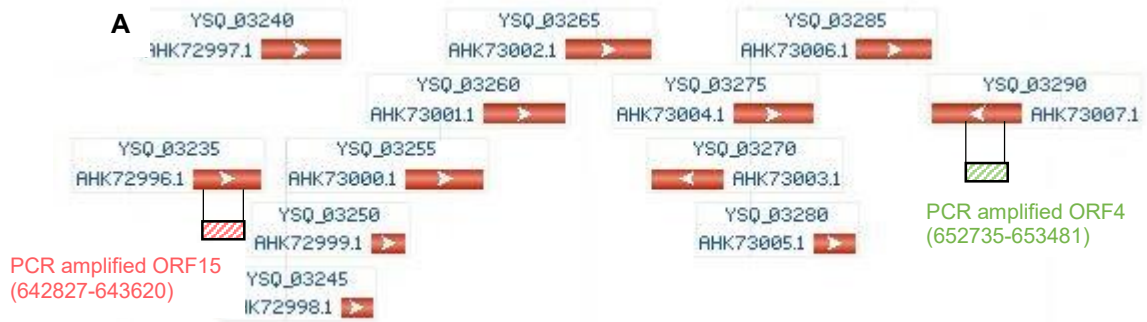
ORF15 (642827-643620)

SacI

CCC<u>GAGCTCT</u>AGTGGTACGAGGCTTTATC→ Primer S1-F

AGTCCGACGTTGTAAACGACGGCCAGTGAATTC<u>GAGCTC</u>TAGTGGTACGAGGCTTTATCCTATCACTTTAACTTTATGTAAGCAGCTTTTGCCAA
TTTATGATAAGCCCATGATTTACTATCCTCTATCTGTTTTGATGTTGGCTAAAATTCGCGAAGTCTTGATTATCTCCACGCCTAAAGATACGAGTA
AATTTCAAGAGCTTTTTGGTGATGGTTCTTGGCTTGGCATGGAAATTTCGTATTGTGTACAAGAGCAGCCAAAAGGCTTGGCAGAAGGACTTAT
TTTAGCGGATAGTTTTATAAAAAATGATGATATAGCTTTAATTTTAGGGGATAATATTTTTTATGGACAAGGATTTAGCGATTTGTTGCAAAATG
CTAAAAATGATTCTAAAAATGGCTATGCTAGTATATTTTCTTATCATGTAAAAGATCCGGATCGTTTTGGTGTAGCTGAAATTTCTAAAAATGGC
GAGGTTTTAAGTTTGGAAGAAAAACCTCAATATCCTAAGAGCAATTATGCGGTAACTGGGCTTTATTTTTATGATAACTCAGCTATTGAAATAGC
AAAAAGTGTAAAACCTAGCTCTAGAGGAGAACTTGAAATTACTGATGTTAATATAGAATATTTGAAACAAAATAGATTAAAGTCGCAAATTTTA
GGACGAGGTTTTGCTTGGATTGATACAGGTACGCATGACAGCTTAATAGAAGCTTCAGAATTTGTGCAAACAATAGAACTTAGACAAGGATACA
AGATTGCATGTTTGGAAGAAATTGCTTTTCGAAATAACTGGATAGATGAGAGAAGATTGTTAGATCGCGCAAAGC<u>CTCGAG</u>CGGCCTATCGAC
AATACTGATAAGATAATATGTAATTAAATACTGT

Primer S1-R ←CAATCTAGCGCGTTTCG<u>GAGCTC</u>GCC

XhoI

ORF4 (652735-653481)

XhoI

CCG<u>CTCGAG</u>TAAATCTTATTGGCGCTTGC → Primer S2-F

CAGACAAATCCGTTAGA<u>CTCGAG</u>TAAATCTTATTGGCGCTTGCAAAAAGAATCATACCAAAATGTATATCTTCATTGATGATATAATTTACAAAT
TTAAGTTTAATTTTCTTTAAAAAAATTAAAATCAATCATCCCACCCCAACTAAAAGAAATATCTCTTGACCCAACCTCTAAAGCTCTTTTAGCATAAT
CTTTTGGGTTTATAATGCGTTCTTCTTTATAGGAGGAAATTTGCATTCTTGTTTTTCGTCCTTTATCTTCAATACCTTTTTCATAAACACACAGATGA
TCAAACCACAATACATCAACGTCTTGCATTCGTTTGGCACATTCTTCAATGCAGTTTAATTCCCAAGAATTATCAGAATCTAAGAAAATTAAATAA
TCAATATCAGGAGCTTCTAGATTTTTTTCTATAAAAGTATTTTTATTTTTGTAAATTTTATAAACATTTTGCGGATTTTCATTTGCAACGGTAAAGG
TGTAAAAACCATCAATTTCATTTGAAAAATGAGTTTCATACTCTTTGTAAAAGAATTCTATCCCTGTGTTTCTAGCTGAACTTAGTCCCCCATTTTC
TTTATCAAATAAAGTAATTCTTTTGTCTTTTAAGGCATATTCCTTAGCTATATTCAATGAATTCTCATCGGTGCTACCATCATTTACAAGTATAATTT
CTAAATTAACATAGGTTTGATTGATGACGCTTTCTAAGCATTCTCTTAAGTATTTTTTCTACATTATAGATAGGGATTACTATGCCTACAGTTC<u>GGT
ACC</u>CGGGGATCCTCTAGAG

Primer S2-R ← CCCTAATGATACGGATGTCAAG<u>CCATGG</u>TGC

KpnI

B

1000 bps
800 bps
600 bps
400 bps
200 bps

795 bps ORF15   758 bps ORF4
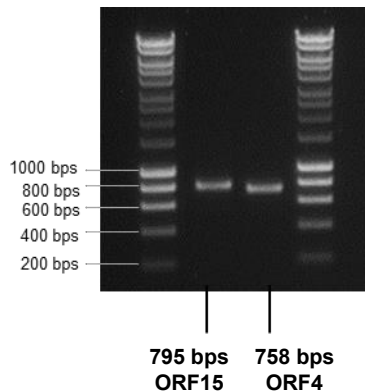
Figure 5.4 (A): A representation of location of ORF15 (sequence from 642827 to 643620; upstream) and ORF4 (sequence from 652735 to 653481; downstream) in the LOS biosynthesis cluster of *C. coli* RM1875 gDNA. (B): PCR amplification of 795 bps ORF15 and 758 bps ORF4.

ORF15 present upstream of the point of mutation was amplified using S1-F (adding *Sac*I restriction site) and S1-R (adding *Xho*I restriction site) primers. ORF4 present downstream of the point of mutation was amplified using S2-F (adding *Xho*I restriction site) and S2-R (adding *Kpn*I restriction site).
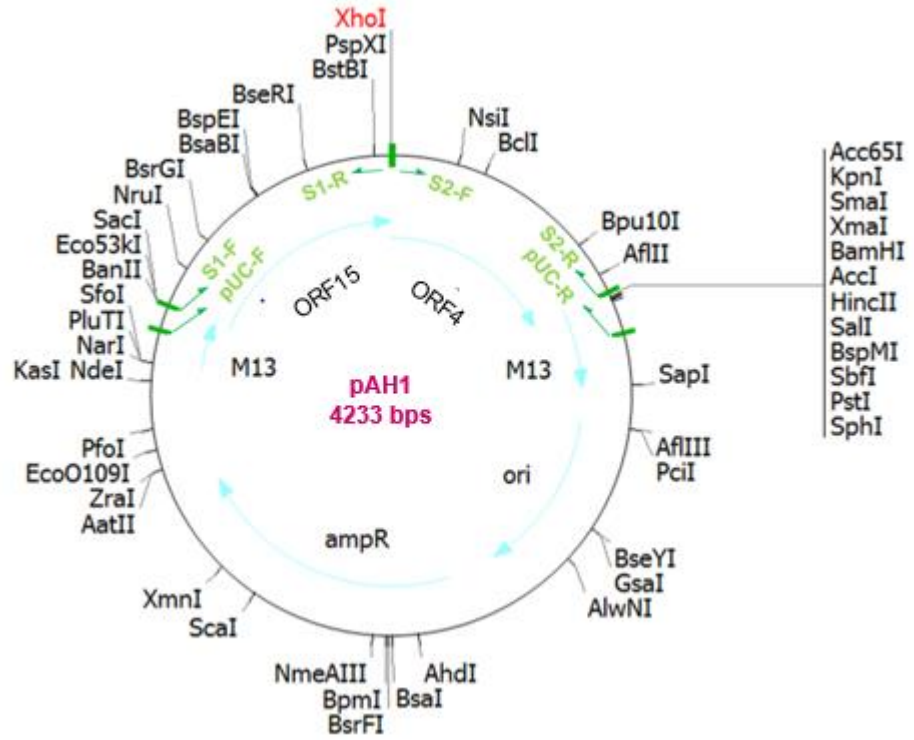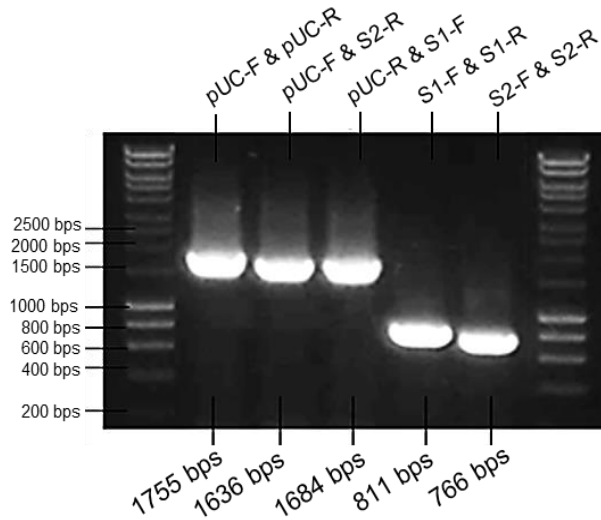
Figure 5.5 (A): A map of 4233 bps pAH1, indicating the annealing positions of different primers (pUC-F, pUC-R, S1-F, S1-R, S2-F, and S2-R) and representing the position of cloned ORF4 and ORF15 in Multiple Cloning Site; **(B): PCR amplicons of expected sizes on gel**, obtained using plasmid specific primer pair (pUC-F & pUC-R), plasmid-gene specific primers sets (S1-F & pUC-R; pUC-F & S2-R), and cloned gene specific primer pairs (S1-F & S1-R; S2-F & S2-R) to confirm the cloning of ORF15 and ORF4 into pUC19.
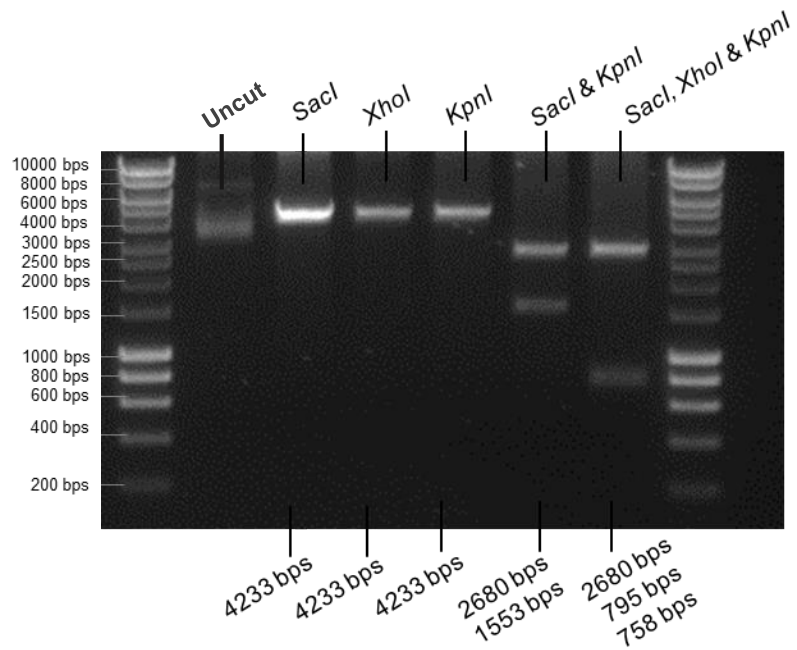
Figure 5.6: Confirmation of ORF15 and ORF4 cloning into pUC19 by pAH1 restriction digest

Single restriction with *Sac*I, *Xho*I and *Kpn*I linearised the plasmid (4233 bps) and digestion with *Sac*I and *Kpn*I gave two fragments (2680 bps pUC19, 1553 bps *Xho*I-ligated ORF15 and ORF4). Restriction of pAH1 with *Sac*I, *Xho*I and *Kpn*I produced three fragments (2680 bps pUC19, 795 bps ORF15, and 758 bps ORF4).

The plasmid pAH2 was also constructed to generate a larger *C. coli* deletion mutant or a mutant with deletion of those LOS biosynthesis genes (Δ*waaD-waaC*) which flanked the smaller deletion (ΔORF15-ORF4) in *C. coli* LOS biosynthesis locus. *Sac*I & *Xho*I restricted *waaD* (620 bps: 636937-637557) and *Xho*I & *Xba*I restricted *waaC* (709 bps: 657041-657750) were ligated into pUC19 to construct a 4007 bps plasmid, pAH2. This was also confirmed by PCR and restriction digests (data not shown).

5.3.1.3. Inserting the Cat-*rpsL* cassette into pAH1 and pAH2

A 1510 bps Cat-*rpsL* cassette (containing *C. jejuni* 11168 *rpsL*^{S} gene promoter, *Helicobacter pylori* strep^S *rpsL* gene and a chloramphenicol acyltransferase encoding Cat gene) was extracted from a plasmid, pRDH315, by restriction of pRDH315 with *Xho*I. Plasmids pAH1 and pAH2 were also restricted with *Xho*I to ligate with *Xho*I digested Cat-*rpsL* cassette. The plasmids, pAH1 and pAH2, after insertion of Cat-*rpsL* cassette were named as pAH3 and pAH4, respectively.

The figure 5.7 (A) represents a map of 5743 bps pAH3 and shows the binding position of different primers (pUC-F, pUC-R, S1-F, S1-R, S2-F, and S2-R), most importantly of catR primer, which was used for the confirmation of cloning and right orientation of Cat-*rpsL* cassette into pAH1. A PCR product of expected size (2111 bps) amplified using plasmid specific pUC-F primer and Cat-*rpsL* cassette specific catR primer (Figure 5.7 B) and restriction digestion of pAH3 with *Sac*I, *Xho*I, and *Kpn*I (Figure 5.8), confirmed the presence of Cat-*rpsL* cassette between ORF15 and ORF4, as well as, its correct orientation into pAH3. The presence of Cat-*rpsL* cassette in pAH4 was confirmed similarly by performing PCR assays and restriction digests (data not shown).

A

B
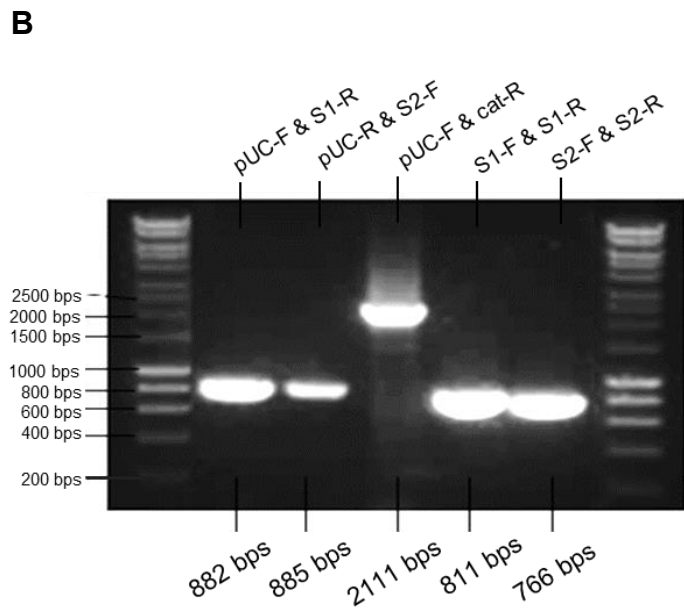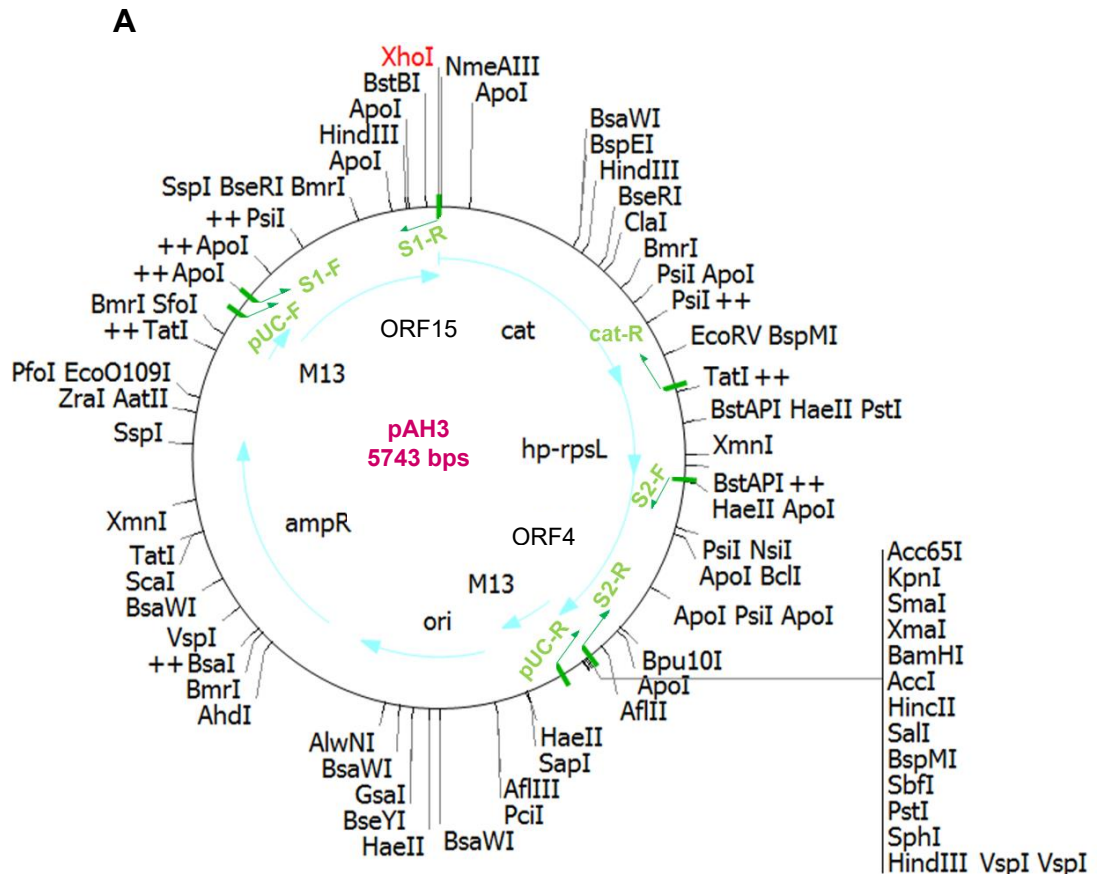
Figure 5.7 (A): A map of 5743 bps pAH3, indicating the position of Cat-*rpsL* cassette between ORF 4 and ORF15 as well as annealing positions of different primers (pUC-F, pUC-R, S1-F, S1-R, S2-F, S2-R, and cat-R); **(B): Confirmation of cloning and orientation of cat-*rpsL* cassette into pAH1 by PCR**, 2111 bps PCR product amplification with pUC-F and cat-R primers confirmed the presence of Cat-*rpsL* cassette in right orientation into pAH1. PCR assays performed with other primer pairs confirmed the intact presence of ORF15 and ORF4 into pAH3.
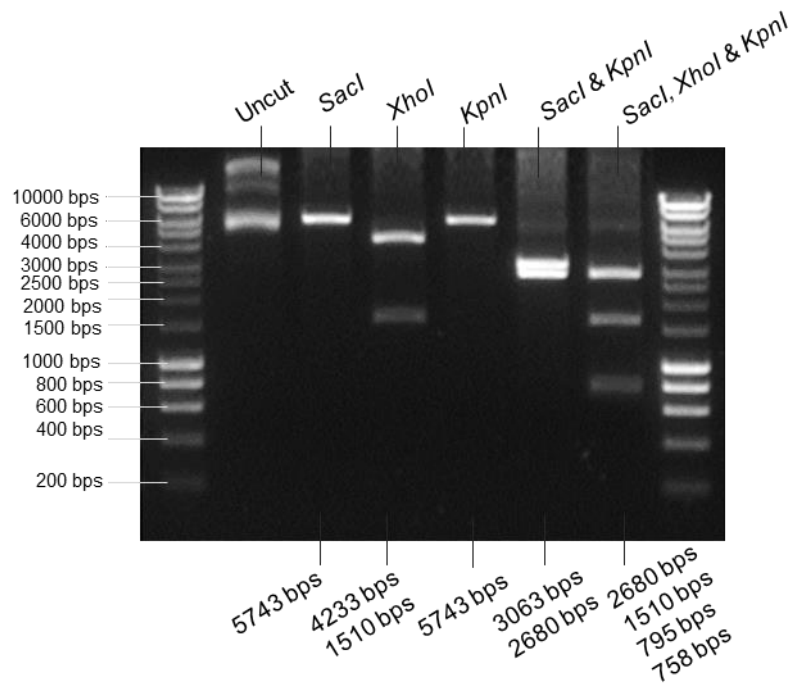
Figure 5.8: Confirmation of Cat-*rpsL* cassette insertion into pAH3 by restriction digest

Single restriction with *Sac*I and *Kpn*I linearised the pAH3 (5743 bps). A single digestion with *Xho*I gave two fragments (4233 bps pAH1 and 1510 bps cat-*rpsL* cassette). Double digestion with *Sac*I and *Kpn*I gave two fragments (2680 bps pUC19, 3063 bps ligated ORF15, ORF4 and cat-*rpsL* cassette). Restriction of pAH3 with *Sac*I, *Xho*I and *Kpn*I produced four fragments (2680 bps pUC19, 1510 bps cat-*rpsL* cassette, 795 bps ORF15, and 758 bps ORF4).

5.3.1.4. Integration of Cat-*rpsL* cassette into WT *C. coli* RM1875

The integration of Cat-*rpsL* cassette into the *C. coli* RM1875 gDNA was confirmed by performing PCR with gDNA of *C. coli* RM1875 mutant strain and Cat-*rpsL* cassette specific primers (cat-F & cat-R) as it is demonstrated in figure 5.9 (B). It was also confirmed by growing the *C. coli* RM1875 mutant strain on MHA with 20 µg/mL chloramphenicol. This chloramphenicol resistant *C. coli* strain should have exhibited strep^S phenotype. Conversely, it was found strep^R when tested at various concentrations of streptomycin (100 µg/mL-1000 µg/mL), which were higher than its MIC (50 µg/mL). Further, a PCR assay with *cat*-F and SS2-R primers showed positive results and indicated the presence of the Cat-*rpsL* cassette upstream of the ORF4 (Figure 5.9 C). However, the position of Cat-*rpsL* cassette next to the ORF15 (as demonstrated in figure 5.9 A) could not be confirmed using SS2-F and cat-R primers even after making many attempts and using alternative primer pairs. By integration of this cassette, 10 LOS biosynthesis genes present between ORF4 and ORF15 should have been deleted. Unexpectedly, all LOS genes were found present in the gDNA of *C. coli* RM1875 mutant strain after PCR based analysis of the gene deletion site. The positive PCR results for the presence of a LOS biosynthesis gene, *YSQ_03250*, are illustrated in figure 5.9 (D); this is only an example to illustrate that LOS genes were not deleted, despite the insertion of pAH3 derived Cat-*rpsL* cassette into the gDNA of *C. coli* RM1875 mutant. It was assumed that Cat-*rpsL* cassette might have located upstream of the ORF4 in the LOS biosynthesis gene cluster of the *C. coli* RM1875 mutant, but without deletion of any LOS biosynthesis gene.

The gDNA of the *C. coli* RM1875 was WG sequenced using the Illumina platform to identify the point of Cat-*rpsL* cassette integration into it. The analysis of the *C. coli* RM1875 mutant sequence (33 contigs) demonstrated that the entire pAH3 plasmid sequence had integrated into the chromosomal DNA. The pairwise alignment between reference WT *C. coli* LOS sequence and mutant *C. coli* sequence confirmed that ampicillin resistance gene (Amp), origin of replication (Ori), cat-*rpsL* cassette, and pAH3 derived ORF4 region, all were present intact in *C. coli* RM1875 WG sequence contig 15. The only exception was pAH3 derived ORF15 whose sequence was found split in contig 15. 1-129 bps at the proximal end of contig 15 sequence were the 1-129 bps of cloned ORF15 and 5078 to

5203 bps present at the distal end of contig 15 were the 669-794 bps of cloned ORF15. These split ORF15 regions flanked the remaining, integrated pAH3 sequence (Figure 5.10 B). ORF15 sequence from base 130 to 668 was missing in the reads of contig 15; this was the reason that LOS deletion screening SS2-F primer could not bind with the gDNA of mutated *C. coli* RM1875 strain and linkage of 5' end of cat-*rpsL* cassette with ORF15 could not confirm by PCR. The PCR results (given in figure 5.9 C), indicating the location of cassette upstream of the ORF4, were positive because of the pAH3 integration into the gDNA.

The exact location of the plasmid integration into the *C. coli* RM1875 gDNA could not be determined as the whole contig 15 had the plasmid sequence. The presence of intact LOS in another contig (contig 1) indicated no disruption in the LOS biosynthesis region of mutated *C. coli* RM1875 strain. A view of contig 1 reads obtained using Artemis software is given in figure 5.10 (A) which shows that the contig 1 sequence reads containing mutant *C. coli* LOS locus sequence align to the reference or WT *C. coli* RM1875 LOS cluster sequence with no gap. This data indicate that rather than introducing a double-crossover event to generate a deletion in the LOS biosynthesis region, the plasmid itself has randomly integrated somewhere else in the gDNA of *C. coli* RM1875 and this requires further investigation.
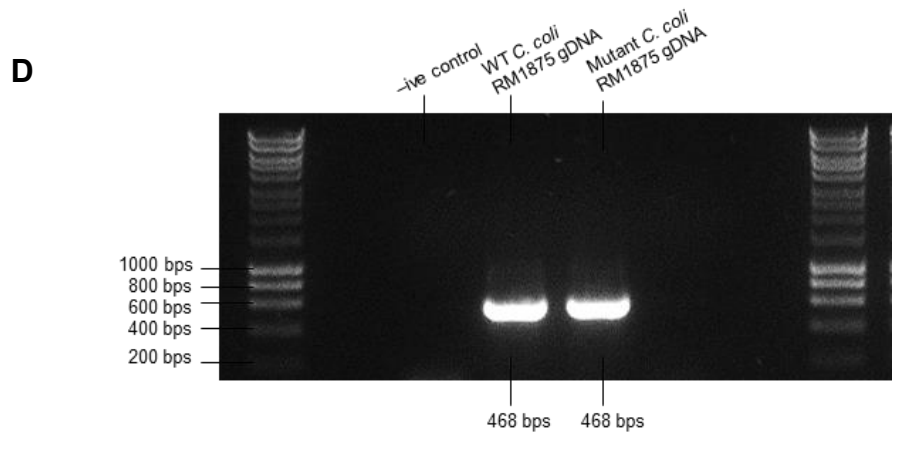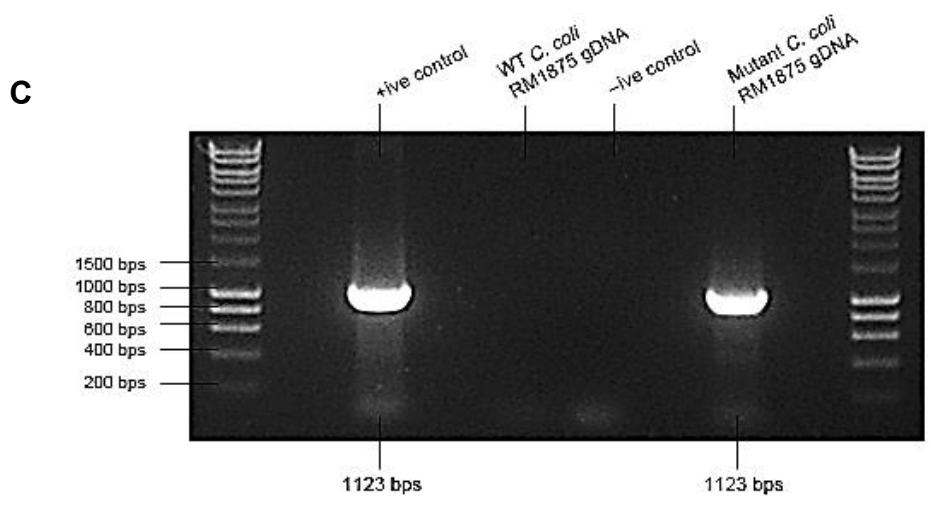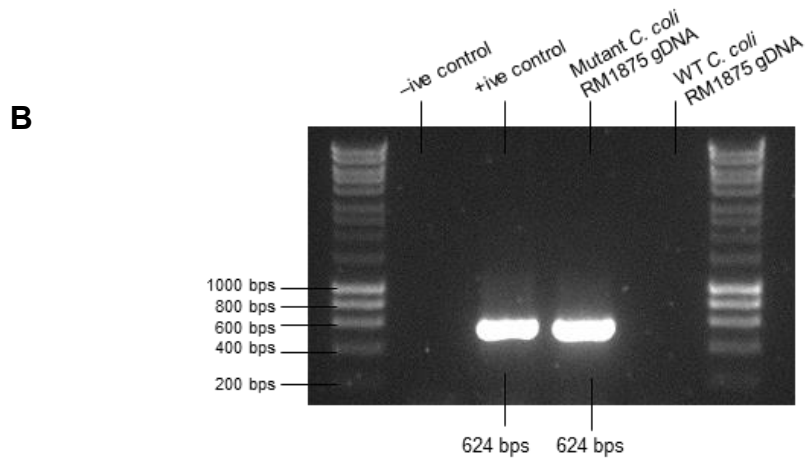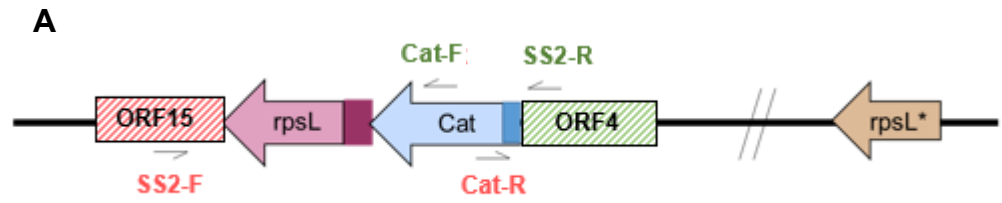
Figure 5.9: Analysis of gDNA of *C. coli* RM1875 mutant for the presence of Cat-*rpsL* cassette and LOS gene deletion.

(A): A representation of expected LOS region deletion (ΔORF15-ORF4) between *C. coli* RM1875 LOS biosynthesis ORF15 and ORF4

(B): Amplification of a PCR product (624 bps) with cat-F and cat-R confirmed the presence of Cat-*rpsL* cassette in the gDNA of *C. coli* RM1875 mutant. Positive control: PCR with pAH3; Negative controls: PCR reactions without any DNA and WT *C. coli* gDNA.

(C): Amplification of a PCR product (1123 bps) with cat-F and SS2-R confirmed the location of Cat-*rpsL* cassette upstream of the ORF4. Positive control: PCR reaction with pAH3; Negative controls: PCR reactions without any DNA and WT *C. coli* gDNA.

(D): The PCR product (468 bps) with primers, 210-F and 210-R, confirmed the presence of a LOS biosynthesis gene (*YSQ_03250*). Positive control: PCR with WT *C. coli* gDNA; Negative control: PCR reaction without any template DNA.
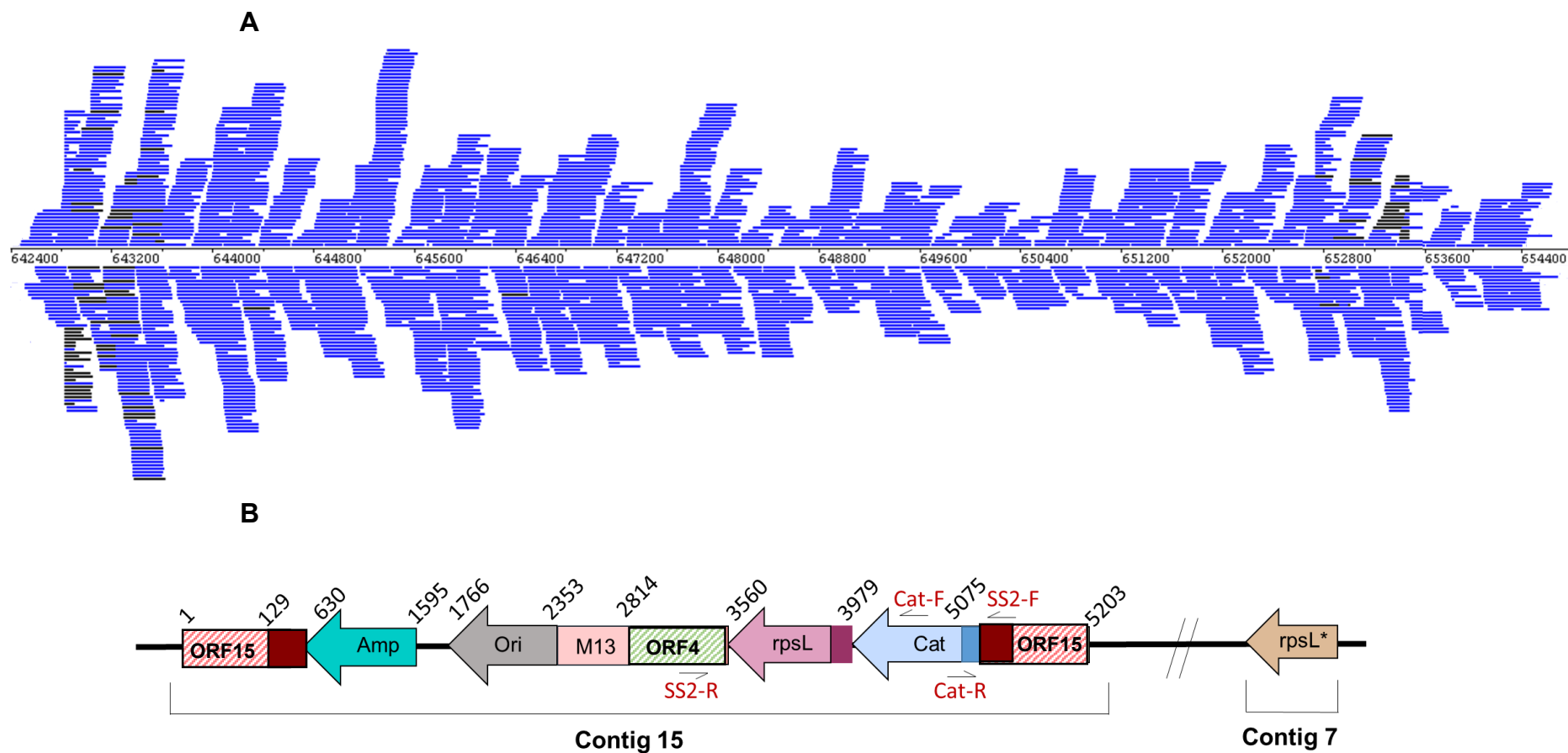
Figure 5.10: Analysis of WG sequence of *C. coli* RM1875 mutant (A): The Artemis view of mutant *C. coli* RM1875 contig 1 showing mapping of its sequence reads (blue bars) against the reference *C. coli* LOS locus sequence (643620-652735 bps); representing that all LOS biosynthesis genes were present in the mutant strain. **(B): A representation of entire plasmid sequence, randomly integrated into the gDNA of *C. coli* RM1875 mutant.** All plasmid genes including Amp, Ori, ORF4, and Cat-*rpsL* cassette were consecutively present in the *C. coli* mutant WG sequence (contig 15: 5203 bps), whereas, ORF15 was split. The split ORF15 sequence flanked the integrated plasmid sequence (contig 15: 1-129 bps upstream; contig 15: 5078-5203 bps downstream). Red boxes with ORF15 indicate the presence of partial ORF15 into the gDNA of *C. coli* RM1875 mutant.

5.3.2. LOS analysis of *C. coli* RM1875 mutant

The LOS extracted from WT *C. coli* RM1875 and mutant *C. coli* RM1875 strains migrated the same distance on 16 % (v/v) SDS-PAGE gel and showed no difference in mobility (Figure 5.11 A). This was expected because no mutation in *C. coli* RM1875 LOS biosynthesis gene cluster was produced. Similarly, no difference was identified when the LOS samples from WT and mutant *C. coli* RM1875 strains were dot blotted on the PVDF membrane using the peanut lectin (Figure 5.11 B).

A
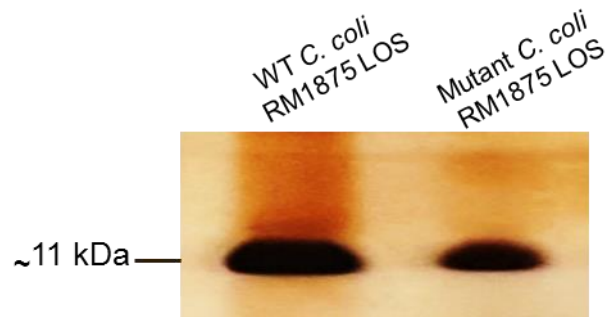
~11 kDa —

B
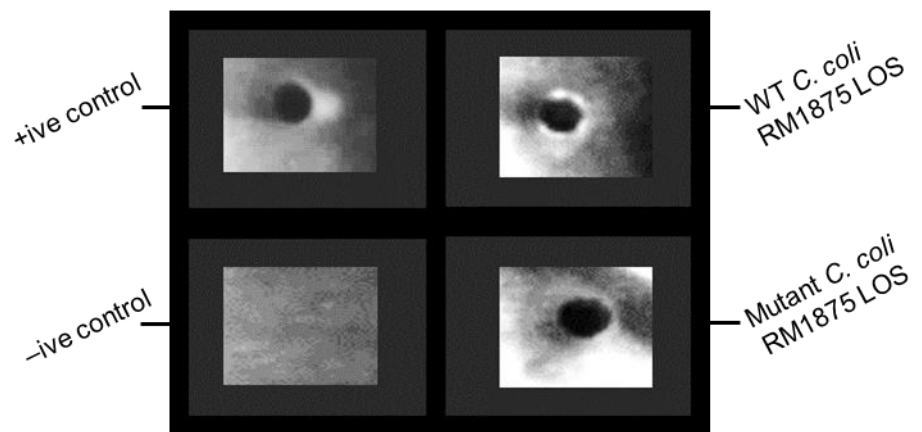
Figure 5.11 (A): Analysis of LOS by SDS PAGE. The figure shows the mobility of WT *C. coli* RM1875 LOS (~11 kDa) and mutant *C. coli* RM1875 LOS (~11 kDa) bands on silver stained 16% (v/v) SDS-PAGE gel; Multicolour low range protein ladder (1.7- 40 kDa) was used to predict the size of LOS bands. **(B): Analysis of LOS by Dot Blot.** The figure shows the binding of WT and mutant *C. coli* RM1875 LOS with peanut lectins. Lipopolysaccharides extracted from *E. coli* DH5α was used as a positive control and endotoxin-free water (available in the Pierce LAL Chromogenic Endotoxin Quantitation Kit) was used as a negative control.

5.3.3. Impact of plasmid integration on *C. coli* RM1875 mutant growth and motility

The *C. coli* RM1875 mutant strain grew differently from the WT *C. coli* RM1875 strain. In comparison to the WT, the mutant *C. coli* entered earlier into the log phase and went into the final decline phase more rapidly (Figure 5.12).

There was a significant difference (*t*-test; $p < 0.001$) in the diameter of motility zones between WT and mutant *C. coli* RM1875 indicating that compared to WT, the mutant was hypermotile (Figure 5.13). To determine whether motility associated *flaA* gene expression had been modulated in the mutant, expression of *flaA* was examined by qPCR. The *flaA* had increased expression in the mutant strain compared with the wild type (Figure 5.14), which might explain the hypermotile phenotype of *C. coli* mutant.
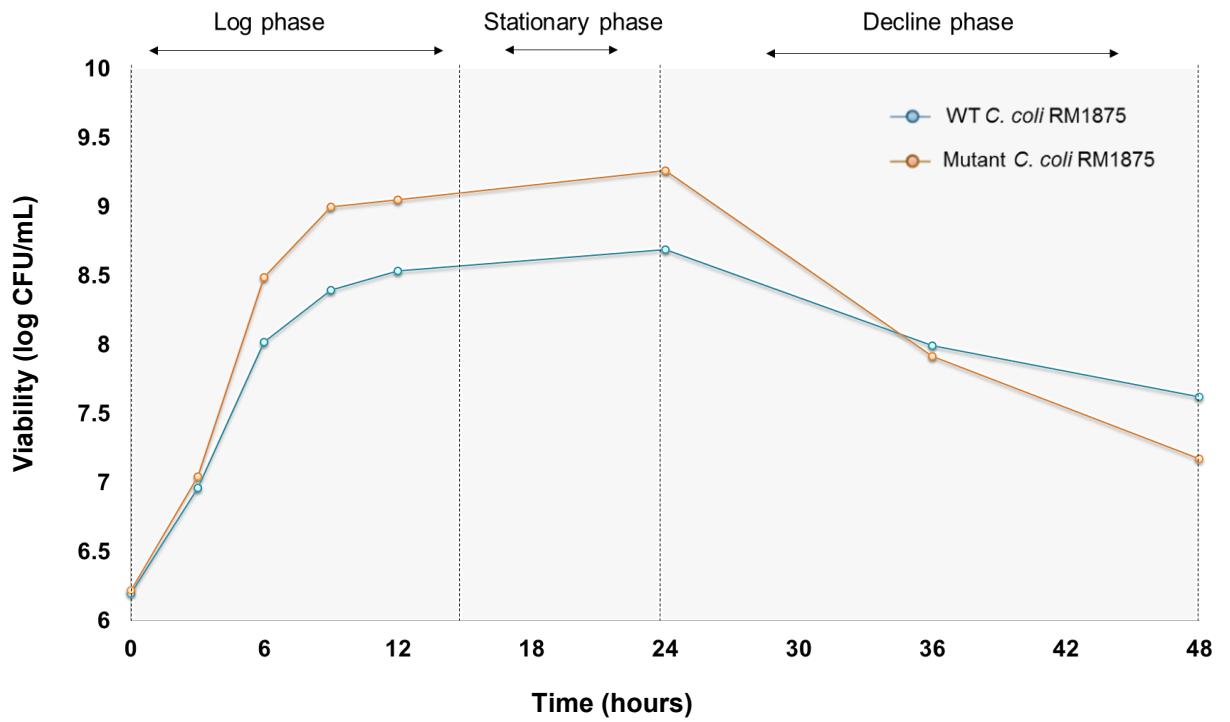
Figure 5.12: Comparison between WT and mutant *C. coli* RM1875 growth.

The values at each time point are the means of three independent experiments performed in triplicate.
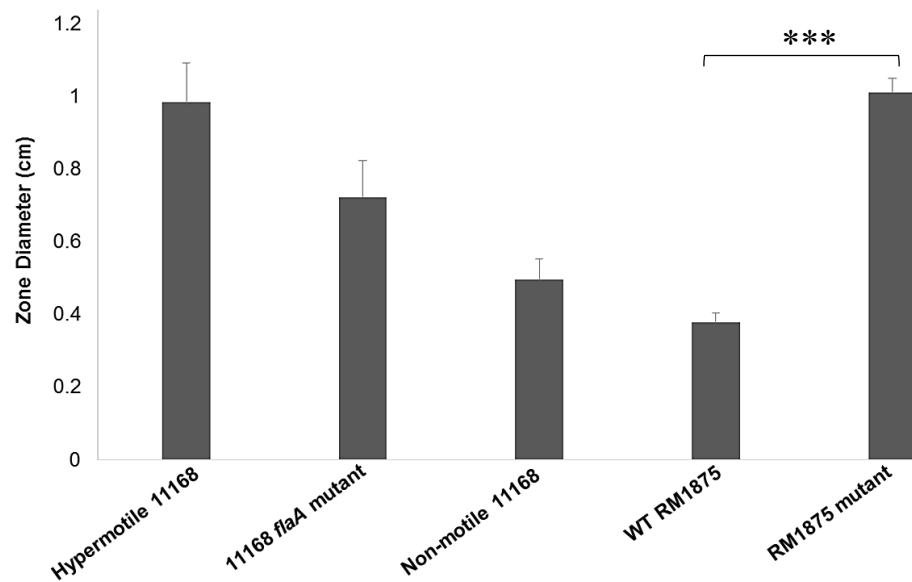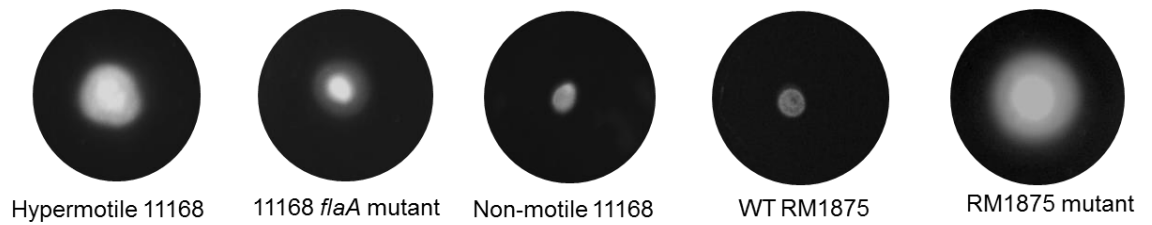
Figure 5.13: Comparison between WT and mutant *C. coli* RM1875 motility.

Hypermotile *C. jejuni* 11168 was used as a positive control. *C. jejuni* 11168 mutant strain with *flaA* mutation and non-motile 11168 strain were used as negative controls. The results are the means $\pm$ SD of three independent experiments performed in triplicate. Student's *t*-test was performed to determine the statistical significance ($p < 0.001$***) between WT and mutant *C. coli* RM1875 strains. The figure showing the motility zones is given for illustration only.
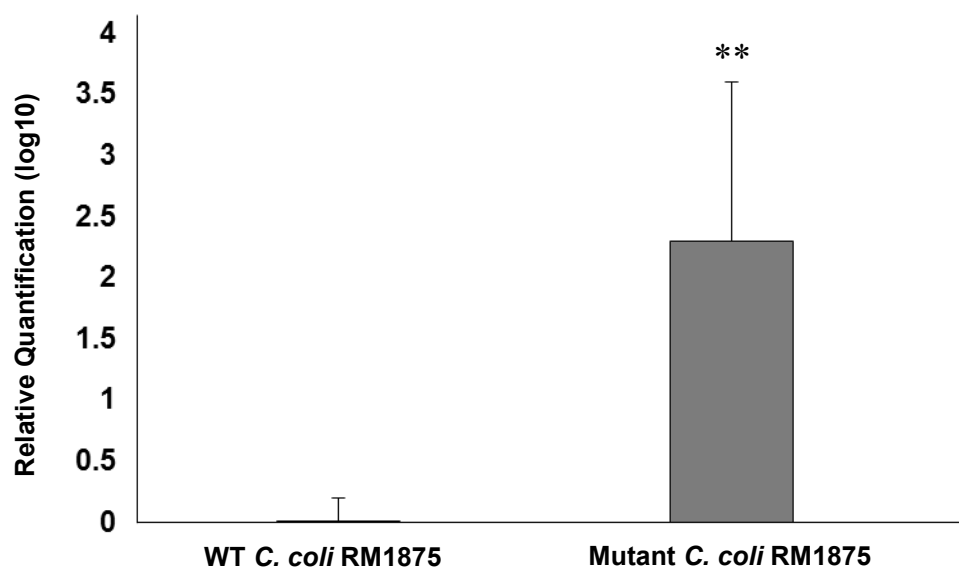
Figure 5.14: The qPCR results showing the relative quantification (RQ) of *flaA* gene expression in WT and mutant RM1875 strains. The *rpsL* gene was used as an endogenous control to normalise the data. Values are the mean $\pm$ SD of three independent experiments performed in triplicate. (Student's *t*-test; $p < 0.01$**).

5.4. Discussion

The first requirement of the mutagenesis strategy was to develop a spontaneous genomic mutation in the *rpsL* gene of that bacterial strain, in order to make it resistant to streptomycin (Hendrixson *et al*., 2001; Marsden *et al*., 2009). In this study, a spontaneous single base mutation in the *rpsL* gene of *C. coli* RM1875 was not required as this strain already had a single nucleotide polymorphism (A to G) in the *rpsL* gene sequence which would result in a substitution a lysine (codon 43) to an arginine residue within the *rpsL* encoded S12 protein and confer streptomycin resistance to *C. coli* RM1875. This SNP in the *rpsL* gene of *Helicobacter pylori* strains, *C. jejuni* 14980A, and *C. coli* 14983A, and its link to streptomycin resistance have been identified previously (Torii *et al*., 2003; Miller *et al*., 2016). These types of spontaneous mutations in chromosomal or integrated *rpsL* genes may also occur even after the transformation or electroporation of bacterial strains, which further leads to the production of false positive strep[S] or strep[R] phenotypes (Torii *et al*., 2003; Tuntufye and Goddeeris, 2011).

A suicide plasmid, such as *E. coli* pUC19, is unable to replicate or propagate in the *Campylobacter* strains (Wood *et al*., 1999). The gene regions having a length of ~ ≥567 bps, cloned in a suicide plasmid, are considered appropriate for the initiation of homologous recombination and regions with 270-286 bps length are unable to initiate the homologous recombination in *C. coli* (Richardson and Park, 1997). In the current study, two gene regions with 758 and 795 base pair lengths were cloned in the suicide plasmid, pAH3. This vector was supposed to create a deletion in LOS biosynthesis region as a consequence of a double-crossover in *C. coli*. Following the electroporation of WT *C. coli* RM1875 with pAH3, the entire pAH3 plasmid sequence was found to have integrated into the genome of *C. coli* RM1875. Generally, a single-crossover between homologous regions (Campbell-like recombination) and a recombination between the non-homologous sequences (illegitimate recombination) can facilitate the integration of an entire plasmid DNA into *Campylobacter* chromosomal DNA (Wang and Taylor, 1990; Richardson and Park, 1997). If the former is the case in the current case of pAH3 integration into *C. coli* RM1875 chromosomal DNA, then the

plasmid DNA should have integrated into the LOS biosynthesis cluster even via a single-crossover between homologous sequences. The pAH3 sequence did not integrate within the LOS biosynthesis cluster and therefore, Campbell-like recombination may not be associated with this integration. The entire DNA of pAH3 is likely to have randomly integrated into the *C. coli* RM1875 gDNA due to the occurrence of illegitimate recombination events. This type of recombination has been observed previously in *C. coli*, where a suicide plasmid-derived tetracycline antibiotic cassette had integrated into the *C. coli* chromosome randomly and at multiple locations, despite having no sequence homology to the chromosomal DNA sequence (Richardson and Park, 1997). Similarly, the suicide plasmid's derived streptomycin and nalidixic acid resistance genes were found fused into the chromosomes of *C. coli* and *C. jejuni* strains (Wang and Taylor, 1990). A fragment of plasmid sequence was also found inserted next to the leucine tRNA genes in the genomic DNA (gDNA) of *C. jejuni* 81-176 (Hofreuter *et al*., 2006). The phenomenon of illegitimate recombination has also been observed in *Rhodococcus fascians* (Desomer *et al*., 1991), *Bacillus subtilis* (Hofmeister *et al*., 1983), *Mycobacterium* (Kalpana *et al*., 1991), and *Streptomyces ambofaciens* (Kuhstoss *et al*., 1989).

Illegitimate recombination involves very short sequences of homology, which may ligate with intracellular nuclease-cleaved plasmid sequences during the DNA repair process (Desomer *et al*., 1991; Kalpana *et al*., 1991; Kusano *et al*., 1997). In the current study, deletion of ~ 538 bps from the plasmid derived or integrated ORF15 point out the involvement of intracellular nuclease activity in the procedure of illegitimate recombination. This is in line with a previous study, where stretches of plasmid sequence were found deleted after integration into the *C. coli* chromosome (Richardson and Park, 1997). The extracellular nucleases, such as DNAse I, do not affect the exchange between a plasmid DNA and gDNA in *C. jejuni* (Oyarzaba *et al*., 2007). The suicide plasmid may recombine with *C. coli* resident plasmids in order to rescue itself (Wang and Taylor, 1990) and the sequence of recombined plasmids can be read possibly with the WG sequence of gDNA. The absence of endogenous *C. coli* plasmid sequence within the *C. coli* mutant WG sequence reads excludes the occurrence of this possibility in the current study.

The *C. coli* RM1875 mutant was resistant to streptomycin despite having pAH3 derived strep^S *rpsL* gene in its gDNA. It is known previously that plasmid derived strep^S *rpsL* can become dominant on mutated gDNA strep^R *rpsL* when these both *rpsL* genes exist simultaneously in the bacterial chromosome (Skorupski and Taylor, 1995; Hendrixson *et al*., 2001). In this study, gDNA strep^S *rpsL* was not mutated to strep^R. Instead, strep^R *rpsL* was constitutively present in *C. coli* RM1875, which might be a reason behind the maintenance of the strep^R phenotype in *C. coli* RM1875 mutant. This may also happen due to the fact that any unknown single-base point mutation in the *rpsL* gene can contribute to the development of a strep^R phenotype. However, no SNPs in the strep^R *rpsL* of *C. coli* RM1875 mutant were detected.

No difference in the gel migration of WT and mutant *C. coli* RM1875 LOS was observed when they were resolved with 16% SDS-PAGE. This result is explained because of the lack of successful deletion of the LOS biosynthesis gene cluster. Only partial sequences of LOS biosynthesis ORF4 and ORF15 were cloned in the plasmid pAH3 and hence, off target plasmid integration into *C. coli* RM1875 has not affected the cell surface LOS structure or its synthesis.

The *C. coli* RM1875 mutant showed a modified growth rate, increased cell motility and higher *flaA* gene expression in comparison to the WT strain, which might have happened due to the insertion of the plasmid into one of the growth and motility regulating genes although this requires confirmation. It may also occur due to the phase variation in motility associated genes (Karlyshev *et al*., 2002), which supports that the modifications in the growth and motility of RM1875 mutant might not be the direct impacts of illegitimate recombination between pAH3 and WT RM1875 gDNA. The assimilation of the chloramphenicol cassette alone into the gDNA does not alter the growth rate in *C. jejuni* (Karlyshev and Wren, 2005). Full characterisation of where the pAH3 plasmid ended up is ongoing, but we speculate it might have affected the motility associated genes based on the results obtained.

5.5. Conclusion

A SNP in WT *C. coli* RM1875 *rpsL* responsible for the strep^R phenotype was confirmed. *C. coli* RM1875 maintained this phenotype even after integration of the cat-*rpsL* cassette in gDNA and unexpectedly, did not develop sensitivity to streptomycin. This may happen due to the lack of dominance of plasmid derived strep^S *rpsL* on WT gDNA strep^R *rpsL* when these both *rpsL* genes are present simultaneously in the chromosome of *C. coli* RM1875. The LOS mutant could not be constructed due to high rate of illegitimate recombination in *C. coli*. However, this study supports this fact that intracellular nuclease activity occurs during the illegitimate recombination. Increase in sequence homology (>1000 bps), required to introduce the double homologous recombination, might decrease the chances of occurrence of illegitimate recombination, and subsequently, this might prove useful for the successful deletion of a long stretch of the *C. coli* LOS biosynthesis gene region.

CHAPTER 6

Induction of Interleukin-1β production in the Human Monocytic Cell Line THP-1 by *Campylobacter*

6.1. Introduction

The main areas of *Campylobacter* localisation in humans is the lower GI tract including the small intestine (ileum and jejunum), caecum, and colon (van Spreeuwel *et al*., 1985; Black *et al*., 1988). A highly viscous mucus layer shielding the innermost layer of lower GI tract is a most favourable habitat for *Campylobacter* due to the low oxygen concentration and availability of nutrients including mucin, glycoproteins, L-fucose, galactose, sialic acid, *N*-acetyl galactosamine (GalNac), *N*-acetyl glucosamine, and mannose (Tu *et al*., 2008; Stahl *et al*., 2011). In addition to colonisation in the mucus layer, *Campylobacter* also adheres to microvilli or crypts present on the surface of the innermost layer of GI tract with the help of flagella and other adhesive proteins such as CadF and JlpA (Jin *et al*., 2001; Konkel *et al*., 2013; Baldvinsson *et al*., 2014). The mucus layer, crypts, and cell layers underneath the mucus layer (epithelium and lamina propria) are the main sites where *Campylobacter* come into contact with a range of human white blood cells or leukocytes (van Spreeuwel *et al*., 1985; Hodgson *et al*., 1998). In the first 7 days, after the onset of *Campylobacter* infection, acute inflammation is characterised by the influx of leukocytes (granulocytes, macrophages and lymphocytes) to the infection sites and disruption of red blood cells (Black *et al*., 1988; Samie *et al*., 2007).

6.1.1. *Campylobacter* interaction with macrophages

C. jejuni and *C. coli* are readily internalised by macrophages upon interaction (Kiehlbauch *et al*., 1985; Banfi *et al*., 1986; Watson and Galán, 2008). Earlier studies have demonstrated that *Campylobacter* cells are rapidly killed by endocytic lysosomes following internalisation (Wassenaar *et al*., 1997; Watson and Galán, 2008; Heikema *et al*., 2013), however, another earlier study showed that they are not rapidly killed after phagocytosis and can survive in

macrophages for 6-7 days (Kiehlbauch *et al*., 1985). It is also proposed that *Campylobacter* has the potential to produce an enzyme, catalase, which is encoded by a *katA* gene and help *Campylobacter* to survive within a macrophage (Day *et al*., 2000). The pathogen recognition receptors (PRRs) of human macrophages become activated to recognise the pathogen-associated molecular patterns (PAMPs) when pathogens develop interaction with a macrophage or survive intramacrophage subsequent to the phagocytosis (Day *et al*., 2000; Bouwman *et al*., 2014). Human macrophages PRRs generally possess cell membrane bound receptors [lectin receptors (LRs) and Toll-like receptors (TLRs)] and cytosolic receptors [Nucleotide-binding oligomerisation protein (NOD)-like receptors *e.g.* inflammasomes] (van Sorge *et al*., 2009; Stephenson *et al*., 2013; Bouwman *et al*., 2014).

Membrane bound receptor activation by *Campylobacter*

Different cellular constituents of *Campylobacter* such as lipoproteins, lipooligosaccharides, flagella, capsule, cell wall lipopolysaccharides (LPS) and DNA can bind to TLRs in human immune cells to activate them (de Zoete *et al*., 2009; Rathinam *et al*., 2009; Stahl *et al*., 2014). *Campylobacter* lipoprotein, JlpA, dependent stimulation of TLR-2 signalling and DNA dependent activation of TLR-9 in intestinal epithelial cells have been described by previous studies (Jin *et al*., 2003; O'Hara *et al*., 2012). *Campylobacter* toxins and lipooligosaccharides are known to activate the TLR signalling in epithelial cells and dendritic cells (DCs) (Hickey *et al*., 2000; Hu *et al*., 2006; Zheng *et al*., 2008). Conversely, *Campylobacter* flagella help *Campylobacter* in evasion from the TLR-5 recognition and its capsule is used as a TLR antagonist (Andersen-Nissen *et al*., 2005; Stahl *et al*., 2014).

Little is known about the correlation of *Campylobacter*-associated molecular patterns specifically with LRs and TLRs of human macrophages. Previously, *C. jejuni N*-linked glycosylated proteins and lipooligosaccharides with terminal GalNac have been reported as ligands of macrophage galactose-type lectin receptors (MGLRs), a type of LRs (van Sorge *et al*., 2009). Another type of macrophage LRs, sialoadhesin (Sn), generally recognises the sialylated ligands

mainly GM1 and GD1-like LOS present on the cell surface of *C. jejuni* to enhance the internalisation process of *C. jejuni* into the macrophages (Klaas *et al*., 2012; Heikema *et al*., 2013). The sialylated LOS, phosphorylated LOS as well as ester/amide linked LOS of *C. jejuni* have different capabilities towards the activation of TLR-4 in human primary monocytes and THP-1 cells, indicating a potential role of LOS in monocyte/macrophage TLR-4 signalling (Stephenson *et al*., 2013). In addition, the purified cell wall polysaccharides of *C. jejuni* also have ability to elicit the TLR-4 responses in murine macrophages (Korneev *et al*., 2018).

NOD-like receptors or inflammasomes induction by *Campylobacter*

Currently, no *C. jejuni* cellular component has been defined as a ligand of human macrophage NOD-like receptors, however, the event of *Campylobacter* internalisation into macrophages itself and a very low intracellular level of K$^+$ as a consequence of cellular damage can trigger signalling via these receptors (Pétrilli *et al*., 2007; Bouwman *et al*., 2014). In response to these type of stimuli generally NLRC4 inflammasomes for Gram-negative bacteria such as *Salmonella* Typhimurium and *Pseudomonas aeruginosa* and NLRP3 inflammasomes in *Neisseria gonorrhoeae* activate (Lara-Tejero *et al*., 2006; Franchi *et al*., 2007; Duncan *et al*., 2009). *C. jejuni* are unable to induce the NLRC4 inflammasomes, instead, they activate the NLRP3 inflammasomes in human macrophages (Bouwman *et al*., 2014). The NLRC4 or NLRP3 inflammasome mediated signalling in almost all gram-negative bacteria consequently leads to the pyroptosis (programmed cell death) of macrophages to enhance the inflammatory responses. In contrast, NLRP3 inflammasome activation in *C. jejuni* does not contribute to apoptosis or pyroptosis of human macrophages (Bouwman *et al*., 2014; Siegesmund *et al*., 2004).

6.1.2. Signalling pathways for cytokines induction in human macrophages

Macrophages respond to infection and produce a range of pro-inflammatory cytokines including TNFα, Interleukin (IL)-1 (surface bound IL-1α, secretory IL-1α, and secretory IL-1β), IL-6, IL-12, IL-18, IL-23, IL-27 (Baer *et al*., 1998;

Schindler *et al*., 2001; Verreck *et al*., 2004; Robinson and Nau, 2008; Fettelschoss *et al*., 2011; Fernando *et al*., 2014) and anti-inflammatory cytokines such as TGFβ and IL-10 (Chadban *et al*., 1998; Gong *et al*., 2012).

Intestinal inflammation is a hallmark of *Campylobacter* infection (Black *et al*., 1988). The signalling pathways (Figure 6.1), which are elicited due to the *Campylobacter*-macrophage interaction, ultimately induce the secretion of several pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, and TNFα from macrophages, indicating a significant role of monocytes or macrophages in the development of intestinal inflammation (Jones *et al*., 2003; Hamza *et al*., 2017). These signalling pathways activate due to PAMPs, specifically LOS in case of *C. jejuni*, binding to TLR1/2/6, TLR-4 and TLR-9 which recruits an adaptor protein, MyD88, to interact with IRK (IL-1 receptor–associated kinase) complex and TNF receptor-associated factor 6 (TRAF6) (Kawai *et al*., 1999; Wesche at al., 2001; Verstak *et al*., 2009). This interaction induces the two closely related kinases: (1). TGF beta-activated kinase 1 (TAK-1); and (2). Mitogen activated protein 3 kinase (MAP3K) (Baud *et al*., 1999; Irie *et al*., 2000). TAK-1 phosphorylates IKK which further phosphorylates the NFκß inhibitor (IKB) and consequently leads to the translocation of NFκß to the nucleus to induce the transcription of pro-inflammatory cytokines (TNFα, IL-6 and IL-1β) (Wesche *et al*., 2001; Verstak *et al*., 2009). The MyD88 activated kinase, MAP3K, further stimulates the MAP-1 kinases (MAP1K) including extracellular signal-regulated kinase (ERK), c-*jun* N-terminal kinase (JNK) and p38 induction. These MAP1K translocate to the nucleus, act as transcription factors, and regulate the transcription of NFκß as well as synthesis of pro-inflammatory cytokines (Baud *et al*., 1999; Feng *et al*., 1999). This type of MyD88 dependent signalling occurs downstream the TLR as well as Sn and is crucial for initiating the transcription of pro-inflammatory cytokines (Kawai *et al*., 1999; Klaas *et al*., 2012).

Macrophage TLRs, mainly TLR-2 and TLR-4, also involve the MyD88 independent signalling (Figure 6.1) where they interact with adaptor proteins, TRAM and TRIF, and activate the interferon regulatory factor 3 (IRF-3) (Kawai *et al*., 1999; Yamamoto *et al*., 2002; Nilsen *et al*., 2014). IRF-3 accumulates in the macrophage nuclei and stimulates the synthesis of pro-inflammatory cytokines (*e.g.* IL-1α) as well as regulates the synthesis of other pro-

inflammatory cytokines via coordination with NFκß (Kawai *et al*., 1999; Nilsen *et al*., 2014).
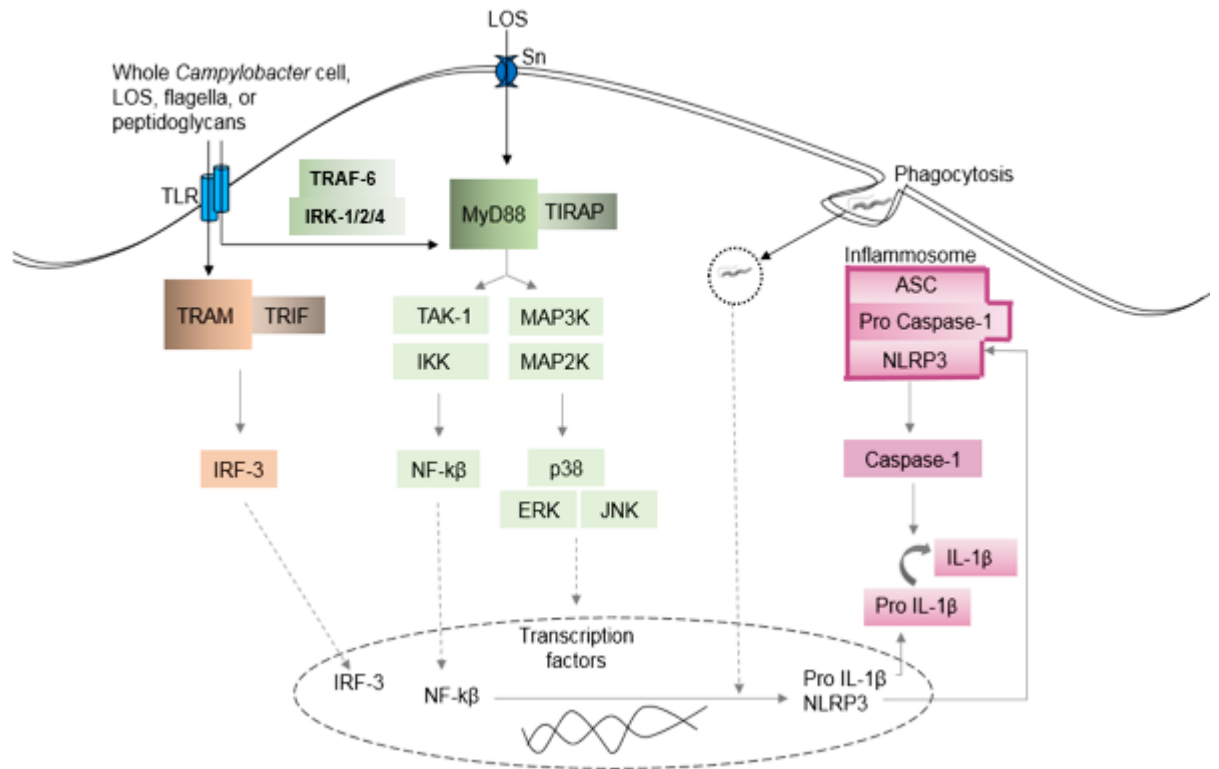
Figure 6.1: A representation of signalling pathways downstream the macrophage cell membrane receptors (TLR & Sn) and cytosolic receptors (NLRP3 inflammasome).

Upon *Campylobacter* infection, TLR signals may be either MyD88 dependent or independent while Sn corresponds via MyD88 only. The NLRP3 inflammasomes activation can occur directly by the phagocytosis process after the initiation of NLRP3 and pro-IL-1β transcription by NFκß.

NLRP3 inflammasome, a NOD-like receptor, is a macromolecular complex of three major proteins; NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC; 22-kDa) and Caspase-1, and is known to induce the pro-inflammatory cytokine IL-1β in human macrophages (Masumoto *et al*., 1999; Agostini *et al*., 2004). The activation of NLRP3 inflammasomes' dependent signalling in human macrophages requires dual signals. Initial signalling, also known as priming, occurs via TLRs. TLRs get stimulation by TLR ligands or PAMPs (*e.g.* LPS) binding, TLR mediated cytokines (*e.g.* TNFα), and host factors (*e.g.* ATP) which further trigger the NFκß to initiate the transcription of

NLRP3 and pro-IL-1β. Following the TLRs stimulation, downstream signalling can be either MyD88 dependent or MyD88 independent (Bauernfeind *et al.*, 2009). The transcribed NLRP3 proteins interact with two other proteins, ASC and Caspase-1, to structurally assemble the NLRP3 inflammasomes. This assembly formation involves an initial interaction between the pyrin domains (death domains) of ASC and NLRP3 and then another interaction of ASC caspase recruitment domain (CARD) with Caspase-1 (Masumoto *et al.*, 1999; Agostini *et al.*, 2004; Bae and Park, 2011). It is demonstrated that phosphorylation of pyrin domains activate the assembled inflammasomes, indicating the crucial role of kinases and phosphatases in the inflammasomes activation (Stutz *et al.*, 2017). The inflammasome remains inactivated in the cytosol and activates by a second signal, which may be the presence of PAMPs or ingested whole bacterial cell into the cytosol (Bauernfeind *et al.*, 2009). The activated NLRP3 inflammasomes then elicit the Caspase-1, which further catalyses the 31 kDa pro-IL-1β (already located into cytosol) into mature, biologically functional 17 kDa IL-1β. The cleaved IL-1β is then secreted outside the cell to mediate the inflammatory responses (Hazuda *et al.*, 1990; Bauernfeind *et al.*, 2009; Bouwman *et al.*, 2014). Secretory IL-1β regulates its own synthesis as well as synthesis of IL-1α (Hiscott *et al.*, 1993; Fettelschoss *et al.*, 2011).

6.1.3. THP-1 cell culture as an *in vitro* model of human macrophages

Macrophage can be differentiated into two forms, classically activated and alternatively activated, depending on the environmental conditions in their surroundings (Porta *et al.*, 2009; Fernando *et al.*, 2014). Classically activated macrophages produce pro-inflammatory cytokines whereas alternatively activated macrophages release the anti-inflammatory cytokines (Porta *et al.*, 2009). Figure 6.2 represents that circular THP-1 cells in suspension resemble human monocytes and can be differentiated into flat, adherent, classically activated macrophages by Phorbol 12-myristate 13-acetate (PMA) treatment (Tsuchiya *et al.*, 1980, 1982; Tedesco *et al.*, 2018). PMA treated THP-1 cells are phenotypically and functionally similar to the human macrophages and can be used as an *in vitro* model to study the human macrophages (Daigneault *et al.*, 2010; Tedesco *et al.*, 2018).

PMA is a strong inducer of Ca2+-activated, phospholipid-dependent protein kinase (PKC), which phosphorylates MAP1K specifically ERK. The phosphorylation of ERK and downstream NFκß activation do not only regulate the expression of p21 (a cyclin kinase inhibitor) to regulate the cell proliferation, but also leads to the expression of cell differentiation associated cell-surface markers (Castagna *et al*., 1982; Herrera *et al*., 1998). ERK signalling also increases the expression of adhesion molecules such as CD29, CD18 and CD11 to enhance the cell to cell interaction (Prieto *et al*., 1994). ERK phosphorylation can be kinase independent where, rather than the MAPK, the high expression of a cell surface marker, CD44, stimulates the ERK phosphorylation (Zhang *et al*., 2014). Briefly, ERK phosphorylation by PMA is a key event which plays an important role in the termination of cell proliferation and initiation of the human monocytes differentiation to macrophages (Herrera *et al*., 1998; Zhang *et al*., 2014).

The differentiated THP-1 cells were used in this study rather than the undifferentiated ones because of two major reasons. First, PMA differentiated THP-1 cells do not require priming as monocyte to macrophage differentiation process itself contributes to the NFκß activation (Takashiba *et al*., 1999). Second, monocytes constitutively express Caspase-1 for IL-1β cleavage and therefore, cannot be used for the identification of inflammasome mediated Caspase-1 activation and IL-1β processing (Netea *et al*., 2009).
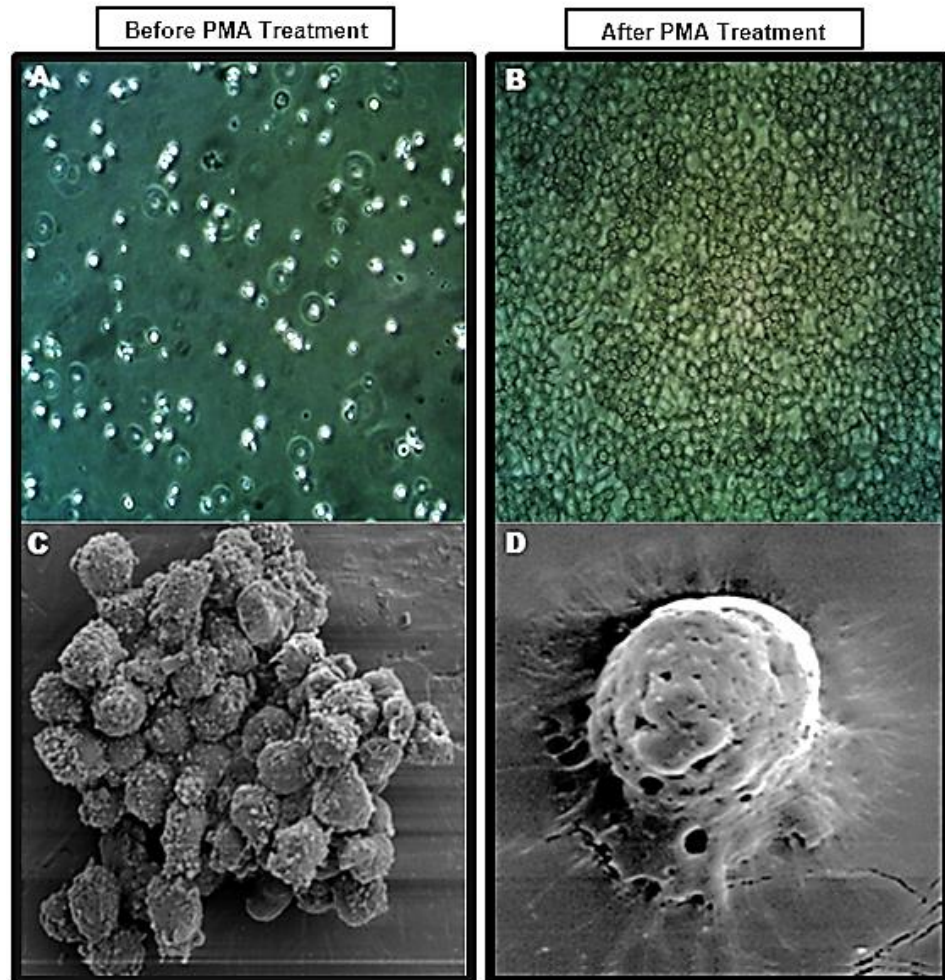
Figure 6.2: THP-1 cells before and after PMA (100 ng/mL) treatment (Current study)

A). Undifferentiated THP-1 cells under inverted microscope; B). Differentiated THP-1 cells under inverted microscope; C). A cluster of undifferentiated THP-1 cells under SEM; D). A differentiated THP-1 cell under SEM. Undifferentiated or monocytes-like cells are in suspension (before PMA treatment), whereas, differentiated or macrophage-like cells are adherent (after PMA treatment).

6.2. Aims and Objectives

The *Campylobacter* LOS can bind to the membrane bound receptors (TLRs, MGLRs, and Sn) of human macrophages (van Sorge *et al*., 2009; Klaas *et al*., 2012; Heikema *et al*., 2013; Stephenson *et al*., 2013), but the role of *Campylobacter* LOS in the stimulation of human macrophages-linked cytosolic receptors or inflammasomes remains unidentified. For this reason, this study aims to elucidate the association of *C. jejuni* and *C. coli* LOS to the activation of inflammasome signalling in human macrophages. To investigate it, the expression of IL-1β and Caspase-1 will be examined as these proteins are the final by-products of inflammasome mediated signalling pathway in macrophages.

The IL-1β and Caspase-1 activation downstream of the NLRP3 inflammasomes dependent signalling in the human macrophages in response to the *Neisseria gonorrhoeae, Neisseria meningitidis*, and *C. jejuni* infections has been previously reported (Duncan *et al*., 2009; Bouwman *et al*., 2014; John *et al*., 2016), but it is currently unknown for *C. coli*. Therefore, this study also aims to investigate whether *C. coli* elicits the NLRP3 inflammasomes mediated IL-1β and Caspase-1 in macrophages and behaves similar to *C. jejuni*.

In order to achieve the above aims, this study will involve the use of a mutant of *C. jejuni* 11168, *C. jejuni* 11168Δ32-52, which expresses a modified LOS with a lipid A-disaccharide backbone (attached to four phosphates, six saturated fatty acid chains, and two Kdo) and lack of core oligosaccharides structure (Marsden *et al*., 2009). In comparison to *C. jejuni* mutant strain, wild-type (WT) *C. jejuni* 11168 LOS consists of a lipid A-disaccharide backbone (attached to two phosphates, six saturated fatty acid chains, and one Kdo), an inner core of tetrasaccharides, and an outer core mimicking the human ganglioside structures (Moran, 1997). In addition, variable LOS expressing *C. coli* strains, RM1875 with non-sialylated LOS and 76339 with sialylated LOS, will also be used.

6.3. Results

6.3.1. IL-1β and Caspase-1 induction in THP-1 cells using extracted LOS from *Campylobacter*

THP-1 cells were incubated with WT *C. jejuni* 11168 LOS, *C. coli* RM1875 LOS (non-sialylated), and *C. coli* 76339 LOS (sialylated), to determine the impact of LOS on the induction of Caspase-1 and IL-1β secretion in macrophages. The modified LOS structures extracted from *C. jejuni* 11168Δ32-52 were also used to further assess whether alteration in the LOS structure can vary the NLRP3 inflammasomes mediated signalling in macrophages. The SDS-PAGE gel in figure 6.3 shows the estimated molecular weight of LOS of *C. jejuni* 11168, *C. jejuni* 11168Δ32-52, *C. coli* RM1875, and *C. coli* 76339.

In comparison to PBS treated THP-1 cells, Caspase-1 was induced in THP-1 cells upon exposure to LOS from *C. jejuni* 11168 ($p < 0.01$), *C. coli* RM1875 ($p < 0.05$), and *C. coli* 76339 ($p < 0.001$). However, increase in the level of Caspase-1 was almost negligible in *C. jejuni* 11168Δ32-52 LOS treated THP-1 cells compared to PBS treated THP-1 cells (Figure 6.4 A). In addition to Caspase-1, WT *C. jejuni* 11168 LOS, *C. jejuni* 11168Δ32-52 LOS, *C. coli* RM1875 LOS, and *C. coli* 76339 LOS, all induced IL-1β production significantly ($p < 0.001$) in THP-1 cells when exposed to purified LOS (Figure 6.4 B).

The modified LOS from *C. jejuni* 11168Δ32-52 induced significantly less Caspase-1 ($p < 0.01$) and IL-1β ($p < 0.001$) from THP1 cells in comparison to the WT *C. jejuni* 11168 LOS (Figure 6.4 A & B), indicating that LOS structural modifications affect the secretion of IL-1β in THP-1 cells.
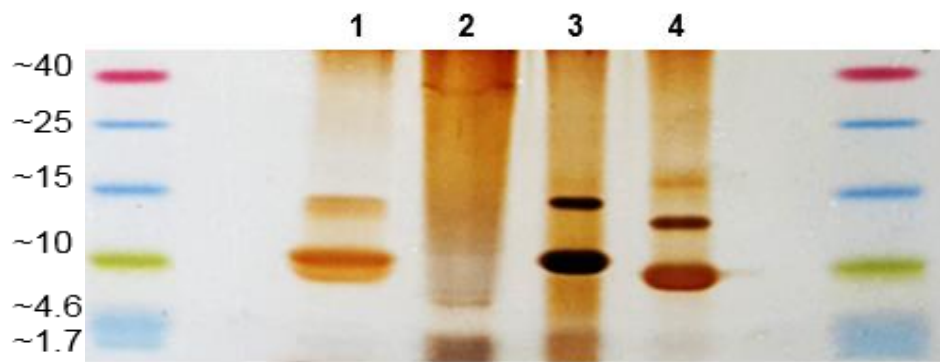
Figure 6.3: Analysis of LOS by SDS PAGE.

Campylobacter extracted LOS migration on silver stained 16% (v/v) SDS-PAGE gel. (1). *C. jejuni* 11168 LOS (~11 kDa); (2). 11168Δ32-52 LOS (No detectable band); (3). *C. coli* RM1875 LOS (~11 kDa). (4). *C. coli* 76339 LOS (~10 kDa). Multicolour low range protein ladder (1.7- 40 kDa) was used to predict the size of LOS bands.

(A)

(B)
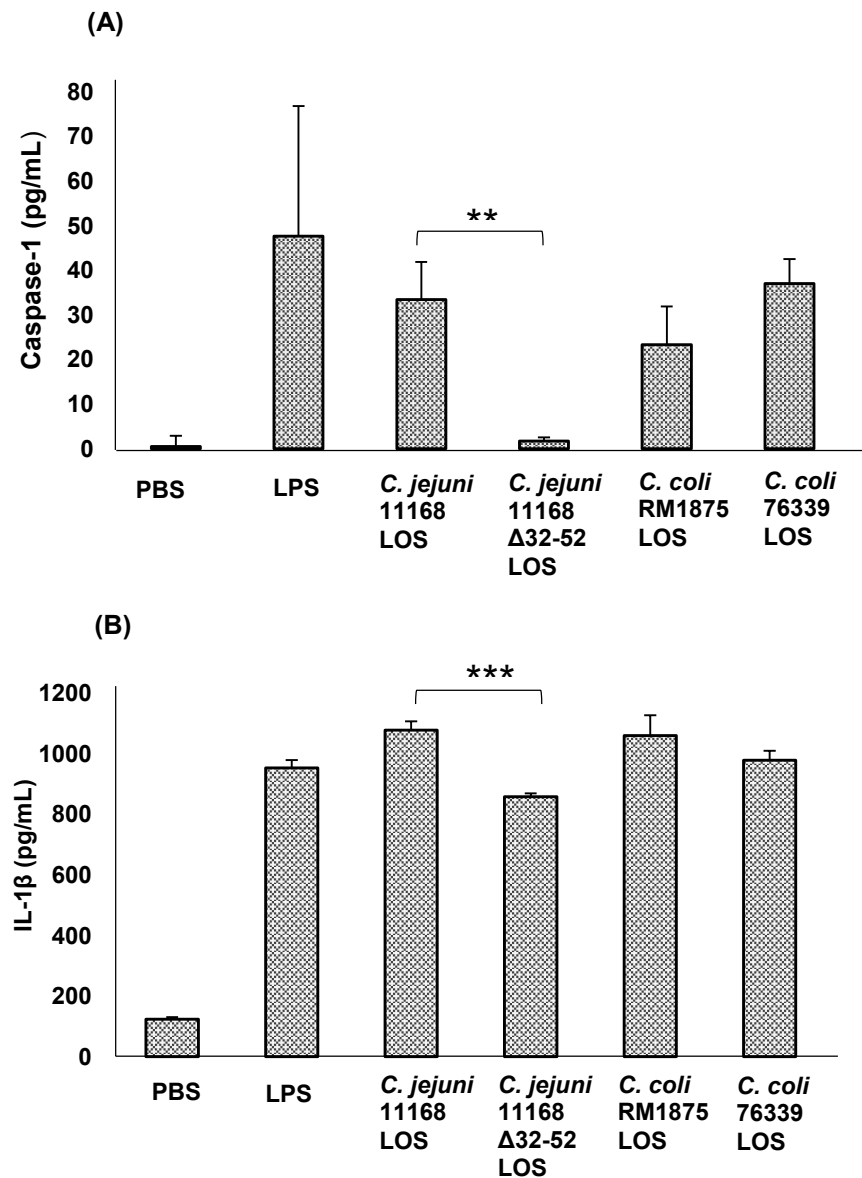
Figure 6.4: (A) Caspase-1 induction in LOS treated THP-1 cells at 12 hours post treatment. Compared to PBS treated THP-1 cells (negative control), the level of Caspase-1 increased in *E. coli* LPS (0.1 µg; positive control) and all *Campylobacter* LOS (1mg) treated THP-1 cells except those who were treated with *C. jejuni* 11168Δ32-52 LOS. **(B) Increase in IL-1β secretion in THP-1 cells upon treatment with LOS of *Campylobacter* strains at 12 hours post treatment.** The quantity of secreted IL-1β also significantly increased in LPS and *Campylobacter* LOS treated THP-1 cells. However, *C. jejuni* 11168Δ32-52 LOS induced significantly less Caspase-1 ($p < 0.01^{**}$) and IL-1β ($p < 0.001^{***}$) from THP-1 cells in comparison to the WT *C. jejuni* 11168 LOS. Values are the mean $\pm$ SD of three independent experiments performed in triplicate. Student's *t*-test was performed to determine the statistical significance between two experimental groups.

6.3.2. IL-1β and Caspase-1 induction using a LOS core deficient *C. jejuni* 11168 mutant

To further confirm the influence of *C. jejuni* LOS on IL-1β and Caspase-1 induction, THP-1 cell culture assays were carried out with *C. jejuni* 11168Δ32-52 disrupted (equivalent to ~20 µg total protein) and live cells (at MOI of 2, 50 and 200). The results were compared with *C. jejuni* 11168 disrupted or live cells inoculated THP-1 cell cultures.

Figure 6.5 shows that both types of *C. jejuni* 11168Δ32-52 cells, disrupted and live, significantly induced IL-1β secretion in comparison to PBS treated THP-1 cells. The IL-1β induction intensified with increase in number of live cells of *C. jejuni* 11168Δ32-52 and was comparable with *C. jejuni* 11168. Compared to *C. jejuni* 11168 infection of THP-1 cells, *C. jejuni* 11168Δ32-52 infection did not show a reduction in IL-1β in THP-1 cells. These results were in contrast to those which were observed with *C. jejuni* 11168Δ32-52 LOS, where *C. jejuni* 11168Δ32-52 LOS induced significantly ($p < 0.001$) reduced IL-1β in THP-1 cells in comparison to *C. jejuni* 11168 LOS.

(A)

(B)
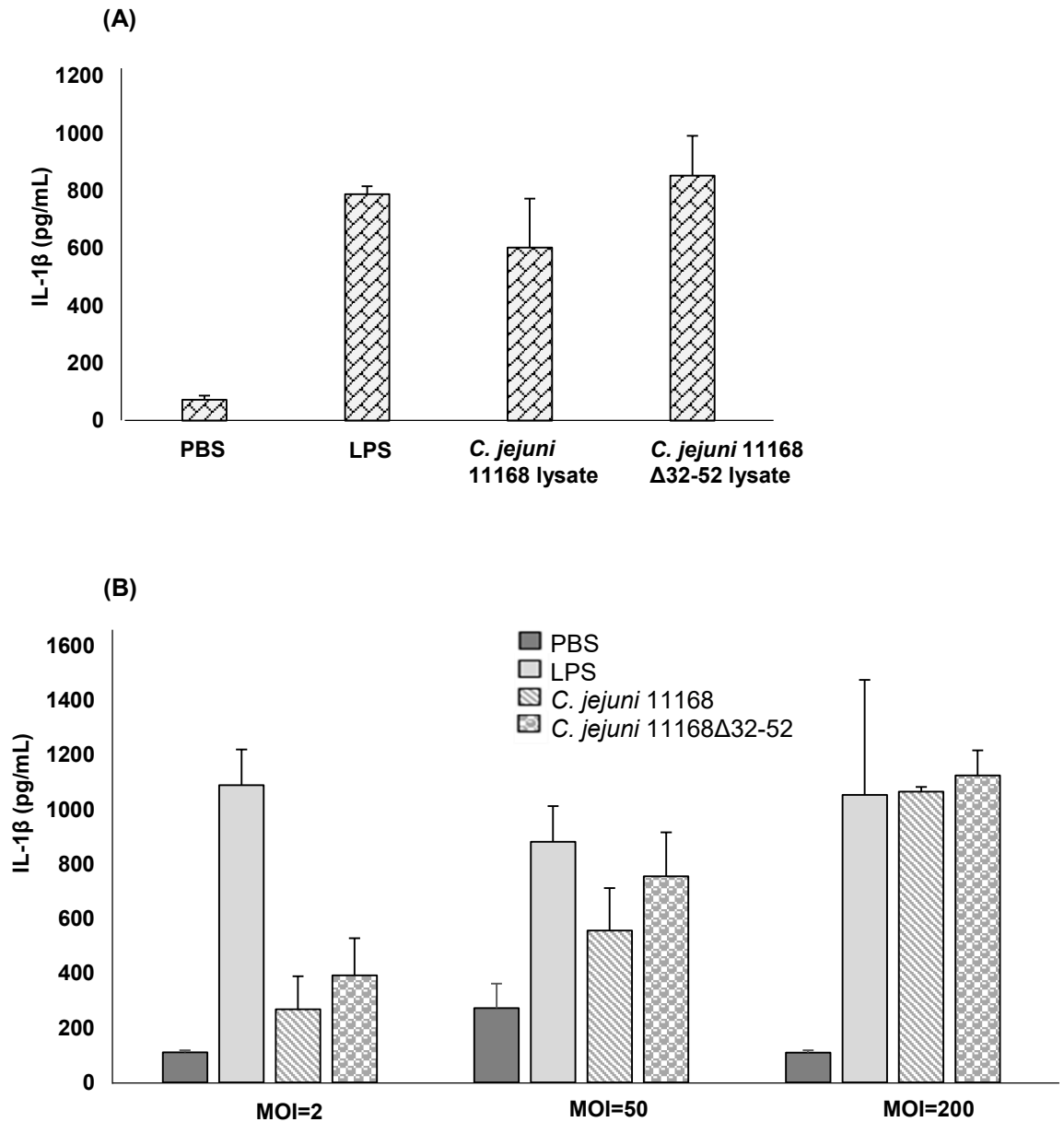
Figure 6.5: Increase in IL-1β induction in THP-1 cells by LOS deficient *C. jejuni* 11168Δ32-52 disrupted cells (A) and live cells (B) at 12 hours post treatment.

The level of secreted IL-1β in ~1x 10^6 differentiated THP-1 cells raised with increase in MOI or number of live *Campylobacter* cells. THP-1 cell culture with PBS only was used as a negative control. THP-1 cells treated with *E. coli* LPS (0.1 μg) and *C. jejuni* 11168 (disrupted or live) were used as positive controls. Values are the mean ± SD of three independent experiments performed in triplicate.

In comparison to PBS treated THP-1 cells, increase in Caspase-1 induction was observed upon infection of THP-1 cells with *C. jejuni* 11168 and 11168Δ32-52 as it is demonstrated in figure 6.6 A.

THP-1 infection assays with *C. jejuni* 11168 and 11168Δ32-52 were performed after inhibition of Caspase-1 and K^+ channels in THP-1 cells. Caspase-1 induction is coupled with the induction of IL-1β in the NLRP3 mediated signalling pathway (Hazuda *et al*., 1990; Bauernfeind *et al*., 2009). Therefore, Caspase-1 inhibitor or Z-VAD-FMK treatment of both *C. jejuni* 11168 and *C. jejuni* 11168Δ32-52 infected THP-1 cells caused decrease in the production of Caspase-1 ($p < 0.05$; Figure 6.6 A) as well as IL-1β ($p < 0.01$; Figure 6.6 B). Inhibition of K^+ channels by glyburide did not pose significant effect on IL-1β induction in *C. jejuni* 11168 infected THP-1 cells. However, it caused significant reduction ($p < 0.05$) in IL-1β induction in *C. jejuni* 11168Δ32-52 infected THP-1 cells (Figure 6.6 B).

Moreover, reduction in IL-1β due to the inhibition of K^+ channels was also observed in those THP-1 cells, which were co-cultured with disrupted cells of *C. jejuni* 11168Δ32-52 (Figure 6.6 C).

(A)

(B)

(C)
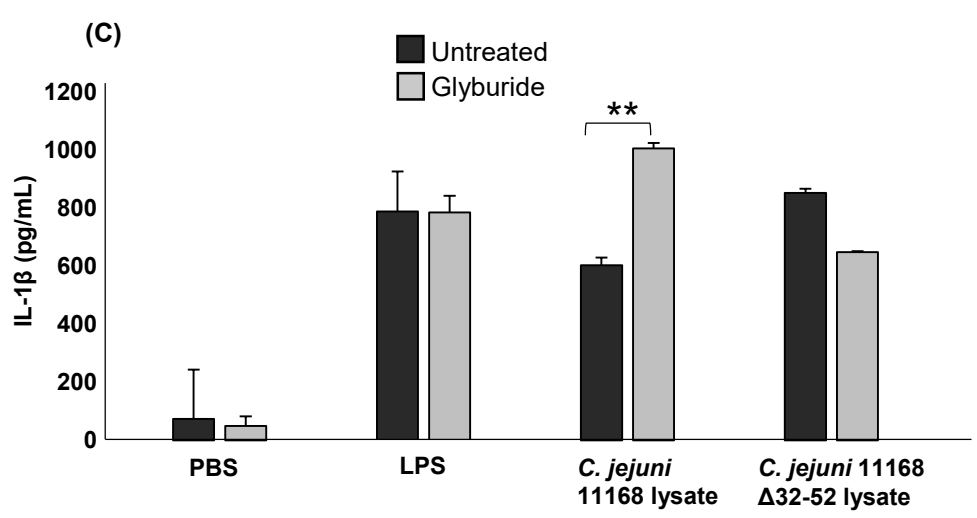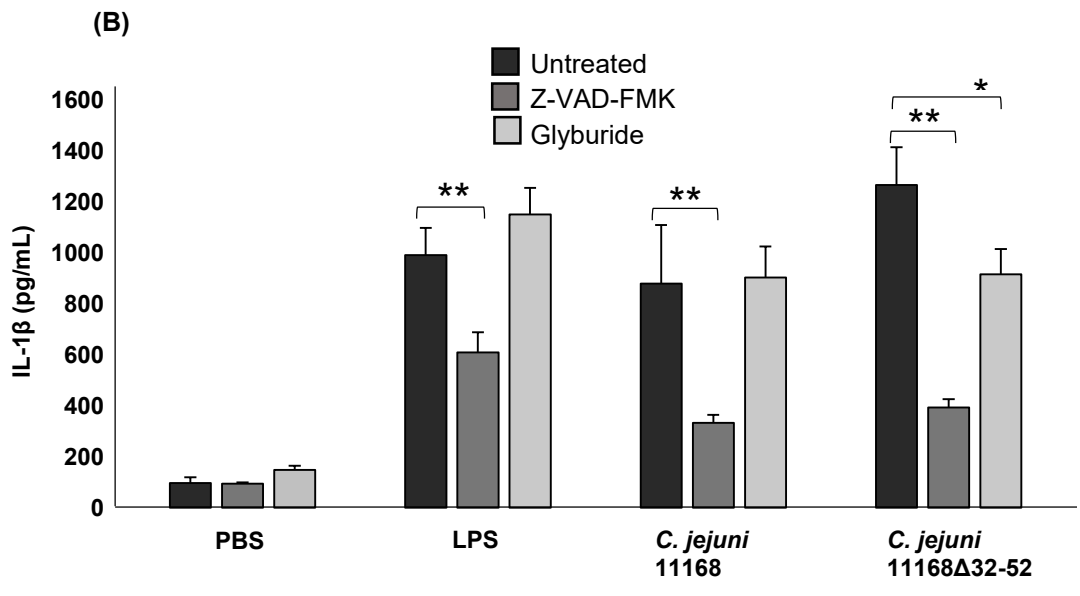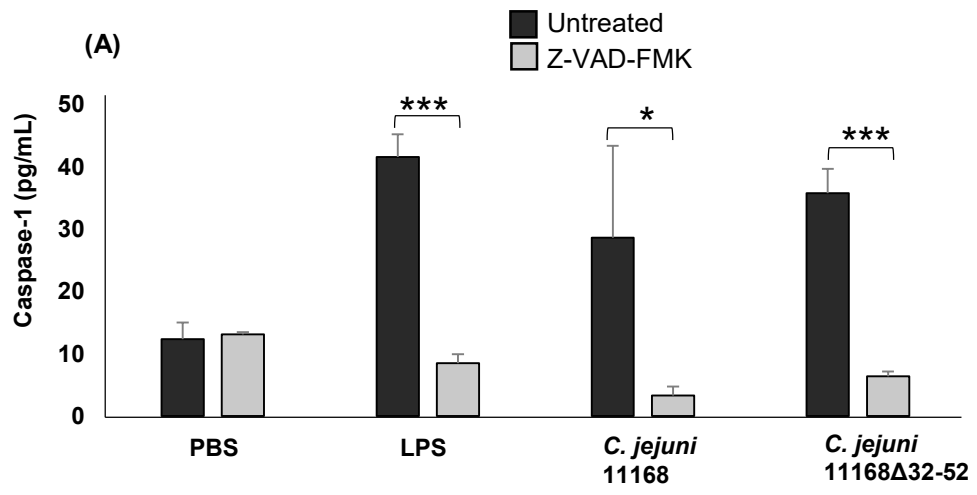
Figure 6.6: (A) Induction of Caspase-1 by live *C. jejuni* 11168 Δ32-52 mutant (MOI=200) in ~1x 10^6 THP-1 cells and its significant reduction upon treating the THP-1 cells with Z-VAD-FMK (10 μM). The quantity of Caspase-1 in Z-VAD-FMK treated THP-1 cells significantly reduced and was almost equivalent to the negative control. **(B) Inhibition of IL-1β secretion in live LOS core deficient *C. jejuni* 11168Δ32-52 mutant (MOI=200) infected ~1x 10^6 THP-1 cells by Z-VAD-FMK (10 μM) and glyburide (50 μM) at 12 hours post inoculation.** Z-VAD-FMK and glyburide, both significantly reduced the IL-1β production in *C. jejuni* 11168Δ32-52 mutant live cells infected THP-1 cells. **(C) Inhibition of IL-1β secretion in *C. jejuni* 11168Δ32-52 mutant disrupted cells infected ~1x 10^6 THP-1 cells by glyburide (50 μM) at 12 hours post inoculation.** Glyburide reduced the IL-1β production in *C. jejuni* 11168Δ32-52 disrupted cells inoculated THP-1 cells.

THP-1 cell culture with PBS only was used as a negative control whereas THP-1 cells stimulated with *E. coli* LPS (0.1 μg) and live *C. jejuni* 11168 cells (MOI=200) were used as positive controls. Values are the mean ± SD of three independent experiments performed in triplicate; (Student's *t*-test; $p < 0.05$*; $p < 0.01$**; $p < 0.001$***).

6.3.3. IL-1β and Caspase-1 induction by *C. coli* infection in THP-1 cells

Differentiated THP-1 cells were inoculated either with *C. coli* disrupted cells (equivalent to ~20 µg total protein) or live cells at different MOI (2, 50 & 200). The following figures, 6.7 and 6.8, illustrate that the live cells of *C. coli* RM1875 and *C. coli* 76339 were able to induce IL-1β secretion in THP-1 cells at levels comparable with *E. coli* LPS and *C. jejuni* 11168 (positive controls). The release of IL-1 β became intensified with increase in number of live cells of *C. coli* RM1875 and *C. coli* 76339. The same findings were observed following the incubation of THP-1 cells with disrupted cells of these *C. coli* strains.
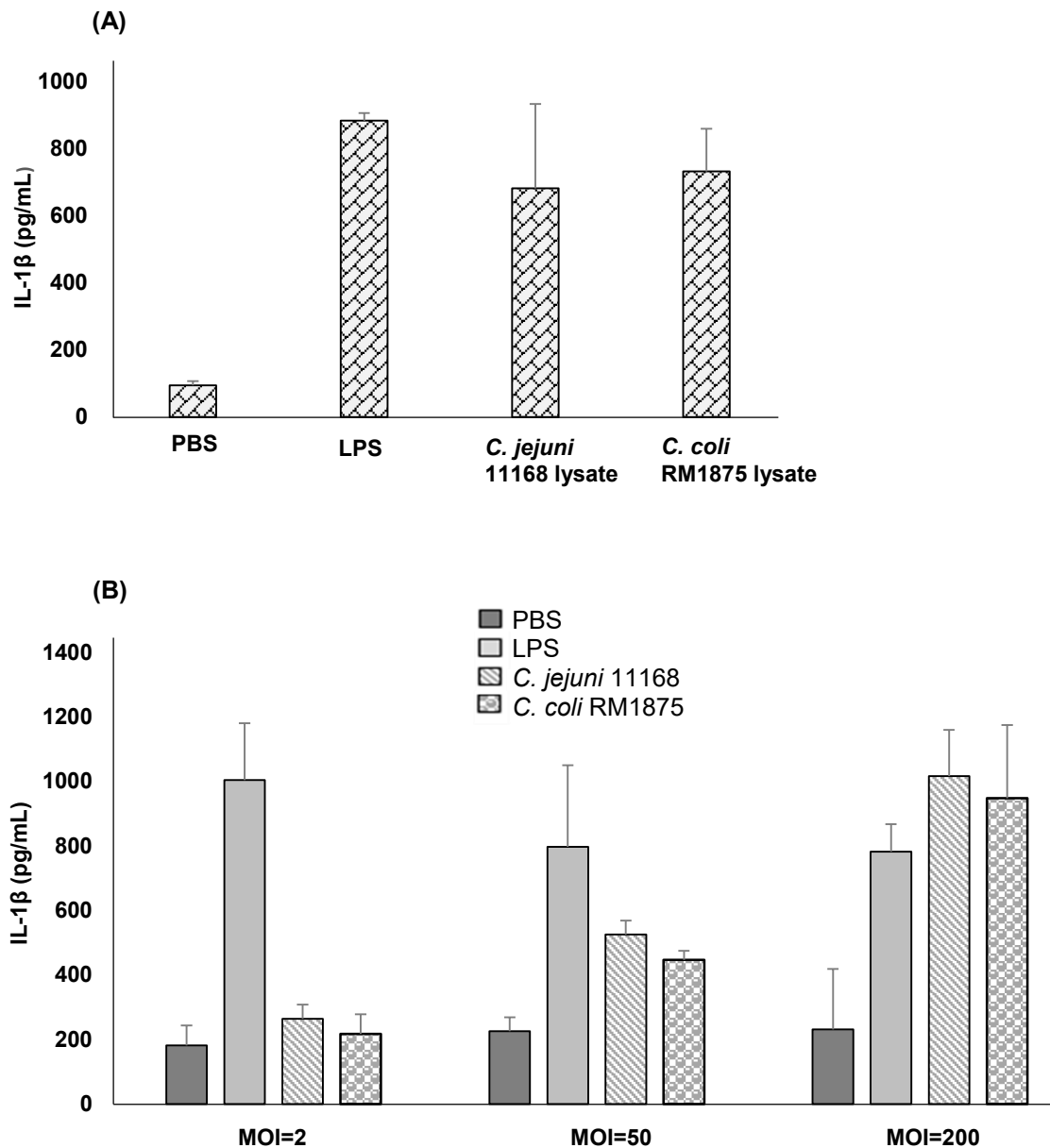
Figure 6.7: Increase in IL-1β induction in THP-1 cells by *C. coli* RM1875 disrupted cells (A) and live cells (B) at 12 hours post treatment.

The level of secreted IL-1β in ~1x 10^6 THP-1 cells raised with increase in MOI or number of live *Campylobacter* cells. THP-1 cell culture with PBS only was untreated and used as a negative control. THP-1 cells stimulated with *E. coli* LPS (0.1 µg) and *C. jejuni* 11168 (disrupted or live) were used as positive controls. Values are the mean $\pm$ SD of three independent experiments performed in triplicate.
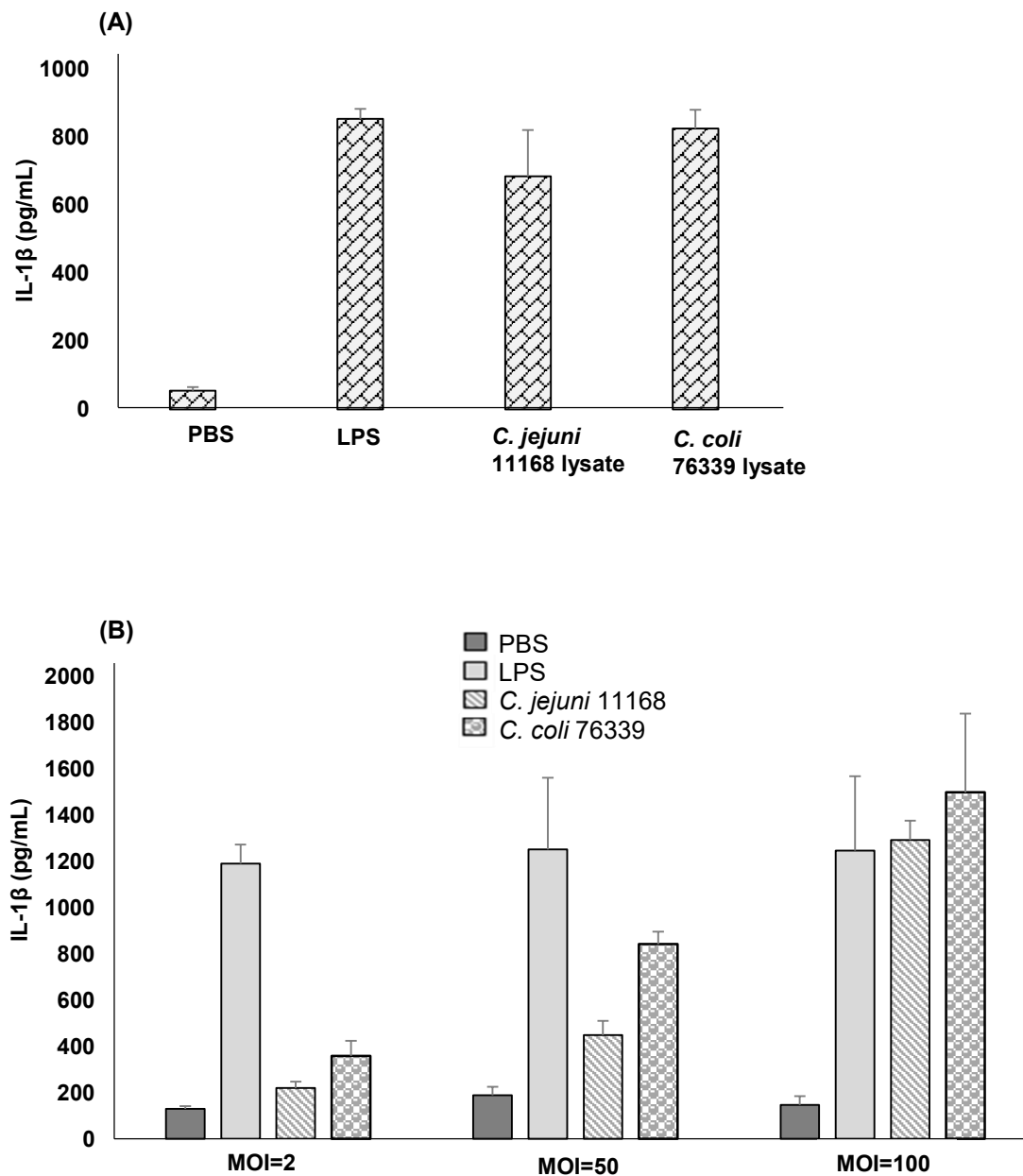
Figure 6.8: Increase in IL-1β induction in THP-1 cells by *C. coli* 76339 disrupted cells (A) and live cells (B) at 12 hours post treatment.

The level of secreted IL-1β in ~1x 10^6 THP-1 cells raised with increase in MOI or number of live *Campylobacter* cells. THP-1 cell culture with PBS only was used as a negative control. THP-1 cells treated with *E. coli* LPS (0.1 µg) and *C. jejuni* 11168 (disrupted or live) were used as positive controls. Values are the mean $\pm$ SD of three independent experiments performed in triplicate.

Figure 6.9 (A) shows that in addition to IL-1β, Caspase-1 was also significantly induced in THP-1 cells upon infection (MOI=200) with live *C. coli* RM1875 (p < 0.05) and *C. coli* 76339 cells (p < 0.01).

THP-1 infection assays with *C. coli* RM1875 and *C. coli* 76339 at MOI=200 were further carried out in the presence of Z-VAD-FMK (10μM) and glyburide (50μM). The expression of Caspase-1 was inhibited in *C. coli* RM1875 (p < 0.01) and *C. coli* 76339 (p < 0.001) infected THP-1 cells when THP-1 cells were treated with Z-VAD-FMK, prior to inoculation with *C. coli* live cells (Figure 6.9 A). Compared to Z-VAD-FMK-untreated THP-1 cells, Z-VAD-FMK-treated THP-1 cells showed reduction in IL-1β also during infection with *C. coli* RM1875 (p < 0.01) and *C. coli* 76339 (insignificant). However, glyburide-treated THP-1 cells following infection with *C. coli* strains did not show any significant change in IL-1β expression than the glyburide-untreated infected cells (Figure 6.9 B).
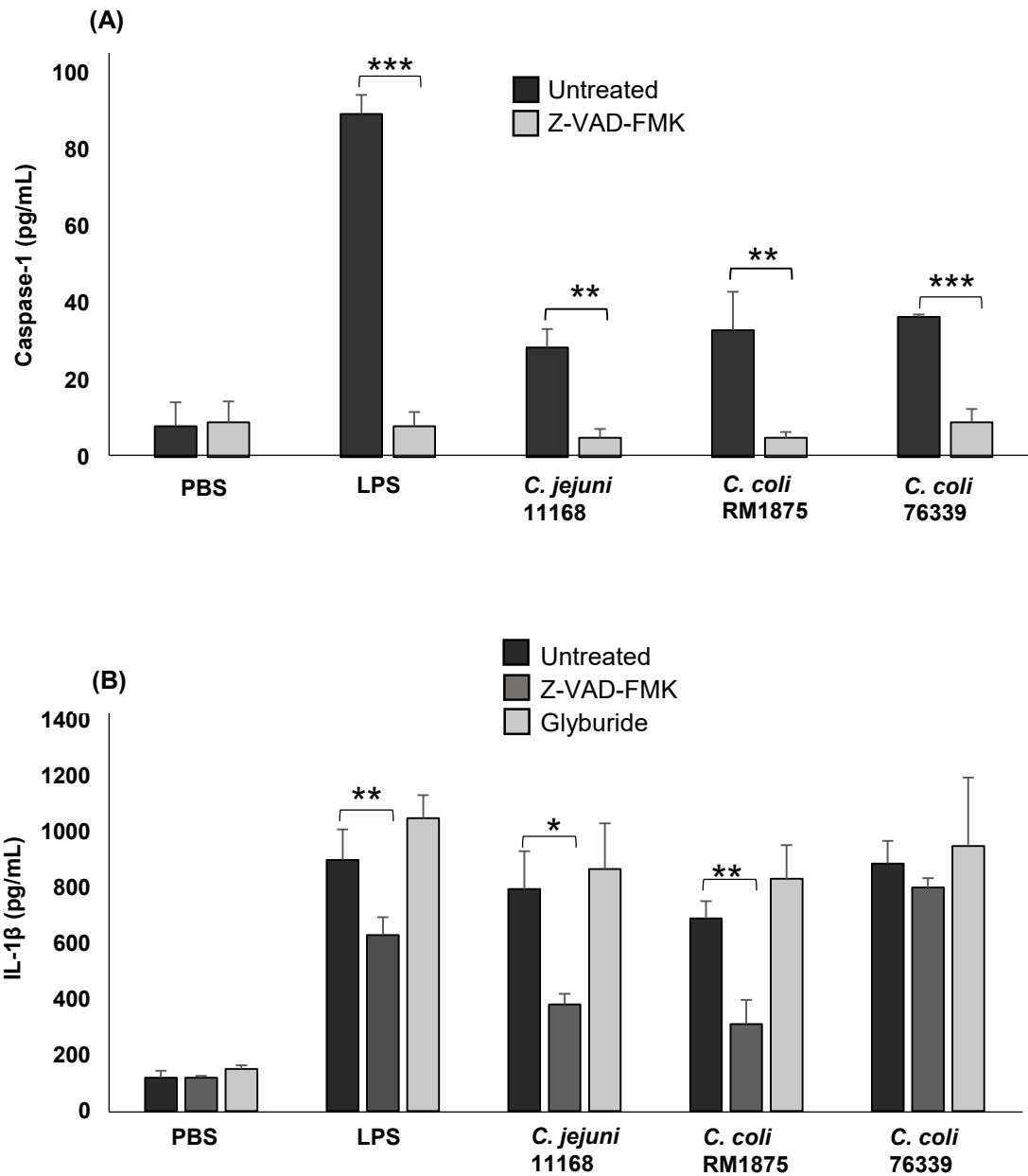
Figure 6.9: (A) Induction of Caspase-1 by live *C. coli* RM1875 and *C. coli* 76339 (MOI=200) in ~1x 10^6 THP-1 cells and its significant reduction upon treating the THP-1 cells with 10 µM Z-VAD-FMK. The quantity of Caspase-1 in Z-VAD-FMK treated THP-1 cells significantly reduced and was almost equivalent to the negative control. **(B) Effects on IL-1β secretion in live *C. coli* RM1875 and *C. coli* 76339 (MOI=200) infected ~1x 10^6 THP-1 cells before and after inhibition with Z-VAD-FMK (10 µM) and glyburide (50 µM) at 12 hours post inoculation.** Z-VAD-FMK significantly reduced the IL-1β production in *C. coli* infected THP-1 cells, however, glyburide did not inhibit the IL-1β secretion in *C. coli* infected THP-1 cells.

THP-1 cell culture with PBS only was used as a negative control. THP-1 cells stimulated with *E. coli* LPS (0.1 µg) and live *C. jejuni* 11168 cells (MOI=200) were used as positive controls. Values are the mean $\pm$ SD of three independent experiments performed in triplicate. (Student's *t*-test; p < 0.05*; p < 0.01**, p < 0.001***).

6.3.4. Increase in the LDH release in live *Campylobacter* infected and LOS treated THP-1 cells

Measurement of increase in LDH release in THP-1 cell culture supernatants provides an estimation of occurrence of cell cytotoxicity (which is another cause of the inflammasome activation) during infection. Therefore, LDH, released in live strains and LOS stimulated THP-1 cell cultures, was measured relative to the LDH release (100%) in LPS treated THP-1 cells (positive control).

In comparison to PBS treated THP-1 cells (negative control), relative LDH level did not increase in live *Campylobacter* infected THP-1 cells and was not in the detectable range (data not shown). However, in comparison to the negative control, a significant increase in relative LDH was present in the supernatants of those THP-1 cells, which were treated with WT *C. jejuni* 11168 LOS ($p < 0.01$), *C. coli* RM1875 LOS ($p < 0.01$), and *C. coli* 76339 LOS ($p < 0.01$) (Figure 6.10).
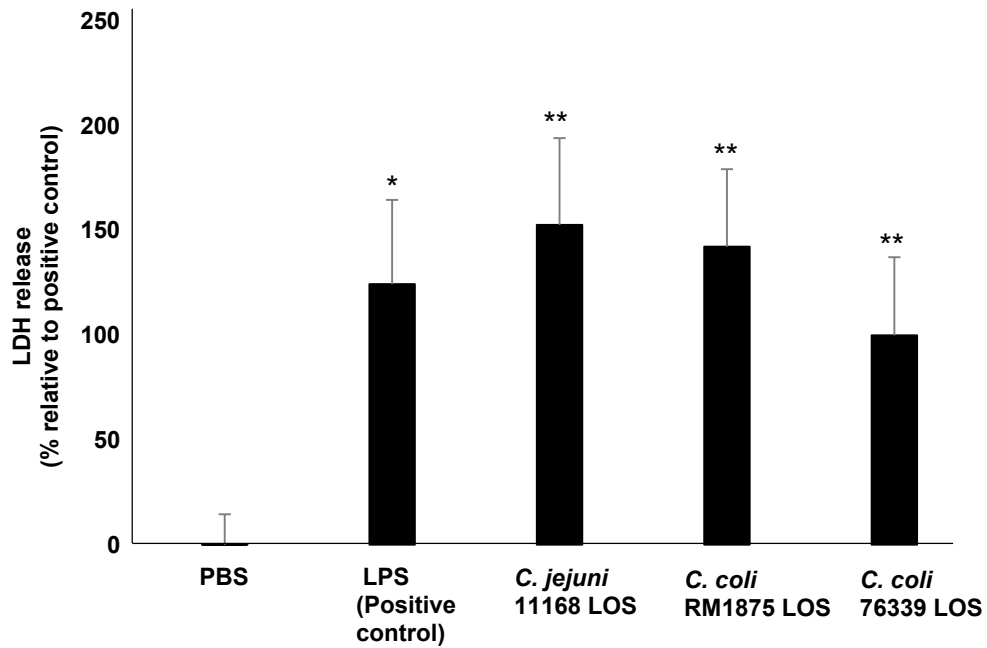
Figure 6.10: A significant increase in LDH release in *Campylobacter* LOS treated THP-1 cells at 12 hours post treatment.

LDH quantity in THP-1 cells was measured relative to the LDH release (100%) in positive control or *E. coli* LPS treated THP-1 cells. Compared to untreated THP-1 cells (negative control), the quantity of LDH significantly increased (Student's *t*-test; $p < 0.05^*$; $p < 0.01^{**}$) in *Campylobacter* LOS (1mg) treated THP-1 cells. Values are the mean $\pm$ SD of three independent experiments performed in triplicate.

6.4. Discussion

6.4.1. Stimulation of the inflammasome dependent IL-1β secretion in human macrophages by *Campylobacter* LOS

To investigate whether *Campylobacter* LOS structures can be presented as a contributing factor in the induction of IL-1β secretion in THP-1 cells, LOS extracted from *C. jejuni* 11168, *C. coli* RM1875 and *C. coli* 76339 were used. Caspase-1 induction and IL-1β secretion are the two major events, which occur downstream the activation of NLRP3 inflammasomes (Bauernfeind *et al*., 2009). Therefore, the Caspase-1 and IL-1β secretion in THP-1 cell cultures was estimated following their incubation with *Campylobacter* LOS extracts. LOS from *C. jejuni* 11168, *C. coli* RM1875 and *C. coli* 76339 strains induced the Caspase-1 and IL-1β from THP-1 cells, signifying a potent link of LOS to NLRP3 inflammasome activation. Previously, purified LOS extracted from *Neisseria gonorrhoeae* and *Neisseria meningitidis* have been reported to induce the NLRP3 inflammasomes in THP-1 cells (Duncan *et al*., 2009; John *et al*., 2016).

Lipid A with hydroxyl-linked acyl chains in *C. jejuni* LOS have been found as more strong inducers of TLR-4 receptors than lipid A with amide-bound acyl chains (Moran, 1997). *C. jejuni* LOS structures with terminal GalNAc residues recognise the MGLRs whereas *C. jejuni* LOS with sialylated residues bind to the Sn on macrophages (van Sorge *et al*., 2009; Klaas *et al*., 2012; Heikema *et al*., 2013). These studies have demonstrated that *C. jejuni* LOS lipid A and core oligosaccharides, both are important in the attenuation of inflammatory responses. In this current study, the LOS of *C. jejuni* mutant 11168Δ32-52 induced the Caspase-1 and IL-1β in THP-1 cells at lower levels than *C. jejuni* 11168 WT strain. *C. jejuni* 11168Δ32-52 LOS were deficient of core oligosaccharides, but had an additional Kdo and two phosphates in the lipid A disaccharide backbone (Marsden *et al*., 2009), which may have caused variation in the NLRP3 inflammasomes activation in THP-1 cells. It has been reported previously that variation in *Neisseria meningitidis* LOS core oligosaccharides do not affect the TLR-4 activation, but the removal of Kdo and phosphate groups from LOS-lipid A, as well as change in the number of lipid A acylated chains can strongly influence the TLR4 activation and cytokine induction in human

macrophages (John *et al*., 2016). Results obtained in the current study revealed that LOS structural modifications can vary the NLRP3 inflammasome-mediated Caspase-1 and functional IL-1β secretion in human macrophages, however, it remains unknown which component of LOS from lipid A, Kdo, and core sugars is actually responsible for this variation.

Experiments with *Campylobacter* LOS extracts were less reliable as these LOS extracts were not very pure and may have carry-over of capsular saccharides and degraded bacterial peptidoglycans (Muramyl dipeptides). Presence of these types of contaminants with LOS in LOS extractions could facilitate the IL-1β induction in THP-1 cells and produce the false-positive results. Previously, the role of bacterial muramyl dipeptides in the activation of inflammasomes in macrophages has been observed (Martinon *et al*., 2004). For this reason, THP-1 assays, performed with *C. jejuni* 11168 WT and mutant LOS, were replicated with *C. jejuni* 11168 WT and mutant live cells.

6.4.2. *Campylobacter* LOS and intracellular K$^+$ depletion independently trigger the inflammasome-mediated IL-1β secretion in human macrophages

In comparison to uninfected THP-1 cells, a significant increase in Caspase-1 and IL-1β production was observed in THP-1 cells infected with either live or disrupted cells of *C. jejuni* 11168Δ32-52. Macrophages do not constitutively express Caspase-1 and are unable to process pro-IL-1β without eliciting the inflammasomes (Netea *et al*., 2009). However, some other proteases such as Cathepsin G, Collagenase, elastase and granzyme may be recruited to process the IL-1β in monocytes (Hazuda *et al*., 1990; Irmler *et al*., 1995). Therefore, to confirm that IL-1β is actually a by-product of Caspase-1 enzymatic activity, THP-1 cells were incubated with a Caspase-1 inhibitor, Z-VAD-FMK, for 3 hours prior to infection. Caspase-1 inhibitor or Z-VAD-FMK treated THP-1 cells secreted significantly less Caspase-1 and IL-1β compared to Z-VAD-FMK untreated THP-1 cells during infection with *C. jejuni* 11168Δ32-52, as expected.

C. jejuni 11168Δ32-52 mutant lacked the core oligosaccharides due to the deletion of LOS region (17 LOS biosynthesis genes deletion from gene *cj1132-gmhB*) and had the kanamycin cassette, inserted in the replacement of deleted LOS gene region (Marsden *et al*., 2009). Despite expressing the altered LOS structures on the cell surface, *C. jejuni* 11168Δ32-52 mutant induced the IL-1β in THP-1 cells almost at the similar level to WT *C. jejuni* 11168. In an earlier study, infections of THP-1 cells with live *C. jejuni* 81116 *waaF* mutant (lacking the core oligosaccharides due to the insertion of chloramphenicol cassette in *waaF* gene) did not cause any reduction in the NLRP3 inflammasomes activation in comparison to its respective parent strain (Bouwman *et al*., 2014). Based on these results, it was assumed that in addition to LOS, some other factors are also involved in the activation of NLRP3 inflammasomes and for this reason, ascertaining the role of LOS in the inflammasome activation in human macrophages can be difficult following the infection of THP-1 cells with *C. jejuni* 11168Δ32-52.

It is well known previously that the K^+ efflux, a highly conserved mechanism in macrophages and a major factor of inflammosome activation, occurs simultaneously with the internalisation of a whole-live bacterial cell, possibly by ATP binding to P2X7 receptors on macrophages. The exact mechanism causing K^+ depletion or efflux in macrophages during infection is yet unknown (Pétrilli *et al*., 2007; Bouwman *et al*., 2014). In this study, glyburide (which inhibits the activation of NLRP3 inflammasomes by inhibition of K^+ channels), was used to assess the *Campylobacter* LOS induced effects after inhibition of K^+ efflux in THP-1 cells. In comparison to the glyburide-untreated infected cells, glyburide-treated THP-1 cells had no reduction in IL-1β levels when they were infected with live WT *C. jejuni* 11168 cells, indicating that other factors in addition to K^+ efflux were present there for the activation of NLRP3 inflammasomes. The same results were expected for live *C. jejuni* 11168Δ32-52 mutant infection. In contrast to the expected results, glyburide-treated THP-1 cells upon exposure to the live cells of *C. jejuni* 11168Δ32-52 mutant showed a significant decrease in IL-1β production. IL-1β reduction was also observed when glyburide-treated THP-1 cells were cultured with disrupted cells of *C. jejuni* 11168Δ32-52 mutant. Blocking of K^+ channels by glyburide and alteration in LOS structure at the same

time decreased the IL-1β production in THP-1 cells, which indicates that K$^+$ efflux and LOS structures are both associated with NLRP3 inflammasome activation in human macrophages. These results also explained that a continuous K$^+$ efflux during the infection with live and disrupted bacterial cells can mask the contribution of LOS structures towards the production of inflammasomes in human macrophages. That is why, in the absence of glyburide, *C. jejuni* 11168Δ32-52 mutant live cells showed similar IL-1β induction to the WT *C. jejuni* 11168 live cells.

C. jejuni 11168Δ32-52 modified LOS showed a clear reduction in Caspase-1 and IL-1β secretion in THP-1 cells in comparison to *C. jejuni* 11168 LOS. However, compared to *C. jejuni* 11168 live cells, *C. jejuni* 11168Δ32-52 live cells did not reduce the Caspase-1 and IL-1β induction from THP-1 cells, suggesting that K$^+$ intracellular depletion or K$^+$ efflux (masking LOS impact) occurred only during infection with live or disrupted cells and this event did not take place when LOS extractions were used.

The NF$_K$ß induction downstream of the TLR signalling is the first step of the inflammasomes activation to initiate the transcription of pro-IL-1β and NLRP3 proteins (Bauernfeind *et al*., 2009). *E. coli* LPS as well as *Campylobacter* heat-killed and disrupted cells, all can activate the TLRs and induce NF$_K$ß in macrophages via MyD88 dependent and independent signalling (Takashiba *et al*., 1999; Mellits *et al*., 2002; Jones *et al*., 2003). In this study, purified LOS and disrupted cells, both stimulated the inflammasomes-mediated signalling in macrophages. Based on our findings and previous data, it can be assumed that TLRs and NF$_K$ß activation may occur either upon initial interaction of *Campylobacter* with macrophage cell surface (LOS interact extracellularly with TLRs) or after the phagocytosis process (lysed cells including LOS interact intracellularly with TLRs) (Figure 6.11). Mellits *et al*. (2002) found that *C. jejuni* LOS preparations do not activate the NF$_K$ß in epithelial cells. Therefore, it may be possible that *Campylobacter* LOS residues directly activate the inflammasomes into the cytosol of macrophages, similar to the gonococcal LOS, which is shed through membrane blebbing by *Neisseria gonorrhoeae* to elicit the NLRP3 inflammasomes (Duncan *et al*., 2009). Hence, it is not known whether
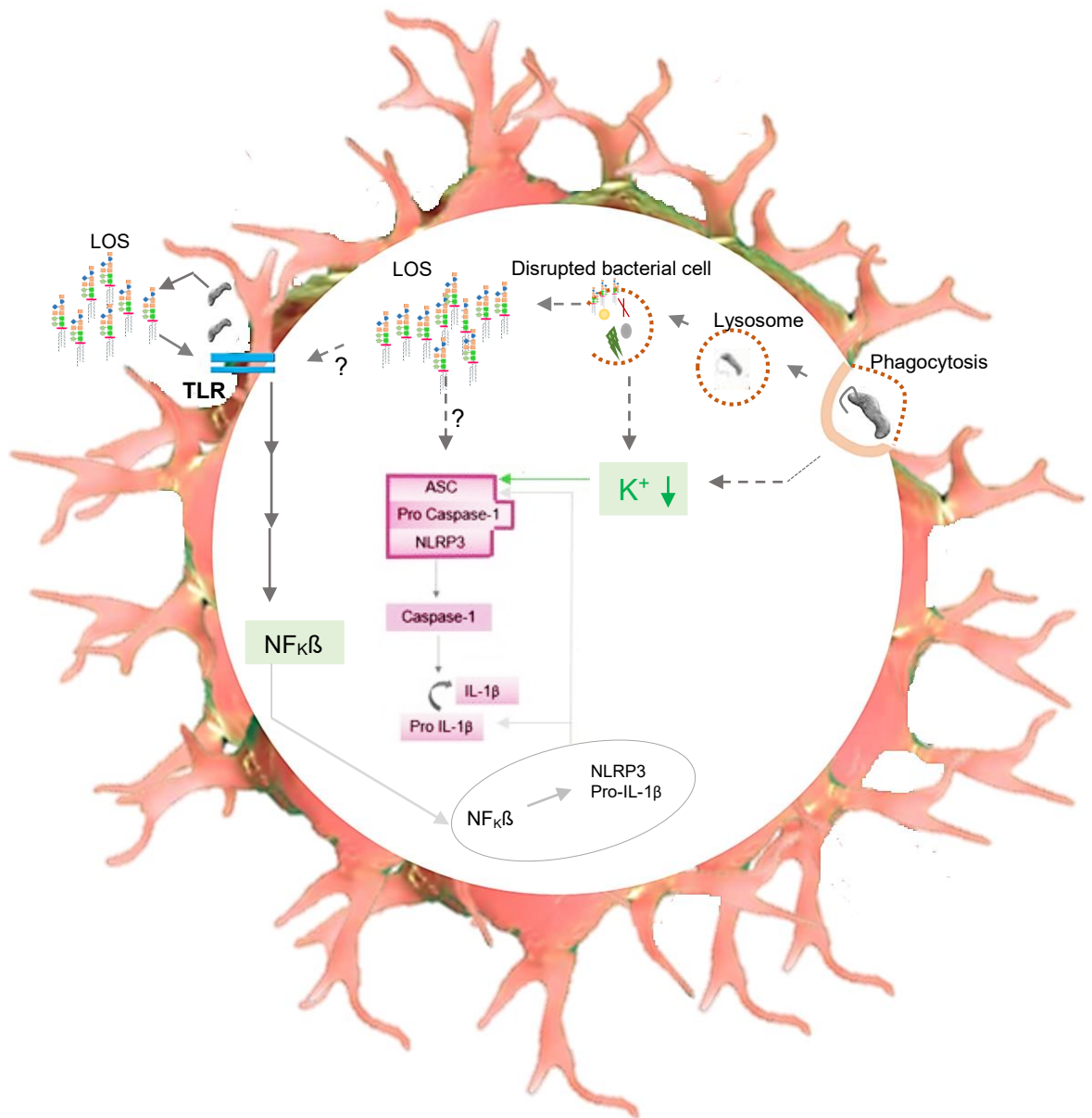
Figure 6.11. A proposed correlation between *Campylobacter* live cells, disrupted cells, and LOS in order to induce the IL-1β secretion in human macrophages.

Live and disrupted cells cause the intracellular K^+ depletion or K^+ efflux. The depletion of K^+ ions stimulate the assembly of NLRP3 inflammasomes by an unknown mechanism. Cell surface LOS structures during the interaction of *Campylobacter* with the macrophage may activate the TLRs extracellularly, whereas, LOS structures released from the phagocytosed or disrupted cells may activate the TLR signalling intracellularly. Subsequently, *Campylobacter* LOS structures intracellularly may activate NF$_K$ß by an undefined mechanism to transcribe the NLRP3 and pro-IL-1β, which is a first step of inflammasome activation. In the second step of inflammasomes dependent signalling, Caspase-1 becomes activated by NLRP3 inflammasome which further cleaves the pro-IL-1β into its mature form, IL-1β. Thus, *Campylobacter* LOS and K^+ depletion both independently induce the inflammasome-mediated IL-1 β secretion. It is not known whether *Campylobacter* LOS elicit the inflammasomes by involving the TLRs or by direct association to the inflammasomes. (Arrows represent the already known events, dashed arrows represent the proposed events, and dashed arrows with question marks represent the unknown signalling pathways).

Campylobacter LOS stimulate the NLRP3 inflammasomes by direct association to the inflammasomes or/and via TLRs activation in human macrophages.

6.4.3. Activation of the inflammasome-mediated IL-1β secretion in the human macrophages by *C. coli* infection

In comparison to uninfected THP-1 cells, a significant increase in IL-1β and Caspase-1 production was observed when cells were treated with live cells of *C. coli* RM1875 and 76339 strains, indicating the NLRP3 inflammasomes activation in response to *C. coli* infection. IL-1β secretion in THP-1 cells induced by the number of *C. coli* live cells in a dose dependent manner. This is in line to the previous studies which explain that THP-1 induced IL-1β secretion depends on the bacterial load (Franchi *et al*., 2007; Duncan *et al*., 2009; Bouwman *et al*., 2014). THP-1 induced IL-1β secretion also upon exposure to *C. coli* disrupted cells, suggesting that neither *C. coli* viability nor invasion is necessary to activate the inflammasomes. These *C. coli* related findings were comparable to *C. jejuni* whose invasion or viability is also not essential for the cytokine production in DCs and macrophages (Jones *et al*., 2003; Hu *et al*., 2006). As expected, Z-VAD-FMK significantly inhibited the Caspase-1 and IL-1β in *C. coli* RM1875 infected THP-1 cells. It also inhibited the Caspase-1 production significantly during the infection of *C. coli* 76339, but not the IL-1β, indicating that *C. coli* 76339 infection may develop the Caspase-1 independent IL-1β stimulation in addition to the Caspase-1 dependent IL-1β activation in macrophages. Previously, Netea *et al*., (2010) described the occurrence of Caspase-1 independent IL-1β stimulation in human neutrophils. The possibility of occurrence of Caspase-1 independent IL-1β stimulation in THP-1 cells upon infection with *C. coli* 76339 requires further investigation by using the inhibitors of other IL-1β activating enzymes, specifically serine protease inhibitors. Overall, these results confirmed that *C. coli* infection activates the NLRP3 inflammasomes in human macrophages.

IL-1β levels in glyburide-treated *C. coli* infected cells were not significantly different than the glyburide-untreated infected cells, specifying that even NLRP3 inflammasomes stimulation was inhibited following inhibition of K^+ efflux, but *C. coli* LOS continued the activation of NLRP3 inflammasome dependent signalling.

C. coli 76339 (contained sialylated LOS) showed higher induction of IL-1β than *C. coli* RM1875 (contained non-sialylated LOS), indicating that *C. coli* LOS and its sialylation are important for IL-1β induction in macrophages.

6.4.4. *Campylobacter* live cells do not cause the cell cytotoxicity

Increase in LDH was absent in *Campylobacter* live cells treated THP-1 cell cultures, which was concordant to the findings of Bouwman *et al*. (2014). However, LDH release was high in *Campylobacter* LOS treated THP-1 cells, indicating LOS may cause cell cytotoxicity in human macrophages. It might not be true as cell cytotoxicity may occur due to the hypersensitivity of THP-1 cells in the presence of LOS and therefore, macrophages in natural settings may not show the same results or any cytotoxic effects. Further investigation is required to find out the association of *Campylobacter* LOS with the cytotoxicity of human macrophages. LDH induction by *Campylobacter* LOS at higher level than LPS or positive control may be linked to the difference in LOS (1mg) and LPS (0.001μg) concentrations, given to stimulate the IL-1β production in THP-1 cells.

6.5. Conclusion

Activation of the NLRP3 mediated inflammatory response in human macrophages is a representation of a host response which develops during the *C. coli* infection. Live and disrupted *C. coli* cells, can both induce the production of NLRP3 inflammasome mediated Caspase-1 and IL-1β. The induction of Caspase-1 and IL-1β secretion depends on the number of *C. coli* live cells and can vary slightly between different *C. coli* strains. This study describes for the first time that *C. coli* is also included in the list of those bacteria which can stimulate the NLRP3 inflammasomes in the human macrophages during infection.

In addition to live and disrupted cells, *C. jejuni* and *C. coli* cell-surface LOS structures can also induce the Caspase-1 and IL-1β secretion in human macrophages. The extent of Caspase-1 and IL-1β induction alters with variation in LOS structures. It is also concluded that during *Campylobacter* infection, *Campylobacter*-associated molecular pattern (*Campylobacter* LOS) and danger-associated molecular pattern (K$^+$ efflux), both independently trigger the activation of NLRP3 inflammasomes. This study is the first description which represents the association of *Campylobacter* LOS with NLRP3 mediated inflammatory response as well as provides new insight into the interaction of *Campylobacter* with human macrophages.

CHAPTER 7

General Conclusions and Future Work

7.1. Major findings and their integration into previous research

7.1.1. Identification of *C. jejuni* LOS loci prevalence by PCR based typing

The first aim of this study was to examine the prevalence of 23 *C. jejuni* LOS locus genotypes in a clinical cohort and analyse the extent of gene variation in the *C. jejuni* LOS biosynthesis gene cluster by LOS locus typing of clinical *C. jejuni* isolates. To achieve this, LOS class specific primer pairs were designed, optimised, and used for *C. jejuni* LOS locus typing of 50 *C. jejuni* clinical strains, isolated from faecal samples of *Campylobacter* infected patients at the Northampton General Hospital (NGH), Northampton, UK. In addition, a simplified LOS classification system to better understand the relationship of *C. jejuni* LOS classes with LOS groups was also presented, where LOS group 1 (with class A, B, C, R, V, M), group 2 (with class E, H, O, P, W), group 3 (with class D, F, K, Q, N, I, J, S), and group 4 (with L, G, T, U) were included.

Previous studies have demonstrated that *C. jejuni* LOS group 1 and its related LOS classes (A, B and C) dominantly circulate amongst clinical *C. jejuni* isolates. The prevalence of this group might be due to their ability to synthesise human gangliosides mimicking LOS structures (Parker *et al*., 2005; Marsden, 2007; Islam *et al.,* 2009; Ellström *et al*., 2013; Islam *et al*., 2014; Ellström *et al*., 2016; Islam *et al*., 2018). This study has also confirmed a high frequency of LOS group 1 (62%) and its related classes (22% B > 20% C > 14% A). Prior published data has been further expanded upon in this study by identifying a hierarchy of LOS subclasses based on prevalence in clinical *C. jejuni* isolates (where B2 > A2 > B1 > A1). The high frequency of *C. jejuni* LOS classes B2 and C might be due to the fact that both LOS locus types exhibit high phase-variation at the gene sequence level and consequently, produce heterogenous LOS epitopes (or ganglioside mimics). For example, LOS locus type B2 present in *C. jejuni* strain 81-176, can change the GM_2 mimicking LOS epitope to GM_3 (GM_{2-3}) and GM_{2-3} to GD (Guerry *et al*., 2002). Similarly, *C. jejuni* 11168 with LOS locus type C can

modify GM_1 to a GM_2 like LOS structure (Linton *et al*., 2000). Moreover, this thesis has also provided a comparison of current findings of *C. jejuni* LOS loci distribution with previous studies, indicating that approximately 50–65% of strains in most of the clinical/enteritis associated *C. jejuni* populations belong to LOS classes A, B or C.

Compared to a high proportion of *C. jejuni* isolates with LOS loci (A, B and C), the prevalence of strains with the other three group 1 related and sialic acid biosynthesis genes containing loci (R, V and M) was negligible. The reasons for this remain unclear. However, class R strains do not produce GBS related ganglioside mimics (GM1, GM2, GD1, GD2) and class M strains are reported not to produce the ganglioside mimicking LOS structures at all despite having sialic acid biosynthesis genes (Houliston *et al*., 2011). A low frequency of these loci (R, V and M) from isolates as well as a low rate of GBS and MFS in *Campylobacter* infected patients (Nachamkin *et al*., 2002; Mortensen *et al*., 2009) despite high predominance of GBS/MFS associated *C. jejuni* LOS A, B, and C loci supports the notion that LOS structure is not a sole cause of GBS/MFS development.

During the collection of clinical *Campylobacter* strains from NGH, *C. coli* strains were much less common than *C. jejuni* isolates from clinical samples. This finding was consistent throughout the year and did not appear to vary with the season. Due to the lack of availability of *C. coli* clinical strains, a PCR based LOS typing assay could not be validated for *C. coli* in this study.

7.1.2. Analysis of *C. jejuni* LOS loci prevalence in GenBank by using bioinformatics approaches

The second aim of this study was to validate a pipeline to use for high-throughput *C. jejuni* LOS locus typing. Therefore, a Galaxy pipeline employing the alignment software MAFFT was designed and used for the *C. jejuni* LOS locus classification and approximately 65% (n=81 of 125) *C. jejuni* strain GenBank sequences had their LOS types categorised. The pipeline allowed successful assignment of ~50% (n=39 of 81) of analysed sequences to

particular LOS classes. In the MAFFT alignment tree or in the output of Galaxy pipeline, unclassified sequences belonging to a particular LOS group clustered together, and therefore, this pipeline indicated a LOS group for each of those remaining 42 of 81 sequences for which a particular LOS class could not be identified. The same LOS class or group for these 81 *C. jejuni* sequences was identified using a manual Megablast approach, which validated the results from Galaxy workflow. The manual approach using Megablast, in addition to validating the results from Galaxy workflow, was also used to LOS classify 622 *C. jejuni* sequences (44 complete and 578 draft), which could not be typed using the Galaxy pipeline. Blastn has been used previously to determine the LOS locus type in *Acinetobacter baumannii* genome sequences (n=234) available in the MLST database (Kenyon *et al*., 2014).

From the LOS locus analysis of 703 *C. jejuni* sequences from GenBank either by the Galaxy pipeline or manual Megablast alignments, the frequency of 23 different *C. jejuni* LOS biosynthesis locus classes (4 LOS groups) present within this collection was determined. The frequency of LOS classes A, B, and C was also high in this repository of *C. jejuni* online sequences [class B (23%) > class C (16%) $\geq$ class A (16%)] similar to the collection of *C. jejuni* clinical isolates [class B (22%) > class C (20%) > class A (14%)]. Other LOS group 1 related classes (R, V and M) were absent from the clinical isolates and this may reflect the fact that even in a large repository sequences belonging to these classes represented only 2.5 % in the online database. The hierarchy of LOS groups prevalence, (group 1 > group 2 > group 3 > group 4), was similar in both *C. jejuni* clinical isolates and from the larger sequence collection. The frequency of sialic acid biosynthesis LOS loci in the online GenBank database was high, although this collection contained other *C. jejuni* sources (*e.g.* animals, birds and farm soil) in addition to humans. This suggests that *C. jejuni* strains having sialylated LOS structures dominantly exist perhaps not just because their LOS structures are important for the pathogenesis, but also because their sialylated LOS phenotype is advantageous for *C. jejuni* general survival and fitness in different niches. Moreover, the *in silico* analysis of *C. jejuni* sequences has identified the class frequency hierarchy in *C. jejuni* LOS group 3 (F > K > S) and group 4 (G > L) for the first time.

7.1.3. Identification of *C. coli* LOS loci prevalence in GenBank by using bioinformatics based approaches

This study also aimed to analyse *in silico* the 564 *C. coli* online sequences available in GenBank to estimate the extent of gene content variation in *C. coli* LOS biosynthesis gene cluster. This study represents the first exploration of LOS locus genotype distribution in *C. coli* GenBank database via *in silico* analysis, where *C. coli* strains possessing LOS class III (41%; n=229 of 564) were the most common in GenBank. This was similar to the findings of Richard *et al*. (2013) and Culebro *et al*. (2018) who also reported class III as the most common class respectively in the collections of 33 US *C. coli* strains and 261 European Nucleotide Archive *C. coli* sequences. The reasons behind the high prevalence of LOS class III in *C. coli* are yet to be investigated. Moreover, the prevalence of *C. coli* LOS genotypes in different niches was estimated by looking at the online published sources from which these *C. coli* strains were isolated. The most common LOS types were III and VIII and tended to come from farm environmental niches (water and soil), as well as, humans and chickens. This suggests that the farm environment, in addition to poultry, is a primary source of *C. coli* transmission to humans. This was in agreement with a previous study that reported agriculture associated *C. coli* as an emerging human pathogen (Sheppard *et al*., 2010, 2013).

7.1.4. *In silico* Identification of novel LOS biosynthesis genes

By *in silico* analysis, LOS biosynthesis genes clusters of two *C. jejuni* strains, *C. jejuni* 414 (Accession no: ADGM01000014.1) and *C. jejuni* 1336 (Accession no: CM000854.1) were found with 5 and 13 novel LOS genes, respectively. The functions of these genes are not known. Four novel LOS genes in *C. jejuni* 1336 locus were predicted to arise from the capsular polysaccharide biosynthesis genes of other *C. jejuni* strains, while, six novel LOS genes of *C. jejuni* 1336 and one gene of *C. jejuni* 414 appeared to have originated from *C. coli*, which indicates the occurrence of intraspecies and interspecies gene recombination in *Campylobacter* species.

Campylobacter LOS biosynthesis genes (*waaC*, *waaM*, *lgtF*, *waaV*, *waaF*, *gmhA*, *waaE*, *waaD*, and *gmhB*) are involved in the biosynthesis of the LOS inner core and remain present with the same order or organisation in almost all *Campylobacter* strains (Parker *et al*., 2008; Richard *et al*., 2013). This is the reason that the inner core of LOS does not vary enormously between *Campylobacter* strains (Gilbert *et al*., 2002; Kolehmainen *et al*., 2019). Previously, organisation of the inner core biosynthesis genes in two *C. jejuni* LOS loci, M and W, was found to be different from all other *C. jejuni* LOS loci. *C. jejuni* class M had a gene between *waaM* and *lgtF* and *C. jejuni* class W contained two genes between *waaF* and *gmhA* (Parker *et al*., 2008; Richard *et al*., 2013). Similarly, this thesis shows that *C. coli* LOS loci, I, II, III, V, and VIII contain an additional gene inserted between *waaF* and *gmhA*. The presence of these LOS genes between *waaF* and *gmhA* was confirmed in 436 *C. coli* GenBank sequences with LOS type I (n=63), II (n=43), III (n=229), V (n=3), and VIII (n=98). Furthermore, a clinical *C. coli* strain, *C. coli* 221089, with class III LOS locus type was WG sequenced using the Illumina platform and submitted in GenBank under the accession number, RJLP00000000 (contigs: RJLP01000001-RJLP01000044). By pairwise alignment of these sequenced contigs with *C. coli* RM2228 (reference LOS class III strain) sequence, a class III gene, located between *waaF* and *gmhA*, was found present (100% query cover; 100% identity score) in *C. coli* 221089 strain.

The identified inner core biosynthesis LOS genes in *C. coli* vary at the sequence and functional level among *C. coli* LOS locus classes (based on data available in GenBank), indicating that, in addition to the LOS outer core, the LOS inner core saccharides also vary amongst *C. coli* strains. The sequence level variation in these genes and their possible putative functions have been determined *in silico* but require further wet-lab based functional characterisation.

7.1.5. Examination of altered motility phenotype in a mutated *C. coli* RM1875 strain

The third aim of this study was to construct and characterise a *C. coli* LOS biosynthesis gene region deletion mutant to determine the impact of LOS gene

deletions on *C. coli* cell phenotype. This aim had not been achieved in the current study, which might be because deleting the LOS locus in *C. coli* was not conducive with survival. However, the whole plasmid, used to generate deletion in LOS, was found integrated in the gDNA of a *C. coli* RM1875 mutant strain at a non-specific site. The exact location of its integration in *C. coli* RM1875 has yet to be identified. This mutant has showed a modified growth rate, increased cell motility and higher *flaA* gene expression in comparison to the WT strain and therefore, it is speculated that plasmid integration might have affected the motility associated genes.

7.1.6. Determination of the impact of LOS variation on IL-1β induction from THP-1 cells

During infection with *Campylobacter*, the occurrence of continuous intracellular K^+ ion depletion or K^+ efflux contributes to the activation of the NLRP3 inflammasome in human macrophages, possibly by ATP binding to macrophage P2X7 receptors (Pétrilli *et al*., 2007; Bouwman *et al*., 2014). The last aim of this study was to determine the *in vitro* impact upon the IL-1β induction of THP-1 cells with variation of the LOS structures in *C. jejuni* and *C. coli*.

The infection of THP-1 cells with a *C. jejuni* 81116 mutant lacking LOS core structures (*waaF:cat*) in a previous study demonstrated that *C. jejuni* LOS variation does not have any impact on the induction of IL-1β from THP-1 cells (Bouwman *et al*., 2014). In this thesis, similar results were observed during infection of THP-1 cells with a *C. jejuni* 11168 mutant (Δ*cj1132c-cj1152c*) lacking LOS core structures. However, replication of this infection assay in the presence of a K^+ channel inhibitor provided contrasting results. Blocking of K^+ efflux with glyburide and simultaneously, with LOS lacking core polysaccharides, caused a significant decrease in IL-1β production in THP-1 cells. This explains that *C. jejuni* LOS variation can affect the extent of NLRP3 dependent IL-1β induction in THP-1 cells, but this minor IL-1β inducing factor (LOS) is difficult to observe due to simultaneous presence of another major IL-1β inducing factor (namely K^+ efflux). The concentration of released IL-1β did not change in WT *C. jejuni* 11168, *C. coli* RM1875 and *C. coli* 76339 infected THP-1 cells following the inhibition of

K$^+$ efflux, which also supports this notion that LOS and K$^+$ efflux both contribute to the activation of NLRP3 inflammasome dependent signalling. This study is the first demonstration of an association between *C. jejuni* LOS and macrophage NLRP3 dependent IL-1β induction.

This study has also demonstrated that, both live and disrupted cells of WT *C. coli* strains, 76339 (present sialylated LOS) and RM1875 (possess non-sialylated LOS), can stimulate the production of IL-1β in THP-1. *C. coli* 76339 infected THP-1 cells induced IL-1β release at high level in comparison to *C. coli* RM1875 infected THP-1 cells, which might be either due to the occurrence of sialylated LOS in *C. coli* 76339 or both Caspase-1 dependent and Caspase-1 independent IL-1β inducing mechanisms in *C. coli* 76339. This data suggests LOS variation can play an important role in modulating innate immune responses mediated through inflammasome activation.

7.2. Research Implications and Future Work

Our understanding of the prevalence of different *C. jejuni* LOS locus genotypes, gained from this study, could be further enhanced by coupling this genetics-based analysis with LOS structural analysis and patient clinical data. Although, the analysis of the extent of gene content variation in *C. jejuni* LOS biosynthesis locus can be helpful for LOS-based *Campylobacter* vaccine related research in future. For example, it might be expected that *C. jejuni* strains with highly frequent LOS types (B and C; identified in this study) exhibit phase variable LOS structures (Linton *et al*., 2000; Guerry *et al*., 2002; Godschalk *et al*., 2004) and therefore might represent a challenging target for LOS based vaccine development (Prendergast *et al*., 2003). A non-phase variable, recombinant LOS structure engineered from the phase variation lacking LOS genes (specifically of highly frequent LOS locus types A, B and C), may be predicted to be good candidates for a *Campylobacter* vaccine.

A limitation of this work is that most of the *C. jejuni* sequences collected were draft sequences from the GenBank database and may not be completely accurate. However, read depth coverage for most samples was >20X. In

addition, most of the strain sequences that have been deposited in the online repository were from Canada, UK or USA, and so may reflect a bias that is different from the overall global frequency of LOS classes/types. Further, the Galaxy pipeline used in this study extracted a 50KB sequence coordinate from the WG sequence (predict to contain LOS sequence), but for some sequences it could not isolate the correct 50Kb window and this is the area of the pipeline that needs improving.

The *rpsL* based mutagenesis strategy used in the current study is complicated, at least for constructing the multiple gene mutation bearing mutants, as single-nucleotide polymorphisms in *rpsL* genes can change the bacterial phenotype for streptomycin action and therefore, are likely to increase the occurrence of false positive results. Additionally, double-crossover events can occur between the plasmid and genomic *rpsL* genes, which can further exacerbate off-target mutations. Although, defined mutants with single gene deletions can be constructed feasibly using this strategy (Hendrixson *et al*., 2001). Single phase variation in an individual *Campylobacter* gene affects its phenotype enormously (Gilbert *et al*., 2002; Godschalk *et al*., 2004) and therefore, phenotypic characterisation of a *Campylobacter* mutant having mutation in multiple genes can be very complex. In the current study, an integration of a foreign plasmid DNA sequence into the chromosomal DNA of *C. coli* RM1875 was observed, which might be a consequence of illegitimate recombination (Richardson and Park, 1997). In addition, integration may be associated with the gain of antibiotic resistance in *C. coli*. A putative link between the rate of illegitimate recombination and gain of antibiotic resistance in *C. coli* can be investigated in future studies.

This study has established a link between LOS and THP-1 induced IL-1β production. However, it remains unknown which LOS structural components are actually responsible for the increase in IL-1β induction and whether *Campylobacter*-induced cell damage during infection facilitates this. The LOS-induced IL-1β secretion can be further investigated in other engineered NLRP3 or Caspase-1 deficient THP-1 cell lines. The Capase-1 independent IL-1β induction in THP-1 cells upon infection with *C. coli* 76339 might be due to increase in expression of proteases and other pro IL-1β processing enzymes during this infection, which also requires further investigation.

7.3. Final Conclusion

This work extends our understanding of the *C. jejuni* LOS locus classification system, validates the potential for a bioinformatic approach to the classification of *C. jejuni* LOS locus types, and provides an overview of *C. jejuni* and *C. coli* LOS loci class frequencies in a worldwide database of *C. jejuni* and *C. coli*. *C. jejuni* strains having ABC LOS loci or potential to produce and modulate the human ganglioside-mimicking sialylated LOS structures continue to be the most prevalent in the online database and amongst our clinical isolates, demonstrating the significance of LOS in multifactorial pathogenesis of *Campylobacter*. This study also highlighted that environmental niches are a major reservoir of *C. coli*, unlike *C. jejuni*, whose major reservoir is in chickens. This work elucidated that the extent of NLRP3 dependent Caspase-1 and IL-1β induction from THP-1 cells alters with variation in LOS structures and demonstrated for the first time that purified LOS from both *C. jejuni* and *C. coli* can induce IL-1β, indicating the importance of LOS in the stimulation of host innate immune responses. Comprehensively, this work has extended our understanding of the *Campylobacter* LOS locus classification system and determined that LOS plays an important role in the development of host immune response during *Campylobacter* infection.

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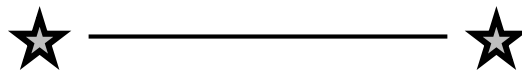
Appendix-I

Table 1: Primers for the identification of *C. jejuni* LOS locus classes (Classes given in brackets in the last column were detectable also with respective primers).

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
Control Primers				
1	Forward Primer: TGGGAGCAAGCCTTATCG Reverse Primer: TCCCAAGGTCTTTTTTTAATC	*waaM*	184	All

Forward primer properties:
%GC= 55   GC clamp= 2
Dimers= 3   3' Dimers= 2
Stability= 1.8   Tm= 56 °C
Runs= 3   Repeats= none
Hairpins = none
Annealing temperature= 50 °C

Reverse primer properties:
%GC= 33   GC clamp= 1
Dimers= 4   3' Dimers= 1
Stability= 2.7   Tm= 52 °C
Runs= 7   Repeats= 3
Hairpins= none
Annealing temperature= 50 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
2	Forward Primer: TCATATCTTCCATTTGGATTAAATT Reverse Primer: AAAGGCATTTTTGCTGGTC	*waaV*	205-220	All

Forward primer properties:
%GC= 24   GC clamp= 0
Dimers= 4   3' Dimers= 4
Stability= 1.7   Tm= 53.1 °C
Runs= 3   Repeats= 2
Hairpins= none
Annealing temperature= 50 °C

Reverse primer properties:
%GC= 42   GC clamp= 1
Dimers= 3   3' Dimers= 1
Stability= 0.7   Tm= 52.4 °C
Runs= 5   Repeats= 2
Hairpins= none
Annealing temperature= 50 °C

First Set of primers for Group 1 (Classes A, B, C, V, R, & M)

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
A1	Forward Primer: AGCTTCTCTTGAAAGCATATTG Reverse Primer: ACAGGATGAAGTTGATTTAGTG	*cgtA* & *cgtB*	1301	A1

Forward primer properties:
%GC= 36   GC clamp= 1
Dimers= 4   3' Dimers= 2
Stability= 3.3   Tm= 54.7 °C
Runs= 3   Repeats= 2
Hairpins= none
Annealing temperature= 49.7 °C

Reverse primer properties:
%GC= 36   GC clamp= 1
Dimers= 2   3' Dimers= 2
Stability= 1.3   Tm= 54.7 °C
Runs= 3   Repeats= none
Hairpins= none
Annealing temperature= 49.7 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
A2	Forward Primer: ATTGCCTGCTATTCAAAGAG Reverse Primer: AGGCTGTTGGTTTAATATCG	*cgtA* & *cgtB*	1090	A2

Forward primer properties:
%GC= 40   GC clamp= 1
Dimers= 3   3' Dimers= 2
Stability= 1.9   Tm= 53.2 °C
Runs= 3   Repeats= 2
Hairpins= none
Annealing temperature= 48.2 °C

Reverse primer properties:
%GC= 40   GC clamp= 2
Dimers= 4   3' Dimers= 2
Stability= 1.9   Tm= 53.2 °C
Runs= 3   Repeats= 2
Hairpins= none
Annealing temperature= 48.2 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
B1	Forward Primer: CCAGCTGCCTTAACTCCTTC Reverse Primer: TCCTAGGGCTATGGCTACTG	*cgtB* & *cst-II*	764	B1

Forward primer properties:
%GC= 55   GC clamp= 1
Dimers= 6   3' Dimers= 1
Stability= 1.6   Tm= 62 °C
Runs= 2   Repeats= none
Hairpins= none
Annealing temperature= 54.4 °C

Reverse primer properties:
%GC= 31   GC clamp= 1
Dimers= 4   3' Dimers= 2
Stability= 2.2   Tm= 56 °C
Runs= 2   Repeats= none
Hairpins= none
Annealing temperature= 54.4 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
B2	Forward Primer: TTAACAAGCACTTCATTCTTAG Reverse Primer: TATAGCAAGGGCAATAGAAAG	*cgtA* & *cgtB*	640	B2

Forward primer properties:
%GC= 52   GC clamp= 2
Dimers= 2   3' Dimers= 1
Stability= 3.3   Tm= 61 °C
Runs= 3   Repeats= none
Hairpins= none
Annealing temperature= 47.9 °C

Reverse primer properties:
%GC= 35   GC clamp= 2
Dimers= 4   3' Dimers= 1
Stability= 1.9   Tm= 55 °C
Runs= 3   Repeats= none
Hairpins= none
Annealing temperature= 47.9 °C

C	Forward Primer: GCTGCTGCTATAGTAGGAAG Reverse Primer: AAATCAAAAAAACCTTTATGCTTTTC	*neuC1* & *neuA1*	931	C or V

%GC= 50	GC clamp= 1
Dimers= 6	3' Dimers= 2
Stability= 1.6	Tm= 57.3 °C
Runs= 2	Repeats= 2
Hairpins= none	
Annealing temperature= 55 °C	

⇒ ⇐

%GC= 23	GC clamp= 1
Dimers= 4	3' Dimers= 1
Stability= 1.2	Tm= 53.8 °C
Runs= 7	Repeats= 3
Hairpins= none	
Annealing temperature= 55 °C	

M	Forward Primer: AGACGCTTTGCAAGTTATAATG Reverse Primer: TGATGGAAGTAGCGATAATAG	*waaM* & Orf51	1149	M

%GC= 36	GC clamp= 1
Dimers= 6	3' Dimers= 2
Stability= 3.0	Tm= 54.7 °C
Runs= 3	Repeats= 2
Hairpins= none	
Annealing temperature=49 °C	

⇒ ⇐

%GC= 38	GC clamp= 1
Dimers= 2	3' Dimers= 1
Stability= 3.1	Tm= 54 °C
Runs= 2	Repeats= none
Hairpins= none	
Annealing temperature=49 °C	

R	Forward Primer: ATAAGCAATCTTCCGAATTCAC Reverse Primer: TGTTTAAGTTATGGCGAAGATG	*cj1145c* & *waaV*	729	R

%GC= 36	GC clamp= 1
Dimers= 6	3' Dimers= 1
Stability= 1.4	Tm= 54.7°C
Runs= 2	Repeats= none
Hairpins= none	
Annealing temperature= 49.7 °C	

⇒ ⇐

%GC= 36	GC clamp= 1
Dimers= 4	3' Dimers= 1
Stability= 2.6	Tm= 54.7 °C
Runs= 3	Repeats= none
Hairpins= none	
Annealing temperature=49.7 °C	

Second Set of primers for Group 2 (Classes E, H, O, P, & W)

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
EHOP	Forward Primer: TTCAAGAGCGTCCAGAAG Reverse Primer: CGTGAGTTCCTGTGTCAATC	Orf22	453	E, H, O, P

%GC= 50	GC clamp= 1
Dimers= 3	3' Dimers= 2
Stability= 2.1	Tm= 53.7 °C
Runs= 2	Repeats= 2
Hairpins= none	
Annealing temperature= 48.7 °C	

⇒ ⇐

%GC= 50	GC clamp= 1
Dimers= 3	3' Dimers= 2
Stability= 2.5	Tm= 57.3 °C
Runs= 2	Repeats= 2
Hairpins= none	
Annealing temperature= 48.7 °C	

26EO	Forward Primer: TTGCCGTTAATTCATTACAG Reverse Primer: ATGTCGCATTTATACCTTTG	Orf26 & Orf27	1017	E, O

%GC= 35	GC clamp= 1
Dimers= 4	3' Dimers= 1
Stability= 2.8	Tm= 51.1 °C
Runs= 2	Repeats= none
Hairpins= none	
Annealing temperature= 60.4 °C	

⇒ ⇐

%GC= 35	GC clamp= 1
Dimers= 4	3' Dimers= 2
Stability= 2.2	Tm= 51.1 °C
Runs= 3	Repeats= 2
Hairpins= none	
Annealing temperature= 60.4 °C	

28EP	Forward Primer: TCAGGTAGAGATGCATTTAG Reverse Primer: CGCCTATGCAATGTTTAACC	Orf28 & Orf29	713	E, P

%GC= 40	GC clamp= 1
Dimers= 6	3' Dimers= 1
Stability= 2.2	Tm= 53.2 °C
Runs= 3	Repeats= 2
Hairpins= none	
Annealing temperature= 57.4 °C	

⇒ ⇐

%GC= 45	GC clamp= 2
Dimers= 4	3' Dimers= 1
Stability= 2.5	Tm= 55.3 °C
Runs= 3	Repeats= none
Hairpins= none	
Annealing temperature= 57.4 °C	

26'HP	Forward Primer: AAAGCAAAGAGAATGGATTAG Reverse Primer: ATGTCGCATTTATACCTTTG	Orf26 & Orf27	904	H, P

%GC= 33	GC clamp= 1
Dimers= 3	3' Dimers= 1
Stability= 2.2	Tm= 52 °C
Runs= 3	Repeats= 2
Hairpins= none	
Annealing temperature= 59.9 °C	

⇒ ⇐

%GC= 35	GC clamp= 1
Dimers= 4	3' Dimers= 2
Stability= 2.2	Tm= 51.1 °C
Runs= 3	Repeats= 2
Hairpins= none	
Annealing temperature= 59.9 °C	

Primer Name	Primer Properties		Target Genes	Amplicon Size (bps)	Class Specificity
W	Forward Primer: GCTTTGGGCTTATGAGAGTG Reverse Primer: GGCGAACAACTACACCCTATAC		Orf53 & Orf54	1502	W

	Forward			Reverse	
	%GC= 50 GC clamp= 1 Dimers= 2 3' Dimers= 1 Stability= 1.9 Tm= 57.3 °C Runs= 3 Repeats= 2 Hairpins= none Annealing temperature=55 °C	→	←	%GC=50 GC clamp=1 Dimers=4 3' Dimers=1 Stability=3.8 Tm=60.3 °C Runs=3 Repeats=2 Hairpins= none Annealing temperature=55 °C	

Third Set of primers for Group 3 (Classes D, K, Q, N, F, I, J, & S)

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
D	Forward Primer: TGGTTGGTGGCCTGATTATG Reverse Primer: CAATGCTTGGAATGGTATAG	Orf3 & Orf17	1221	D, (I)

%GC= 50   GC clamp= 1
Dimers= 4   3' Dimers= 1
Stability= 3.4   Tm= 57.3 °C
Runs= 2   Repeats= none
Hairpins= none
Annealing temperature= 48.2 °C

→ ←

%GC= 40   GC clamp= 1
Dimers= 4   3' Dimers= 2
Stability= 2.6   Tm= 53.2 °C
Runs= 2   Repeats= 2
Hairpins= none
Annealing temperature= 48.2 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
F	Forward Primer: ATATCAAGATCCACCCATAC Reverse Primer: CTCGATGCTTGTGAAATAAC	Orf 16 & *waaV*	886	F, (D)

%GC= 40   GC clamp= 1
Dimers= 4   3' Dimers= 1
Stability= 2.2   Tm= 53.2 °C
Runs= 3   Repeats= 2
Hairpins= none
Annealing temperature= 51.5 °C

→ ←

%GC= 40   GC clamp= 1
Dimers= 4   3' Dimers= 2
Stability= 2.0   Tm= 53.2 °C
Runs= 3   Repeats= 2
Hairpins= none
Annealing temperature= 51.5 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
IS	Forward Primer: GCAAATGGGAAATCTTGATAGG Reverse Primer: GGCCCTCAGATAAACTACCC	Orf41 & Orf42	973	I, S

%GC= 40   GC clamp= 2
Dimers= 3   3' Dimers= 1
Stability= 1.8   Tm= 56.5 °C
Runs= 3   Repeats= none
Hairpins= none
Annealing temperature= 51.5 °C

→ ←

%GC= 55   GC clamp= 3
Dimers= 4   3' Dimers= 1
Stability= 2.0   Tm= 59.4 °C
Runs= 3   Repeats= none
Hairpins= none
Annealing temperature= 51.5 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
J	Forward Primer: TCATCAATACGCTTTAAATTCC Reverse Primer: GGCCCTCAGATAAACTACCC	Orf40 & Orf42	1206	J

%GC= 31   GC clamp= 2
Dimers= 6   3' Dimers= 1
Stability= 1.9   Tm= 52.8 °C
Runs= 3   Repeats= none
Hairpins= none
Annealing temperature=47.8 °C

→ ←

%GC= 55   GC clamp= 3
Dimers= 4   3' Dimers= 1
Stability= 2.0   Tm= 59.4 °C
Runs= 3   Repeats= none
Annealing temperature=51.7 °C

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
K	Forward Primer: ACATAATACTCCTTGCAATC Reverse Primer: TCCCAAGAATCTATAATATCAG	Orf49 & *waaV*	3118	K

%GC= 35   GC clamp= 1
Dimers= 6   3' Dimers= 2
Stability= 1.2   Tm= 51.1 °C
Runs= 2   Repeats= none
Hairpins= none
Annealing temperature=46.1 °C

→ ←

%GC= 31   GC clamp= 1
Dimers= 4   3' Dimers= 2
Stability= 1.5   Tm= 52.8 °C
Runs= 3   Repeats= 2
Annealing temperature=51.7 °C

Q	Forward Primer: TGTTGCTAATTTGGCTAATTC Reverse Primer: AAACGGGATTTATGGTAGTTTG	Orf46 & Orf16	1130	Q

%GC= 33	GC clamp= 1		%GC= 36	GC clamp= 1
Dimers= 4	3' Dimers= 1		Dimers= 4	3' Dimers= 1
Stability= 2.6	Tm= 52 ℃		Stability= 2.4	Tm= 54.7 ℃
Runs= 3	Repeats= none		Runs= 3	Repeats=none
Hairpins= none			Hairpins= none	
Annealing temperature=47 ℃			Annealing temperature=47 ℃	

N	Forward Primer: GCACCAACTCCCAAATGTC Reverse Primer: GAAAGCAGCGATGATACCC	Orf38 & *waaV*	1253	N

%GC= 52	GC clamp= 1		%GC= 52	GC clamp= 3
Dimers= 2	3' Dimers= 1		Dimers= 2	3' Dimers= 1
Stability= 1.9	Tm= 56.7 ℃		Stability= 1.3	Tm= 56.7 ℃
Runs= 3	Repeats= none		Runs= 3	Repeats= none
Hairpins= none			Hairpins= none	
Annealing temperature=51.7 ℃			Annealing temperature=51.7 ℃	

Fourth Set of primers for Group 4 (Classes G, L, T, & U)

Primer Name	Primer Properties	Target Genes	Amplicon Size (bps)	Class Specificity
G	Forward Primer: TCTGATTGATACAACTTTCTATT Reverse Primer: AAGATGCAAATGAAATCATACC	Orf37 & Orf16	776	G

%GC= 26	GC clamp= 0		%GC= 31	GC clamp= 2
Dimers= 3	3' Dimers= 2		Dimers= 4	3' Dimers= 1
Stability= 1.6	Tm= 55 ℃		Stability= 1.3	Tm= 57 ℃
Runs= 3	Repeats= none		Runs= 3	Repeats= none
Hairpins= none			Hairpins= none	
Annealing temperature= 50.5 ℃			Annealing temperature= 50.5 ℃	

T	Forward Primer: TTGGCAAGATGATTGAAATTTTAGG Reverse Primer: TTGTGAAATAGCGTTTAAAGAG	Orf37 & *waaV*	1773	T, (G, U)

%GC= 32	GC clamp= 2		%GC= 31	GC clamp= 1
Dimers= 6	3' Dimers= 1		Dimers= 6	3' Dimers= 1
Stability= 2.2	Tm= 56.4 ℃		Stability= 2.1	Tm= 52.8 ℃
Runs= 4	Repeats= 2		Runs= 3	Repeats= 2
Hairpins= none			Hairpins= none	
Annealing temperature= 47 ℃			Annealing temperature= 47 ℃	

U	Forward Primer: TATTCTTTGCTGCCAACC Reverse Primer: TTGTGAAATAGCGTTTAAAGAG	Orf38 & waaV	1499	U, (G)

%GC= 44	GC clamp= 2		%GC= 31	GC clamp= 1
Dimers= 3	3' Dimers= 1		Dimers= 6	3' Dimers= 1
Stability= 1.3	Tm= 51.4 ℃		Stability= 2.1	Tm= 52.8 ℃
Runs= 3	Repeats= none		Runs= 3	Repeats= 2
Hairpins= none			Hairpins= none	
Annealing temperature= 46.5 ℃			Annealing temperature= 46.5 ℃	

L	Forward Primer: TTTGCTTCTTCTATAAGTAATTTTC Reverse Primer: TTGTGAAATAGCGTTTAAAGAG	Orf16 & *waaV*	1027	L, (F)*

%GC= 24	GC clamp= 1		%GC= 31	GC clamp= 1
Dimers= 4	3' Dimers= 1		Dimers= 6	3' Dimers= 1
Stability= 1.6	Tm= 55 ℃		Stability= 2.1	Tm= 57 ℃
Runs= 4	Repeats= 2		Runs= 3	Repeats= 2
Hairpins= none			Hairpins= none	
Annealing temperature= 47.8 ℃			Annealing temperature= 47.8 ℃	

*- Classes F and L share ORF16 and waaV, therefore Primer L was found to be positive for both class F and L associated strains. However, it does not bind to the class F reference strain, RM1221.

Table 2: Primers used for generating deletion in *C. coli* RM1875 and motility assay

Primer	Primer Sequence (5'-3')
pUC-F	CTGCAAGGCGATTAAGTTGG
pUC-R	TTATGCTTCCGGCTCGTATG
rpsL-F	AACAATGCTGTGTTCTTGTAGG
rpsL-R	AAGGAATTATTGTGCCTACC
S1-F	CCCGAGCTCTAGTGGTACGAGGCTTTATC
S1-R	CCGCTCGAGGCTTTGCGCGATCTAAC
S2-F	CCGCTCGAGTAAATCTTATTGGCGCTTGC
S2-R	CGTGGTACCGAACTGTAGGCATAGTAATCCC
cat-F	TCTATGATACCGTGGACAAG
cat-R	CACTAATGCAGGTGATTTGG
SS2-F	GATACAGGTACGCATGACAG
SS2-R	GCTTTAGAGGTTGGGTCAAGAG
210-F	CCGCTCGAGAATGATACATAAAATGTCAGATGTGCAAAG
210-R	CGCGCGTGGTACCTCTAAAAAACTTTTAATATTTTACAATAATAAG
flaA-F	CCAATGTCGGCTCTGATTTG
flaA-R	GCGCAGGAAGTGGATTTTC

Appendix-II

Table 1: LOS types of *C. jejuni* complete (n=125) and draft sequences (n=597)
Complete sequences: LOS Group 1; Classes A1, A2, B1, B2, C & V

No.	*C. jejuni* Strain	Accession no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
1	00-6200	NZ_CP010307.1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Human, Canada
2	RM3196	NZ_CP012690.1					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Human, South Africa
3	RM3197	NZ_CP012689.1													A1	Human, South Africa
4	RM3420	NZ_CP017456.1					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Human, Canada
5	00-1597	NZ_CP010306.1					99/98	99/100	91/100	98/100	98/99		97/100	95/100	A2	Human, Canada
6	HF5-4A-4	NZ_CP007188.1					100/100	99/100	100/100	100/100	100/100		100/100	100/100	A1	Farm, UK
7	FDAARGOS_262	NZ_CP022076.1					99/100	96/100	96/100	98/100	98/99		98/100	97/100	A2	Bovine, US
8	TS1218	NZ_CP017860.1					99/100	99/100	99/100	98/100	99/99		97/100	98/99	A1	Chicken, US
9	32488	NC_021834.1					99/100	99/100	95/100	97/100	97/100		97/100	95/100	A2 WITH DOBLE Orf neuA1	Human, US
10	M129	NZ_CP007749.1					99/100	99/100	95/100	97/100	98/99		97/100	95/100	A2	Human, US
11	FDAARGOS_422	NZ_CP023867.1					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Human, US
12	MTVDSCj13	NZ_CP017032.1					98/100	99/100	99/100	98/100	98/100	99/100	98/100	98/100	B2	Chicken, US
13	RM3194	NZ_CP014344.1					98/100	99/100	99/100	98/100	98/100	99/100	98/100	98/100	B2	Human, South Africa
14	MTVDSCj16	NZ_CP017033.1					96/95				98/100	99/100		97/100	B1	Chicken, US
15	NCTC11351	NZ_LN831025.1					99/100	100/100	99/100	100/100	99/100	99/100	99/100	99/100	B1	Not Known
16	FORC_046	NZ_CP017229.1					98/100	100/100	99/100	98/100	98/99		98/100	99/100	B2	Human, South Korea
17	YH001	CP010058.1					98/100	98/100	93/100	99/100	98/100		98/100	99/100	B1	Beef, US
18	Cj3	NZ_KK365768.1					98/100	98/100	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, Thailand
19	FDAARGOS_265	NZ_CP022079.1						94/87	91/100	99/100	99/100	99/100	97/100	98/100	B1	Human, US
20	81-176_G1_B0	NZ_CP022440.1					99/100	99/100	95/100	98/100		99/100	100/100	99/100	B2	Human, UK
21	14980A	NZ_CP017029.1					96/95				98/100	99/100	98/100	97/100	B1	Turkey, US
22	11168-BN148	NC_018521.1			100/100	100/100		100/100	100/100	100/100	100/100		100/100	100/100	C	Unknown, Finland
23	00-2538	NC_022351.2			100/100	100/100		100/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
24	00-2544	NC_022353.2			100/100	100/100		100/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
25	00-2426	NC_022352.2			100/100	100/100		100/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
26	00-2425	NC_022362.2			100/100	100/100		100/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
27	NCTC 11168-K12E5	NZ_CP006685.1			100/100	100/100		99/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
28	NCTC 11168-Kf1	NZ_CP006686.1			100/100	100/100		99/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
29	D42a	CP007751.1			100/100	100/100		100/100	100/100	100/100	100/100		100/100	99/100	C	Chicken, US
30	NCTC 11168-mcK12E5	NZ_CP006688.1			100/100	100/100		99/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
31	NCTC 11168-GSv	NZ_CP006689.1			100/100	100/100		100/100	100/100	100/100	100/100		100/100	99/100	C	Human, Canada
32	00-0949	NZ_CP010301.1			100/100	99/100		100/100	100/100	100/100	100/100		99/100	99/100	C	Human, Canada
33	WP2202	NZ_CP014742.1			100/100	100/100		99/100	100/100	100/100	100/100		99/100	99/100	C	Chicken, US
34	ZP3204	NZ_CP017856.1			100/100	100/100		99/100	100/100	100/100	100/100		99/100	99/100	C	Chicken, US
35	NS4-5-1	NZ_CP007192.1			100/100	99/100		99/100	100/100	99/100	100/100		100/100	99/100	C	Farm, UK
36	NS4-9-1	NZ_CP007193.1			100/100	99/100		100/100	100/100	99/100	100/100		100/100	99/100	C	Farm, UK
37	CFSAN032806	NZ_CP023543.1			99/100	99/100		99/100	100/100	100/100	100/100		99/100	99/100	C	Chicken, US
38	01-1512	NZ_CP010072.1			100/100	99/100		100/100	100/100	100/100	100/100		99/100	99/100	C	Human, Canada
39	RM1285	NZ_CP012696.1			100/100	100/100		99/100	100/100	100/100	100/100		99/100	99/100	C	Chicken, US
40	FDAARGOS_263	NZ_CP022077.1			100/100	100/100		100/100	100/100	100/100	100/100		100/100	100/100	C	Human, US
41	YQ2210	NZ_CP017859.1			100/100	100/100		99/100	100/100	100/100	100/100		99/100	99/100	C	Turkey, US
42	11168H/lacY	NZ_CP022439.1			100/100	100/100		100/100	100/100	100/100	100/100		100/100	100/100	C	Human, UK
43	MTVDSCj07	NZ_CP017031.1			100/100	100/100		99/100	100/100	100/100	100/100		99/100	99/100	C	Chicken, US
44	YH002	NZ_CP020776.1			100/100	100/100		99/100	100/100	100/100	100/100		99/100	99/100	C	Calf, US
45	NS4-1-1	NZ_CP007191.1			100/100	99/100		100/100	100/100	99/100	100/100		100/100	99/100	C	Farm, UK
46	11168H/araE	NZ_CP022559.1			100/100	100/100		100/100	100/100	100/100	100/100		100/100	99/100	C	Human, UK
47	NCTC 12664	NZ_CP028912.1			100/100	100/100		100/100	100/100	100/100	100/100		99/100	99/100	C	Chicken, UK
48	IA3902	NC_017279.1			100/100	99/100		100/100	100/100	100/100	100/100		99/100	99/100	C	Sheep, US
49	12567	NZ_CP028909.1			100/100	99/100		99/100	100/100	100/100	100/100		100/100	99/100	C	Chicken, UK
50	NCTC 12660	NZ_CP028910.1			100/100	99/100		99/100	100/100	100/100	100/100		100/100	99/100	C	Chicken, UK
51	OD267	NZ_CP014744.1			100/100			99/91	100/100	100/100	100/100		99/100	99/100	V	Chicken, US
52	PT14	NC_018709.4			100/100	100/100		99/100	100/100	100/100	100/100		99/100	99/100	C WITH ORF 48L INSERTION	Unknown, UK

Complete sequences: LOS Group 2; Classes P, H, E, O & W

No.	*C. jejuni* Strain	Accession no.	21-25	26	26'	27	28	29-34	Class	Host, Country
1	CG8421	NZ_CP005388.1	98/98		99/100	99/92	99/100	98/99	P	Human, US
2	4031	NC_022529.1	98/98		100/100	99/92	99/100	99/99	P	Unknown, Finland
3	MTVDSCj20	NZ_CP008787.1	98/100	99/100		100/100		100/100	O	Chicken, US
4	IF1100	NZ_CP017863.1	98/98		99/100	98/100		99/100	H	Chicken, US
5	RM1246-ERRC	NZ_CP022470.1	98/98		100/100	99/100		99/100	H	Human, US
6	FDAARGOS_266	NZ_CP022080.1	96/100	99/100		98/92	99/100	98/99	E	Unknown, US
7	CJM1cam	NZ_CP012149.1	99/85	99/100		99/99		98/100	W	Human, UK
8	CJ677CC519	NZ_CP010471.1	98/100	99/100		98/100		98/100	O	Faeces, Finland
9	CJ677CC002	NZ_CP010472.1	98/100	99/100		98/100		98/100	O	Human, Finland
10	CJ677CC534	NZ_CP010473.1	98/100	99/100		98/100		98/100	O	Human, Finland
11	CJ677CC536	NZ_CP010474.1	98/100	99/100		98/100		98/100	O	Human, Finland
12	CJ677CC073	NZ_CP010475.1	98/100	99/100		98/100		98/100	O	Human, Finland
13	CJ677CC521	NZ_CP010476.1	98/100	99/100		98/100		98/100	O	Human, Finland
14	CJ677CC526	NZ_CP010477.1	98/100	99/100		98/100		98/100	O	Human, Finland
15	CJ677CC036	NZ_CP010479.1	98/100	99/100		98/100		98/100	O	Human, Finland
16	CJ677CC524	NZ_CP010480.1	98/100	99/100		98/100		98/100	O	Human, Finland
17	CJ677CC016	NZ_CP010481.1	98/100	99/100		98/100		98/100	O	Human, Finland
18	CJ677CC041	NZ_CP010482.1	98/100	99/100		98/100		98/100	O	Human, Finland
19	CJ677CC535	NZ_CP010483.1	98/100	99/100		98/100		98/100	O	Human, Finland
20	CJ677CC092	NZ_CP010488.1	98/100	99/100		98/100		98/100	O	Human, Finland
21	CJ677CC530	NZ_CP010489.1	98/100	99/100		98/100		98/100	O	Human, Finland
22	CJ677CC532	NZ_CP010490.1	98/100	99/100		98/100		98/100	O	Human, Finland
23	CJ677CC529	NZ_CP010491.1	98/100	99/100		98/100		98/100	O	Human, Finland
24	CJ677CC531	NZ_CP010492.1	98/100	99/100		98/100		98/100	O	Human, Finland
25	CJ677CC062	NZ_CP010493.1	98/100	99/100		98/100		98/100	O	Human, Finland
26	CJ677CC059	NZ_CP010494.1	98/100	99/100		98/100		98/100	O	Human, Finland
27	CJ677CC032	NZ_CP010496.1	98/100	99/100		98/100		98/100	O	Human, Finland
28	CJ677CC033	NZ_CP010497.1	98/100	99/100		98/100		98/100	O	Human, Finland
29	CJ677CC537	NZ_CP010498.1	98/100	99/100		98/100		98/100	O	Human, Finland
30	CJ677CC542	NZ_CP010499.1	98/100	99/100		98/100		98/100	O	Human, Finland
31	CJ677CC528	NZ_CP010500.1	98/100	99/100		98/100		98/100	O	Human, Finland
32	CJ677CC538	NZ_CP010495.1	98/100	99/100		98/100		98/100	O	Human, Finland
33	CJ677CC520	NZ_CP010501.1	98/100	99/100		98/100		98/100	O	Human, Finland
34	CJ677CC014	NZ_CP010502.1	98/100	99/100		98/100		98/100	O	Human, Finland
35	CJ677CC039	NZ_CP010503.1	98/100	99/100		98/100		98/100	O	Human, Finland
36	CJ677CC085	NZ_CP010504.1	98/100	99/100		98/100		98/100	O	Human, Finland
37	CJ677CC052	NZ_CP010505.1	98/100	99/100		98/100		98/100	O	Human, Finland
38	CJ677CC527	NZ_CP010506.1	98/100	99/100		98/100		98/100	O	Human, Finland
39	CJ677CC078	NZ_CP010507.1	98/100	99/100		98/100		98/100	O	Human, Finland
40	CJ677CC523	NZ_CP010508.1	98/100	99/100		98/100		98/100	O	Human, Finland
41	CJ677CC540	NZ_CP010509.1	98/100	99/100		98/100		98/100	O	Human, Finland
42	CJ677CC040	NZ_CP010510.1	98/100	99/100		98/100		98/100	O	Human, Finland
43	CJ677CC061	NZ_CP010511.1	98/100	99/100		98/100		98/100	O	Human, Finland
44	CJ677CC539	NZ_CP010457.1	98/100	99/100		98/100		98/100	O	Human, Finland
45	CJ677CC533	NZ_CP010458.1	98/100	99/100		98/100		98/100	O	Human, Finland
46	CJ677CC047	NZ_CP010459.1	98/100	99/100		98/100		98/100	O	Human, Finland
47	CJ677CC058	NZ_CP010460.1	98/100	99/100		98/100		98/100	O	Human, Finland
48	CJ677CC013	NZ_CP010461.1	98/100	99/100		98/100		98/100	O	Human, Finland
49	CJ677CC100	NZ_CP010462.1	98/100	99/100		98/100		98/100	O	Human, Finland
50	CJ677CC522	NZ_CP010463.1	98/100	99/100		98/100		98/100	O	Human, Finland
51	CJ677CC094	NZ_CP010464.1	98/100	99/100		98/100		98/100	O	Human, Finland
52	CJ677CC008	NZ_CP010465.1	98/100	99/100		98/100		98/100	O	Human, Finland
53	CJ677CC541	NZ_CP010466.1	98/100	99/100		98/100		98/100	O	Human, Finland
54	CJ677CC024	NZ_CP010467.1	98/100	99/100		98/100		98/100	O	Human, Finland
55	CJ677CC064	NZ_CP010468.1	98/100	99/100		98/100		98/100	O	Human, Finland
56	CJ677CC525	NZ_CP010469.1	98/100	99/100		98/100		98/100	O	Human, Finland
57	CJ677CC026	NZ_CP010470.1	98/100	99/100		98/100		98/100	O	Human, Finland
58	CJ677CC034	NZ_CP010484.1	98/100	99/100		98/100		98/100	O	Human, Finland
59	CJ677CC086	NZ_CP010485.1	98/100	99/100		98/100		98/100	O	Human, Finland
60	CJ677CC095	NZ_CP010486.1	98/100	99/100		98/100		98/100	O	Human, Finland
61	CJ677CC012	NZ_CP010487.1	98/100	99/100		98/100		98/100	O	Human, Finland
62	CJ677CC010	CP010478.1	98/98	99/100		98/100		98/99	O	Human, Finland

Complete sequences: LOS Group 3; Classes D, F & K

No.	*C. jejuni* Strain	Accession no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	R14	CP005081.1	99/100	99/100	100/100	100/100							100/100	D	Unknown, UK
2	S3	NC_017281.1	99/100	98/94	99/100	99/100								F	Unknown, US
3	ATCC 35925	NZ_CP020045.1	99/100	96/94	98/100	97/100								F	Pigeon, Sweden
4	35925	NZ_CP010906.1	99/100	96/94	98/100	97/100								F	Human, Sweden
5	FJ3124	NZ_CP017862.1	99/100		96/91									F	Chicken, US
6	NCTC12662	NZ_CP019965.1	99/100	96/94	98/100	97/100								F	Unknown, UK
7	NCTC 12661	NZ_CP028911.1	99/100	96/94	98/100	97/100								F	Avian, UK
8	FDAARGOS_421	NZ_CP023866.1	99/100	98/94	99/100	99/100								F	Chicken, US
9	F38011	NZ_CP006851.1	99/100	94/100	95/98							99/100	96/100	K	Unknown, US
10	HF5-5-1	NZ_CP007189.1	99/100	93/100	95/98							99/100	96/100	K	Farm, UK
11	HF5-7-1	NZ_CP007190.1	99/100	93/100	95/98							99/100	96/100	K	Farm, UK

Draft sequences: LOS Group 1; Classes A1, A2, B1, B2, C, V, M & R

Class A1

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
1	ICDCCJ07002	APNP01000000	2					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Human, China
2	HN-CJD07035	ARYE01000000	6					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Human, China
3	BJ-CJD101	ARWV01000000	8					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Human, China
4	CVM 41974	JAKS01000000	4,8					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Human, US
5	HB-CJGB-QYT	ATBM01000000	3					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Human, China
6	BJ-CJGB96G25	ASXL01000000	3					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Human, China
7	BJ-CJGB95377	ASXK01000000	2					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Human, China
8	BJ-CJGB96114	ASXM01000000	2					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Human, China
9	BJ-CJGB96299	ASXN01000000	3					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Human, China
10	CF93-6	AANJ01000000	7					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Unknown
11	LMG 23216	AIOA01000000	5					100/100	99/100	99/100	98/100	99/100		98/97		A1	Chicken, Belgium
12	OXC6626	CUVM01000000	1					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Faeces, UK
13	OXC6414	CUNR01000000	1					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Faeces, UK
14	OXC6408	CUNK01000000	1					99/100	95/99	97/100	98/100	99/100		98/100	99/100	A1	Faeces, UK
15	OXC6305	CUJN01000000	1					100/100	100/100	100/100	100/100	100/100		100/100	100/100	A1	Faeces, UK
16	OXC6301	CUJJ01000000	1					100/100	100/100	100/100	100/100	100/100		100/100	100/100	A1	Faeces, UK
17	OXC6406	CUNI01000000	1					100/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Faeces, UK
18	OXC6535	CUSF01000000	1					100/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Faeces, UK
19	OXC6397	CUNA01000000	1					100/100	100/100	100/100	100/100	100/100		100/100	100/100	A1	Faeces, UK
20	OXC6534	CUSG01000000	1					100/100	100/100	100/100	100/100	99/100		100/100	100/100	A1	Faeces, UK
21	OXC6641	CUWB01000000	1					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Faeces, UK
22	OXC6494	CUQN01000000	2					100/100	99/100	100/100	100/100	100/100		100/100	100/100	A1	Faeces, UK
23	OXC6264	CUHV01000000	2					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Faeces, UK
24	OXC6482	CUPZ01000000	2					99/100	95/99	97/100	97/100	97/100		98/97		A1	Faeces, UK
25	OXC6273	CUIF01000000	1					100/100	100/100	100/100	98/100	99/100		99/100	98/100	A1	Faeces, UK
26	OXC6479	CUPX01000000	1					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Faeces, UK
27	Faeces	CZJF01000000	12					100/100	100/100	100/100	99/100	99/100		100/100	100/100	A1	Chicken, Spain
28	Neck Skin	CZJM01000000	2						100/100	100/100	99/100	100/100		100/100	100/100	A1	Chicken, Spain
29	Faeces	CZHY01000000	12					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Chicken, Spain
30	BCW_6893	MJYX01000000	16					98/100	96/100	96/100		97/100		97/97		A1	Crow, US
31	BCW_3804	MKAL01000000	41					100/100	100/100	100/100	98/100	99/100		99/100	98/100	A1	Crow, US
32	BCW_4328	MKAN01000000	3					98/100	96/100	96/100	96/100	97/100		97/97		A1	Crow, US
33	CDPHFDLB-F12M00560	MOVU01000000	1					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Cow, US
34	CDPHFDLB-M00214	MOVP01000000	1					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Cow, US
35	CDPHFDLB-M00224	MOVO01000000	9					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Cow, US
36	CDPHFDLB-F12M00566-a2	MOVL01000000	7					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Cow, US
37	BCW_6884	MKEW01000000	59					98/100	96/100	96/100	96/100	97/100		97/97		A1	Crow, US
38	BCW_4324	MKET01000000	30					99/100	99/100	98/100	97/100	97/100		96/97		A1	Crow, US
39	CJ096CC21	MLDN01000000	6					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Human, Finland
40	W20	NFNM01000000	65					99/100	95/100	96/100	97/100	98/100		97/100	99/100	A1	Environment, Canada
41	W16	NFNR01000000	35					99/100	95/100	96/100	97/100	98/100		97/100	99/100	A1	Environment, Canada
42	Po_2	CCDE01000000	46					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Unknown, Finland

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
43	Ma_B	CCDD01000000	55					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Unknown, Finland
44	Le_204R	CCDB01000000	45					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Unknown, Finland
45	Ma_1	CCCZ01000000	47					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Unknown, Finland
46	Le_755	CCDC01000000	21, 52					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Unknown, Finland
47	Po_1	CCDA01000000	68					99/100	99/98	99/100	98/100	99/100		98/100	98/100	A1	Unknown, Finland
48	P10-2209	JYEC01000000	5					99/100	95/99	97/100	97/100	97/209		98/97		A1	Pigeon, Japan
49	P3-2209	JYEA01000000	1					99/100	95/99	97/100	97/100	97/209		98/97		A1	Pigeon, Japan
50	P5-2209	JYEB01000000	6					99/100	95/99	97/100	97/100	97/209		98/97		A1	Pigeon, Japan
51	OXC6521	CURR01000000	1, 16					100/100	100/100	100/100	99/100	100/100		100/100	100/100	A1	Faeces, UK
52	OXC6525	CURV01000000	1					100/100	100/100	100/100	100/100	99/100		100/100	100/100	A1	Faeces, UK
53	BCW_3797	MJVM01000000	31					100/100	100/100	100/100	98/100	99/100		99/100	98/100	A1	Crow, US
54	BCW_3799	MJVO01000000	9					98/100	96/100	96/100	96/100	97/100		97/97		A1	Crow, US
55	ICDCCJ07001	CP002029.1	-					99/100	99/100	97/100	99/100	98/100		98/100	99/100	A1	Unknown, China
56	Faeces	CZIN01000000	58, 64					99/100	99/100	99/100	99/100	99/100		100/100	100/100	A1	Chicken, Spain
57	BCW_3797	MJVM01000000	31					100/100	100/100	100/100	98/100	99/100		99/100	98/100	A1	Crow, US
58	OXC6525	CURV01000000	1					100/100	100/100	100/100	100/100	99/100		100/100	100/100	A1	Faeces, UK
59	CVM N15870	JOVW01000000	2					99/100	95/100	96/100	97/100	98/100		98/100		A1	Turkey, US
60	Water	CZJO01000000	32					100/100	100/100	100/100	100/100	99/100		100/100	100/100	A1	Environment, Spain
61	Meat	CZJI01000000	43					100/100	100/100	100/100	100/100	100/100		100/100	100/100	A1	Chicken, Spain
62	CDPHFDLB-F12M00560	MOVT01000000	8					99/100	99/100	99/100	98/100	99/100		98/100	98/100	A1	Cow, US

Class A2

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
1	60004	AIOE01000000	20					100/100	100/100	100/100	100/100	100/100		99/100	95/100	A2	Chicken, US
2	86605	AIOJ01000000	4					99/100	98/99	95/100	98/100	97/100		99/100	95/100	A2	Chicken, US
3	2008-872	AIOR01000000	5, 31					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Human, France
4	1997-7	AIOX01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	98/100	A2	Human, US
5	87459	AIPE01000000	35					99/100	98/99	99/100	98/100	99/100		99/100	95/100	A2	Chicken, US
6	1798	AIPI01000000	21, 36					99/100	99/100	95/100	99/100	97/100		98/100	98/100	A2	Cow, US
7	OXC6453	CURA01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
8	OXC6306	CUJO01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
9	OXC6579	CUTT01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
10	OXC6346	CULG01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
11	OXC6490	CUQJ01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
12	OXC6468	CUPL01000000	1					99/100	99/100	96/100	98/100	98/100		97/100	95/100	A2	Faeces, UK
13	OXC6402	CUNF01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
14	OXC6570	CUTJ01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	98/100	A2	Faeces, UK
15	OXC6283	CUIN01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
16	OXC6268	CUHZ01000000	1					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
17	OXC6505	CUQZ01000000	1					99/100	98/100	95/100	97/100	98/100		97/100	95/100	A2	Faeces, UK
18	OXC6340	CULA01000000	1					99/98	99/100	91/100	98/100	98/100		98/100	96/100	A2	Faeces, UK
19	Water	CZJL01000000	12					99/100	99/100	100/100	99/100	97/100		98/100	98/100	A2	Environment, Spain
20	CVM 41900	JAKC01000000	4					99/98	99/100	91/100	98/100	98/100		98/100	96/100	A2	Human, US
21	CVM N534	JOUW01000000	7					99/98	99/100	91/100	98/100	97/99		97/100	95/100	A2	Chicken, US
22	CCN25	FBJN01000000	28					99/98	99/100	91/100	98/100	98/100		98/100	96/100	A2	Poultry Environment, UK
23	CVM N9016	JOVD01000000	12					99/100	99/100	93/100	98/100	97/99		98/100	97/100	A2	Chicken, US
24	CVM N9095	JOVH01000000	4					99/98	99/100	99/100	98/100	98/100		97/100	95/100	A2	Chicken, US
25	CDPHFDLB-F15M03173	MOTS01000000	8					99/100	99/100	100/100	98/100	98/100		98/100	98/100	A2	Cow, US
26	CDPHFDLB-F15M03174	MPBJ01000000	2					99/100	99/100	100/100	98/100	98/100		98/100	98/100	A2	Milk, US
27	BCW_5913	MKES01000000	74					99/100	98/100	91/100	98/100	97/100		98/100	99/100	A2	Monkey, US
28	W33	NFNA01000000	92					99/100	98/100	98/100	98/100	97/100		98/100	99/100	A2	Environmental water, Canada
29	W11	NFNW01000000	15					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Environmental water, Canada
30	W10	NFNX01000000	25, 45					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Environmental water, Canada
31	S1	NFOH01000000	16					99/100	98/100	98/100	98/100	97/100		98/100	99/100	A2	Environmental water, Canada
32	H34	NFOO01000000	25, 189					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Human, Canada
33	H22	NFPB01000000	41, 67					99/100	99/100	100/100	99/100	97/100		98/100	99/87	A2	Human, Canada
34	H17	NFPG01000000	40, 42					99/98	99/100		98/100	98/100		98/100	96/100	A2	Human, Canada
35	B2	NFQF01000000	41					99/100	99/100	100/100	99/100	97/100		98/100	98/100	A2	Cow, Canada
36	OXC6589	CUUD01000000	1, 14					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Faeces, UK
37	BCW_4727	MKAZ01000000	10					99/100	99/100	100/100	99/100	97/100		98/100	99/100	A2	Unknown, US
38	CDPHFDL-F15M03174-C2	MOTQ01000000	2					99/100	99/100	100/100	98/100	98/100		98/100	98/100	A2	Cow Milk, US
39	CVM N1630	JOUH01000000	20					99/100	99/100	93/100	98/100	98/100		98/100	96/100	A2	Chicken, US

Class B1

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
1	D2600	AGTF01000000	47					97/86	96/83	91/100	99/100	99/100	99/100	98/100	98/100	B1	Human, US
2	Cj5	AUUK01000000	29					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, Thailand
3	Cj2	AUUM01000000	34					98/100	97/83	94/100	99/100	98/100	99/100	99/100	99/100	B1	Human, Thailand
4	P110B	AEIO01000000	2, 3					98/100		93/81			99/36	98/100	99/100	B1	Chicken, New Zealand
5	LMG 23210	AIPN01000000	30. 48					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Chicken, Belgium
6	30286	AUUH01000000	4					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, Viet Nam
7	OXC6640	CUWA01000000	2					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
8	OXC6554	CUSQ01000000	2					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
9	OXC6509	CURF01000000	1					97/86	96/83	91/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
10	OXC6341	CULB01000000	2					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
11	OXC6304	CUJM01000000	1					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
12	OXC6262	CUHT01000000	2					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
13	OXC6484	CUQC01000000	2					99/100	99/100		99/100	99/100	99/100	99/100	99/100	B1	Faeces, UK
14	OXC6572	CUTL01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
15	OXC6618	CUVD01000000	1					98/100	94/87	91/100	98/100	98/100	99/100	99/100	99/100	B1	Faeces, UK
16	OXC6621	CUVH01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
17	OXC6359	CULL01000000	2					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
18	OXC6475	CUPS01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
19	OXC6560	CUSY01000000	2					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
20	OXC6352	CUNL01000000	2					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
21	OXC6528	CURZ01000000	1					97/86	95/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
22	OXC6584	CUTZ01000000	1					97/86	96/83	91/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
23	OXC6288	CUIT01000000	2					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
24	OXC6541	CUSN01000000	2					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
25	OXC6544	CUSS01000000	1					97/86	96/83	91/100	99/100	98/100	99/100	99/100	99/100	B1	Faeces, UK
26	OXC6392	CUMV01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
27	OXC6581	CUTV01000000	2					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
28	OXC6323	CUKJ01000000	1					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
29	OXC6361	CULN01000000	1					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
30	OXC6485	CUQD01000000	2					99/100	99/100	99/100	99/100	99/100	99/100	99/100	99/100	B1	Faeces, UK
31	OXC6349	CUME01000000	2					99/100	99/100	99/100	99/100	99/100	100/100	100/100	100/100	B1	Faeces, UK
32	OXC6455	CURX01000000	1					98/100	96/83	91/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
33	Faeces	CZIA01000000	46					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Chicken, Spain
34	Neck	CZIG01000000	19					97/86	96/83	91/100	99/100	99/100	99/100	98/100	98/100	B1	Chicken, Spain
35	CVM N9016	JOVD01000000	12					98/100	97/83	94/100	99/100	99/100	99/100	100/100	100/100	B1	Chicken, US
36	CVM N9095	JOVH01000000	4					97/86	94/87	95/100	99/100	98/100	99/100	97/100	98/100	B1	Chicken, US
37	CVM N534	JOUW01000000	7					97/86	94/87	91/100	99/100	99/100	99/100	98/100	97/100	B1	Chicken, US
38	BCW_6910	MJZF01000000	43					99/100	99/100	99/100	99/100	99/100	99/100	100/100	100/100	B1	Faeces, US
39	BCW_6956	MJZQ01000000	25					99/100	99/100	99/100	99/100	99/100	99/100	100/100	100/100	B1	Faeces, US
40	BCW_5122	MKBQ01000000	34						100/22	95/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
41	BCW_5129	MKBW01000000	47					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
42	BCW_5135	MKCA01000000	47					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
43	BCW_5136	MKCB01000000	17					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
44	BCW_5144	MKCG01000000	48					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
45	BCW_5148	MKCK01000000	36					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
46	BCW_5151	MKCM01000000	53					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
47	CDPHFDLB-F15M00516E1	MOUW01000000	2					99/100	99/100	100/100	99/100	99/100	100/100	100/100	100/36	B1	Environment, US
48	BCW_5154	MKEZ01000000	31					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
49	CAM970	BDRZ01000000	7					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, Japan
50	W46	NFMN01000000	13					99/100	99/100	100/100	99/100	99/100	100/64			B1	Environmental water, Canada
51	Isolate_1	NFQL01000000	23, 111					97/86	96/83	92/55			99/37	98/100	97/100	B1	Retail Chicken, canada
52	OXC6391	CUMW01000000	1, 2					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
53	OXC6574	CUTO01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
54	OXC6466	CUPJ01000000	2					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
55	OXC6550	CUUZ01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
56	OXC6413	CUNQ01000000	1					97/86	94/87	91/100	99/100	99/100	99/100	97/100	98/100	B1	Faeces, UK
57	OXC6635	CUVW01000000	2					98/100	94/87	93/100	98/100	98/100	99/100	98100	98/100	B1	Faeces, UK
58	OXC6458	CUPA01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK
59	OXC6555	CUST01000000	1					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Faeces, UK

No.	C. jejuni Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
60	OXC6501	CUQU01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
61	BJ-CJD120	LISM01000000	1					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, China
62	BCW_5121	MKBP01000000	1					98/100	94/87	93/100	98/100	99/100	100/100	99/100	99/100	B1	Human, US
63	BCW_5146	MKCI01000000	18					98/100	96/83	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
64	BCW_5159	MKFE01000000	38					98/100	94/87	93/100	98/100	98/100	99/100	98/100	98/100	B1	Human, US
65	FDAARGOS_264	NBTT01000000	2					97/86	96/83	91/100	99/100	99/100	99/100	97/100	98/100	B1	Human, US
66	CVM 41922	JAKL01000000	27					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Human, US
67	MEAT	CZJD01000000	34					98/100	91/81	93/100	99/100	98/98	99/100	98/100	99/100	B1	Chicken, Spain
68	BCW_4735	MKBD01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Unknown, US
69	BCW_4755	MKBN01000000	24					98/100	89/85	93/100	99/100	98/100	99/100	98/100		B1	Unknown, US
70	Neck Skin	CZIZ01000000	23					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Chicken, Spain
71	OXC6477	CUPV01000000	4					98/100	89/85	93/100	99/100	98/100	99/100	98/100		B1	Faeces, UK
72	OXC6430	CUOK01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
73	OXC6454	CURN01000000	5					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
74	OXC6295	CUJC01000000	1, 54					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
75	H25	NFOY01000000	106					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Human, Canada
76	C12	NFQA01000000	59, 69					98/100	91/81	93/100	99/100	98/100	99/100	99/99		B1	Faeces, Canada
77	BCW_4749	MKBK01000000	16					98/100	91/81		99/100	98/100	99/100	98/100		B1	Unknown, US
78	CCN443	FBHK01000000	16					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Poultry farm, UK
79	Neck Skin	CZIT01000000	12					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Chicken, Spain
80	Faeces	CZIP01000000	17					98/88	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Chicken, Spain
81	Neck Skin	CZHH01000000	31					98/100	89/85	93/100	99/100	98/100	99/90	98/100	99/100	B1	Chicken, Spain
82	Faeces	CZHM01000000	15					98/100	91/81	92/91	99/100	98/100	99/91	98/100	99/100	B1	Chicken, Spain
83	Meat	CZHQ01000000	44					98/100	89/85	93/100	99/100	98/100	99/92	98/100	99/100	B1	Chicken, Spain
84	OXC6580	CUTU01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100		B1	Faeces, UK
85	OXC6495	CUQO01000000	2					98/100	89/85	93/100	99/100	98/100	99/100	98/100		B1	Faeces, UK
86	OXC6403	CUNE01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
87	OXC6470	CUPN01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
88	OXC6488	CUQH01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
89	OXC6487	CUQG01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
90	OXC6556	CUSU01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
91	OXC6465	CUPH01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
92	OXC6547	CUTX01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
93	OXC6427	CUOE01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
94	OXC6272	CUIE01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
95	OXC6269	CUIC01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
96	OXC6348	CULQ01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
97	OXC6382	CUMK01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
98	OXC6632	CUVS01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
99	OXC6284	CUIS01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
100	OXC6549	CUUQ01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
101	OXC6638	CUVY01000000	1					98/100	89/85	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
102	OXC6422	CUOA01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
103	OXC6354	CUOG01000000	1					98/100	91/81	93/100	99/100	98/100	99/100	98/100	99/100	B1	Faeces, UK
104	OXC6462	CUPE01000000	1										99/100			B1	Faeces, UK
105	OXC6411	CUNO01000000	1										99/100			B1	Faeces, UK
106	OXC6389	CUMS01000000	1										99/100			B1	Faeces, UK
107	OXC6421	CUNZ01000000	1										99/100			B1	Faeces, UK
108	OXC6356	CULI01000000	1										99/100			B1	Faeces, UK
109	OXC6345	CULF01000000	1										99/100			B1	Faeces, UK
110	OXC6265	CUHW01000000	1										99/100			B1	Faeces, UK
111	OXC6377	CUMF01000000	1										99/100			B1	Faeces, UK
112	OXC6252	CUIL01000000	1										99/100			B1	Faeces, UK
113	OXC6328	CUKM01000000	1										99/100			B1	Faeces, UK
114	LMG 23218	AIOB01000000	3										99/100			B1	Chicken, Belgium
115	ATCC 33560	AIOL01000000	51, 83							99/100			99/100			B1	Bovine, Belgium
116	2008-988	AIOS01000000	46, 48										99/100			B1	Human, France
117	1997-4	AIOW01000000	45, 62										99/100			B1	Human, France
118	140-16	AIPF01000000	31, 53										99/100			B1	Human, US
119	1893	AIPK01000000	12										99/100			B1	Cow, US

Class B2

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country

No.	Strain	Accession no.	Contig no.									Class	Host, Country
1	10227	AUUI01000000	2	97/100	99/100	99/100	99/100	98/100	99/100	98/100	98/100	B2	Human, Viet Nam
2	Cj1	AUUL01000000	1	97/100	99/100	99/100	99/100	98/100	99/100	98/100	98/100	B2	Human, Thailand
3	129-258	AINY01000000	19, 62	99/100	99/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Bovine, US
4	81-176-UMCW7	AZNS01000000	16	99/100	99/100	99/100	100/100	100/100	100/100	100/100	100/100	B2	Chicken, US
5	OXC6333	CUKS01000000	1	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	B2	Faeces, UK
6	OXC6417	CUNU01000000	1	99/100	100/100	99/100	100/100	100/100	99/100	100/100	99/100	B2	Faeces, UK
7	OXC6415	CUNS01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	B2	Faeces, UK
8	OXC6409	CUNM01000000	2	99/100	100/100	99/100	100/100	100/100	99/100	100/100	99/100	B2	Faeces, UK
9	OXC6569	CUTI01000000	1	98/100	100/100	99/100	100/100	99/100	99/100	100/100	99/100	B2	Faeces, UK
10	OXC6350	CUMO01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	B2	Faeces, UK
11	OXC6423	CUOC01000000	2	97/100	99/100	99/100	99/100	98/100	99/100	98/100	98/100	B2	Faeces, UK
12	OXC6388	CUMR01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	B2	Faeces, UK
13	OXC6271	CUID01000000	1	97/100	99/100	99/100	98/100	98/100		99/100	99/100	B2	Faeces, UK
14	Neck	CZJC01000000	16	99/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Chicken, Spain
15	Meat	CZJJ01000000	17	99/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Chicken, Spain
16	BCW_6891	MJYW01000000	15	97/100	99/100	99/100	99/100	98/100	99/100	98/100	98/100	B2	Chicken, US
17	BCW_6929	MKAU01000000	1	97/100	99/100	99/100	99/100	98/100	99/100	98/100	98/100	B2	Caprine, US
18	BCW_5166	MKHU01000000	26	97/100	99/100			98/100	99/100	98/100	98/100	B2	Human, US
19	CDPHFDLB-F15M00521-2	MOUT01000000	12	99/100	99/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Environment, US
20	CDPHFDLB-F15M00592	MOUR01000000	1	99/100	99/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Cow, US
21	CDPHFDLB-F15M00601	MOUO01000000	1	99/100	99/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Cow, US
22	CDPHFDLB-F15M00602	MOUN01000000	1	99/100	99/100	99/100	100/100	100/100	99/100	100/100	99/100	B2	Cow, US
23	CDPHFDLB-F15M00565-1	MOVQ01000000	2, 4	99/100	99/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Cow, US
24	CDPHFDLB-F15M00565-3	MOVN01000000	3	99/100	99/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Cow, US
25	BCW_5155	MKFA01000000	58	97/100	100/100	99/100	99/100	98/100	99/100	98/100	98/100	B2	Human, US
26	C3	NFPV01000000	20	97/100	99/100	99/100	99/100	98/100	99/100	98/100	98/100	B2	Faeces, Canada
27	BCW_3803	MJVR01000000	19	98/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Crow, US
28	BCW_4456	MKAI01000000	29	97/100	-	94/100	98/100	97/100	99/100	98/100	99/100	B2	Faeces, US
29	BCW_4734	MKBC01000000	18, 20	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	B2	Unknown, US
30	BCW_5170	MKHW01000000	36	97/100	99/100	99/100	99/100		99/100	98/100	98/100	B2	Human, US
31	Faeces	CZJE01000000	14	99/100	100/100	99/100	100/100	100/100	100/91	100/100	99/100	B2	Chicken, Spain
32	OXC6512	CURH01000000	3, 4	99/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	B2	Faeces, UK

Class C

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
1	FDAARGOS_263	CP022077.1				100/100	100/100		100/100	100/100	99/100	100/100		100/100	100/100	C	Human, US
2	6399	CAFT01000000	19, 22			100/100	99/100					100/17		100/100	99/100	C	Unknown, Germany
3	OXC6532	CUSD01000000	1			100/100	99/100		100/100	100/100		100/100		99/100	99/100	C	Faeces, UK
4	OXC6633	CUVT01000000	1			100/100	99/100		99/100	100/100		100/100		100/100	99/100	C	Faeces, UK
5	OXC6508	CURD01000000	1			100/100	99/100		100/100	100/100	99/34	100/100		100/100	99/100	C	Faeces, UK
6	OXC6538	CUSK01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
7	OXC6257	CUKO01000000	1			100/100	99/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
8	OXC6573	CUTN01000000	1			100/100	99/100		99/100	100/100		100/100		100/100	99/100	C	Faeces, UK
9	OXC6625	CUVL01000000	1			100/100	99/100		100/100	100/100		100/100		100/100	99/100	C	Faeces, UK
10	OXC6516	CURM01000000	1			100/100	99/100		99/100			100/100		100/100	99/100	C	Faeces, UK
11	G113	AQPK01000000	8			100/100	100/100		99/100	100/100		100/100		100/100	99/100	C	Unknown
12	G1	JRLT01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Human, UK
13	OXC6564	CUTD01000000	1			100/100	99/100		99/100	100/100		99/100		100/100	99/100	C	Faeces, UK
14	OXC6543	CUSR01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
15	OXC6527	CURY01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
16	OXC6387	CUMQ01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
17	OXC6266	CUHX01000000	1			100/100	100/100		100/100	100/100		100/100		99/100	99/100	C	Faeces, UK
18	OXC6590	CUUC01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
19	OXC6522	CURS01000000	1			100/100	99/100		99/100			100/100		99/100	99/100	C	Faeces, UK
20	OXC6636	CTRT01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
21	OXC6615	CUVC01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
22	OXC6449	CUPU01000000	1			100/100	100/100		99/100	99/100		100/100		99/100	99/100	C	Faeces, UK
23	OXC6429	CUOI01000000	1			100/100	99/100		100/100	100/100		100/100		99/100	99/100	C	Faeces, UK
24	OXC6394	CUMU01000000	1			100/100	100/100		100/100	100/100		100/100		99/100	99/100	C	Faeces, UK
25	OXC6456	CUSI01000000	2			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
26	OXC6303	CUJL01000000	1			100/100	100/100		99/100	100/100		100/100		99/100	99/100	C	Faeces, UK
27	OXC6539	CUSL01000000	1			100/100	99/100		99/100	100/100		100/100		100/100	99/100	C	Faeces, UK

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
28	OXC6602	CUUP01000000	1				100/100	99/100		100/100	100/100	100/100		100/100	99/100	C	Faeces, UK
29	OXC6370	CULX01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
30	OXC6553	CUSP01000000	1				100/100	99/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
31	OXC6464	CUPG01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
32	OXC6530	CUSB01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
33	OXC6563	CUTC01000000	1				100/100	99/100		99/100	100/100	100/100		100/100	99/100	C	Faeces, UK
34	OXC6629	CUVP01000000	1				100/100	99/100		100/100	100/100	100/100		100/100	99/100	C	Faeces, UK
35	OXC6603	CUUR01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
36	OXC6500	CUQV01000000	2				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
37	OXC6571	CUTK01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
38	OXC6251	CUIA01000000	1				100/100	99/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
39	OXC6292	CUIZ01000000	1				100/100	100/100		100/100	100/100	100/100		99/100	99/100	C	Faeces, UK
40	OXC6565	CUTE01000000	1				100/100	100/100		99/100	100/100	99/100		99/100	99/100	C	Faeces, UK
41	OXC6524	CURU01000000	1				100/100	100/100		100/100	100/100	100/100		99/100	99/100	C	Faeces, UK
42	OXC6282	CUIP01000000	1				100/100	99/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
43	OXC6600	CUUN01000000	1				100/100	100/100		99/100	100/100	99/100		99/100	99/100	C	Faeces, UK
44	OXC6277	CUIJ01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
45	OXC6616	CUVB01000000	1				100/100	99/100		99/100	100/100	99/100		100/100	99/100	C	Faeces, UK
46	OXC6310	CUJT01000000	1				100/100	99/100		99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
47	CVM 41943	JAKQ01000000	3				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Human, US
48	CDPHFDLB-F15M00600	MOUP01000000	1				100/100	100/100		100/100	99/100	100/100		99/100	99/100	C	Cow, US
49	CDPHFDLB-F15M01862-D	MOUA01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Goat Milk, US
50	CDPHFDLB-F15M01873-A	MOTZ01000000	2				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Goat Milk, US
51	CJ003CC21	MLDJ01000000	6				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Human, Finland
52	CJ070CC21	MLDM01000000	5				100/100	100/100		100/100	100/100	99/39	100/100	99/100	99/100	C	Human, Finland
53	CJ035CC21	MLDL01000000	6				100/100	100/100		100/100	100/100	100/100		99/100	99/100	C	Human, Finland
54	CJ507CC21	MLDP01000000	3				100/100	99/100		99/100	100/100	99/85	100/100	99/100	99/100	C	Human, Finland
55	CJ069CC21	MLDQ01000000	8				100/100	99/100		100/100	100/100	100/100		99/100	99/100	C	Human, Finland
56	CJ502CC21	MLDU01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	100/100	C	Human, Finland
57	CJ503CC21	MLDV01000000	1				100/100	100/100		99/100	100/100	100/100		99/100	100/100	C	Human, Finland
58	CJ506CC21	MLDY01000000	9				100/100	100/100		100/100	100/100	100/100		99/100	99/100	C	Human, Finland
59	CJ505CC21	MLDX01000000	6				100/100	99/100		99/100	100/100	100/100		99/100	99/100	C	Human, Finland
60	CJ097CC21	MLDZ01000000	1				100/100	100/100		99/100	99/100	100/100		99/100	99/100	C	Human, Finland
61	CJ504CC21	MLDW01000000	14				100/100	99/100		99/100	100/100	100/100		99/100	99/100	C	Human, Finland
62	D7331	MORJ01000000	8				100/100	99/100		99/100	100/100	100/100		99/100	99/100	C	Stool, US
63	BCW_5157	MKFC01000000	21, 47				100/100	100/100		99/100	100/100	100/100		99/100	99/100	C	Human, US
64	Water	CZJK01000000	2				100/100	100/100								C	Environment, Spain
65	CDPHFDLB-F15M01909	MOTW01000000	1				100/100	100/94								C	Goat, US
66	OXC6393	CUMX01000000	1		99/100					100/100	100/100	100/100		99/100	99/100	C	Faeces, UK
67	OXC6459	CUPC01000000	1		99/100					99/100	100/100	100/100		99/100	99/100	C	Faeces, UK
68	BCW_6920	MKAC01000000	2		100/99					100/100	100/100	100/100		99/100	99/100	C	Bovine, US
69	BCW_6922	MKAE01000000	4		99/99					99/100	100/100	100/100		99/100	99/100	C	Ovine, US
70	BCW_6925	MKAG01000000	7		99/99					99/100	100/100	100/100		99/100	99/100	C	Ovine, US
71	BCW_6933	MKIB01000000	18		100/99					100/100	100/100	100/100		99/100	99/100	C	Sheep, US
72	BCW_6931	MKIC01000000	5		100/99					99/100	100/100	100/100		99/100	99/100	C	Sheep, US
73	BCW_6932	MKID01000000	33		100/99					99/100	100/100	100/100		99/100	99/100	C	Sheep, US
74	11168H	FPEE01000000	36, 37		99/100					100/100	100/100	100/100		100/100		C	Laboratory, UK
75	OXC6392	CUKN01000000	1, 17		99/100					100/100	100/100	100/100		99/100	100/100	C	Faeces, UK
76	OXC6519	CURQ01000000	1, 19		99/100					99/100	100/100	100/100		100/100	99/100	C	Faeces, UK
77	BCW_6924	MKAF01000000	16, 20		100/99					99/100	100/100	100/100		99/100	97/100	C	Ovine, US
78	BCW_6934	MKIA01000000	1, 19		99/99					99/100	100/100	100/100		99/100		C	Goat, US
79	BCW_5156	MKFB01000000	22, 42		100/99					99/100	100/100	100/100		99/100	99/100	C	Human, US

Class M

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
1	BCW_6896	MJYY01000000	34	99/36	96/100					96/100	98/100	99/100		97/100	98/99	M	Crow, US
2	BCW_4223	MJXU01000000	36, 70	95/95												M	Crow, US
3	BCW_6459	MJXH01000000	37, 84	95/95												M	Crow, US
4	BCW_6453	MJXC01000000	5, 68	99/98	96/99											M	Crow, US
5	OXC6561	CUSZ01000000	1	100/100	100/100					100/100	100/100	100/100		99/100	99/100	M	Faeces, UK
6	OXC6639	CUVZ01000000	1	100/100	100/100					100/100	100/100	100/100		99/100	99/100	M	Faeces, UK
7	OXC6364	CULS01000000	1	100/100	100/100					100/100	100/100	100/100		99/100	99/100	M	Faeces, UK

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Class R

No.	*C. jejuni* Strain	Accession no.	Contig no.	51	17	14	15	5	6	7	8	9	5-II	10	11	Class	Host, Country
1	OXC6327	CUKK01000000	1							96/100		96/100		97/100	97/100	R	Faeces, UK
2	BCW_6880	MJYO01000000	17									97/94		98/99	97/100	R	Crow, US
3	BCW_4324	MKET01000000	30					99/100	95/99	96/100	99/100	99/100		99/100	98/100	R	Crow, US
4	BCW_3791	MJVJ01000000	53							95/100	98/100	98/100		98/100	97/100	R	Crow, US
5	BCW_3807	MJVT01000000	28							96/100		98/100		98/100	97/100	R	Crow, US
6	BCW_6458	MJXG01000000	41					97/100	95/99	96/100	98/100	98/100		99/100	98/100	R	Crow, US
7	LMG 23211	AIPO01000000	10					100/100	100/100	100/100	100/100	100/100		100/100	100/100	R	Chicken, Belgium
8	CVM N15262	JOUG01000000	2						100/30	100/100		100/100		99/100	99/100	R	Chicken, US
9	CDPHFDLB-F15M01873-B	MOTY01000000	1						99/83	100/100		100/100		99/100	99/100	R	Goat Milk, US
10	BCW_3799	MJVO01000000	9					97/100	95/99	96/100	98/100	98/100		99/100	98/100	R	Crow, US

Draft sequences: LOS Group 2; Classes P, H, E, O & W

Class P

No.	*C. jejuni* Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
1	HB-CJGB -LL	ATBJ01000000	12	98/98		100/100	99/92	100/100	97/99	P	Human, China
2	51494	AINZ01000000	9	97/98		100/100	99/92	99/100	97/99	P	Chicken, US
3	51037	AIPB01000000	12,37	98/98		100/100	99/92	99/100		P	Chicken, US
4	OXC6609	CUUU01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
5	OXC6278	CUIK01000000	2	98/98		100/100	99/92	99/100	99/99	P	Faeces, UK
6	OXC6302	CUJK01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
7	OXC6366	CULT01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
8	OXC6620	CUVF01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
9	OXC6481	CUPY01000000	1	98/98		100/100	99/92	100/100	97/99	P	Faeces, UK
10	OXC6313	CUJW01000000	2	98/98		100/100	99/92	99/100	99/99	P	Faeces, UK
11	OXC6440	CUOT01000000	1	98/98		100/100	99/92	100/100	97/99	P	Faeces, UK
12	OXC6311	CUJU01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
13	OXC6293	CUJA01000000	1	98/98		100/100	99/92	99/100	99/99	P	Faeces, UK
14	OXC6342	CULC01000000	1	98/98		100/100	99/92	100/100	97/99	P	Faeces, UK
15	OXC6365	CULR01000000	1	98/98		100/100	99/92	99/100	99/99	P	Faeces, UK
16	OXC6503	CUQX01000000	1	98/98		99/100	99/92	100/100	97/99	P	Faeces, UK
17	OXC6339	CUKY01000000	1	98/98		100/100	99/92	99/100	99/99	P	Faeces, UK
18	OXC6357	CULJ01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
19	OXC6351	CUMZ01000000	1	98/98		100/100	99/92	99/100	99/99	P	Faeces, UK
20	OXC6396	CUNB01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
21	OXC6259	CUHO01000000	1	98/98		100/100	99/92	99/100	99/99	P	Faeces, UK
22	Neck Skin	CZIL01000000	9			100/100	99/92	100/100		P	Chicken, Spain
23	Neck Skin	CZIB01000000	11,15			100/100	99/92	99/100	97/99	P	Chicken, Spain
24	BCW_3782	MJYK01000000	17	98/98		100/100	99/92	99/100	99/99	P	Crow, US
25	BCW_6475	MJYM01000000	2	96/98		99/100	99/92	99/100	98/99	P	Crow, US
26	BCW_5132	MKBY01000000	29	98/98		100/100	99/92	100/100	97/99	P	Human, US
27	BCW_5131	MKBX01000000	101	98/98		99/100	99/92	99/100	98/99	P	Human, US
28	BCW_5147	MKCJ01000000	117	98/98		99/100	99/92	99/100	98/99	P	Human, US
29	CDPHFDLB-F15M00591	MOUS01000000	1	98/98		100/100	99/92	99/100	99/99	P	Cow, US
30	CDPHFDLB-F15M00554-a2	MOVZ01000000	1	98/98		100/100	99/92	99/100	97/99	P	Water, US
31	CDPHFDLB-F15M00554-a3	MOVY01000000	1	98/98		100/100	99/92	99/100	97/99	P	Water, US
32	CDPHFDLB-F12M00558	MOVX01000000	1	98/98		100/100	99/92	99/100	99/99	P	Cow, US
33	OXC6316	CUJZ01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
34	OXC6274	CUIG01000000	1	98/98		100/100	99/92	99/100	97/99	P	Faeces, UK
35	BCW_4224	MJWH01000000	18	98/98		100/100	99/92	99/100	99/99	P	Crow, US
36	BCW_4757	MKBO01000000	47	98/98		100/100	99/92	99/100	99/99	P	Unknown
37	BCW_5150	MKCL01000000	69	98/98		99/100	99/92	99/100	98/99	P	Human, US

No.	C. jejuni Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
38	W22	NFNK01000000	4	98/98		100/100	99/92	99/100	99/99	P	Water, Canada
39	C16	NFPX01000000	20	98/98		100/100	99/85	99/100	99/99	P	Chicken, Canada
40	A1	NFQG01000000	4			100/100	99/89	99/77		P	Chicken, Canada
41	H23	NFPA01000000	15	97/97		99/100	99/92	99/100	97/99	P	Human, Canada
42	K5	AUUP01000000	15, 450, 451	-		99/100	99/92	99/100	97/99	P	Human, Pakistan

Class H

No.	C. jejuni Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
1	30318	AUUJ01000000	26	98/98		99/100	99/100		99/100	H	Human, Viet Nam
2	HB-CJGB-LC	ASXO01000000	1	98/98		100/100	99/100		98/100	H	Human, China
3	2008-894	AIOQ01000000	2	98/98		100/100	99/100		99/100	H	Human, France
4	1997-10	AIOY01000000	42, 101	98/98		99/100	99/100		99/99	H	Human, US
5	1854	AIPJ01000000	14	99/99		99/100	99/100		98/100	H	Cow, US
6	OXC6622	CUVG01000000	2	98/98		99/100	99/100		99/100	H	Faeces, UK
7	OXC6497	CUQR01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
8	OXC6628	CUVO01000000	1	99/99		99/100	99/100		98/100	H	Faeces, UK
9	OXC6260	CUHR01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
10	OXC6566	CUTF01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
11	OXC6520	CURP01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
12	OXC6478	CUPW01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
13	OXC6611	CUUW01000000	2	98/98		99/100	99/100		99/100	H	Faeces, UK
14	OXC6473	CUPP01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
15	OXC6585	CUUA01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
16	OXC6438	CUOS01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
17	OXC6575	CUTP01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
18	OXC6591	CUUE01000000	1	98/98		99/100	99/100		99/100	H	Faeces, UK
19	Faeces	CZIS01000000	78			100/97	99/92			H	Chicken, Spain
20	Meat	CZHK01000000	31	99/98		100/99	99/100		98/100	H	Chicken, Spain
21	Meat	CZHZ01000000	4			100/99			98/99	H	Chicken, Spain
22	Faeces	CZIV01000000	34	99/96		100/99	99/100		98/100	H	Chicken, Spain
23	Meat	CZHN01000000	1			99/100	99/100		99/99	H	Chicken, Spain
24	CVM 41921	JAKK01000000	34	99/96		99/100	99/100			H	Human, US
25	BCW_4230	MJWJ01000000	32	98/98		99/100	99/100		99/100	H	Crow, US
26	BCW_7692	MKAB01000000	26	98/98		99/100	99/100		99/100	H	Human, US
27	BCW_5126	MKBU01000000	52	98/98		99/100	99/100		99/100	H	Human, US
28	CDPHFDLB-F12M00584-a2	MOVC01000000	3	99/98		99/100	99/100		98/100	H	Cream, US
29	CDPHFDLB-F12M00584-a1	MOVD01000000	1	99/98		99/100	99/100		98/100	H	Cream, US
30	CDPHFDLB-F12M00585-a1	MOVB01000000	1	99/98		99/100	99/100		98/100	H	Cream, US
31	CDPHFDLB-F12M00585-a2	MOVA01000000	1	99/98		99/100	99/100		98/100	H	Cream, US
32	CDPHFDLB-F12M00589-a2	MOUY01000000	1	99/98		99/100	99/100		98/100	H	Cream, US
33	CDPHFDLB-F12M00589-a1	MOUZ01000000	1	99/98		99/100	99/100		98/100	H	Cream, US
34	CDPHFDLB-F12M00436-A	MOWK01000000	1	99/98		99/100	99/100		98/100	H	Cow, US
35	CDPHFDLB-F12M00436-D	MOWJ01000000	1	99/98		99/100	99/100		98/99	H	Cream, US
36	CDPHFDLB-F12M00436-H	MOWI01000000	2	99/98		99/100	99/100		98/99	H	Cream, US
37	CDPHFDLB-F12M00521-3g3a	MOWH01000000	1	99/98		99/100	99/100		98/100	H	Milk, US
38	CDPHFDLB-F12M00521-3g3b	MOWG01000000	2	99/98		99/100	99/94			H	Milk, US
39	CDPHFDLB-F12M00521-3k2a	MOWF01000000	1	99/98		99/100	99/100		98/100	H	Milk, US
40	CDPHFDLB-F12M00521-3k2b	MOWE01000000	1	99/98		99/100	99/100		98/100	H	Milk, US
41	CDPHFDLB-F12M00521-8g1a	MOWD01000000	4	99/98		99/100	99/100		98/100	H	Milk, US
42	CDPHFDLB-F12M00521-8g1b	MOWC01000000	1	99/98		99/100	99/100		98/100	H	Milk, US
43	CDPHFDLB-F12M00521-8k1a	MOWB01000000	1	99/98		99/100	99/100		98/100	H	Milk, US
44	CDPHFDLB-F12M00521-8k1a	MOWA0100000	17	99/98		99/99	99/100		98/100	H	Milk, US
45	CDPHFDLB-F12M00562-b7a1	MOVS01000000	1	99/98		99/100	99/100		98/100	H	Milk, US
46	CDPHFDLB-F12M00562-b7b1	MPBL01000000	31	99/98		99/99	99/100			H	Milk, US
47	CDPHFDLB-F12M00580-a2	MOVJ01000000	1	99/98		99/100				H	Milk, US
48	CDPHFDLB-F12M00582-a1	MOVG01000000	1	99/98		99/100	99/100		98/100	H	Milk, US
49	CDPHFDLB-F12M00582-a2	MOVF01000000	1	99/98		99/100				H	Milk, US
50	CDPHFDLB-F12M00583-b1	MPBK01000000	4	99/98		99/99	99/78			H	Milk, US
51	BCW_6902	MKEU01000000	12	98/98		100/100	99/100		99/100	H	Crow, US
52	S3	NFOF01000000	35	98/98		99/100	99/100		99/100	H	Environment water, Canada
53	H27	NFOW01000000	23, 31	98/98		99/100	99/100		98/99	H	Human, Canada
54	H10	NFPM01000000	87, 98	99/98		99/100	99/100			H	Human, Canada

No.	C. jejuni Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
55	C1	NFQC01000000	47	98/97			100/100	99/100	99/100	H	Chicken, Canada
56	W4	NFMU01000000	30, 80	99/98	99/100		99/95			H	Environment water, Canada
57	H32	NFOQ01000000	8, 72	99/98	99/100		99/100			H	Human, Canada
58	OXC6586	CTRS01000000	1	97/98	99/100		99/100		99/100	H	Faeces, UK
59	OXC6353	CUNV01000000	1	99/98	100/99		99/100		98/100	H	Faeces, UK
60	OXC6314	CUJX01000000	1	99/98	99/100		97/100		98/100	H	Faeces, UK
61	OXC6507	CURC01000000	2	98/98	99/100		99/100		99/100	H	Faeces, UK
62	RC429	CYRQ01000000	33	98/98	99/100		99/98			H	Chicken, UK
63	12502	CYRV01000000	11	98/98	99/100		99/100		99/100	H	Chicken, UK
64	BCW_4221	MJWI01000000	5	98/98	99/100		99/100		99/100	H	Crow, US
65	BCW_5125	MKBT01000000	14	98/98	99/100		99/100		99/100	H	Human, US
66	BCW_5145	MKCH01000000	42	98/98	99/100		99/100		99/100	H	Human, US
67	BCW_5161	MKHS01000000	33	98/98	99/100		99/100		99/100	H	Human, US
68	BCW_5162	MKHT01000000	31	98/98	99/100		99/100		99/100	H	Human, US
69	OXC6588	CUUG01000000	1	98/98	99/100		99/100		99/100	H	Human, US
70	BH-01-0142	ABKD01000000	1	98/98	99/100		99/100		99/100	H	Human, Thailand

Class E

No.	C. jejuni Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
1	1997-14	AIPA01000000	2, 30	98/100	99/100		98/92	99/100		E	Human, US
2	OXC6373	CUMA01000000	1	96/100	99/100		98/92	99/100	98/99	E	Faeces, UK
3	OXC6330	CUKP01000000	1	96/100	99/100		98/92	99/100	98/99	E	Faeces, UK
4	OXC6294	CUJB01000000	1	96/100	99/100		98/92	99/100	98/99	E	Faeces, UK
5	OXC6510	CURE01000000	1	96/100	99/100		98/92	99/100	98/99	E	Faeces, UK
6	OXC6511	CURG01000000	2	95/100	99/100		98/92	99/100	98/99	E	Faeces, UK
7	OXC6437	CUOQ01000000	1	96/100	99/100		98/92	99/100	98/99	E	Faeces, UK
8	OXC6369	CULV01000000	2	96/100	99/100		98/92	99/100	98/99	E	Faeces, UK
9	Faeces	CZIK01000000	72	96/98	99/100		98/92	99/77		E	Chicken, Spain
10	Neck Skin	CZIW01000000	31	96/98	99/100		98/92	99/100		E	Chicken, Spain
11	BCW_6898	MJZA01000000	29	96/100	99/100		98/92	99/100	98/99	E	Chicken, Spain
12	BCW_6899	MJZB01000000	17	96/100	99/100		98/92	99/100	98/99	E	Chicken, Spain
13	W38	NFMV01000000	12	96/97	99/100		98/92	99/100	98/99	E	Environment water, Canada
14	W32	NFNB01000000	24	96/100	99/100		98/92	99/100	98/99	E	Environment water, Canada
15	BCW_4743	MKBH01000000	18	96/100	99/100		98/92	99/100	98/99	E	Human, US

Class O

No.	C. jejuni Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
1	LMG 9872	AIPM01000000	11, 18	98/100	99/100		98/100		98/99	O	Human, Sweden
2	OXC6536	CUSH01000000	3	99/100	100/100		100/100		100/100	O	Faeces, UK
3	OXC6332	CUKR01000000	1	98/100	99/100		98/100		98/100	O	Faeces, UK
4	5070	CCXG01000000	6	98/100	99/100		98/100		98/100	O	Chicken, Finland
5	BCW_4753	MKBM01000000	8	99/100	100/100		100/100		99/100	O	Unknown, US
6	W27	NFNH01000000	5	96/97	99/100		98/75			O	Environment water, Canada
7	OXC6321	CUKG01000000	2	99/100	100/100		100/100		100/100	O	Faeces, UK

Class W

No.	C. jejuni Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
1	7092_1	CUPI01000000	1	99/85	100/100	99/99		99/100		W	Faeces, UK
2	CVM 41910	JAKG01000000	18	99/85	99/100	99/99		98/100		W	Human, US
3	CVM 41914	JAKI01000000	10	99/85	99/100	99/99		98/100		W	Human, US
4	CVM 41933	JAKN01000000	2	98/85	99/100	99/99		99/100		W	Human, US
5	CVM 41973	JAJF01000000	9	99/85	99/100	99/97				W	Human, US
6	CVM 41964	JAKR01000000	2	99/85	99/100	99/99		98/100		W	Human, US
7	CVM 41985	JAKU01000000	14		99/100	99/99		98/100		W	Human, US
8	BCW_4452	MJWG01000000	16	99/85	100/100	99/99		99/100		W	Faeces, US
9	BCW_6882	MJYR01000000	6	95/85	99/100	99/100		99/100		W	Crow, US
10	BCW_6886	MJYU01000000	64	95/85	99/100	99/100		99/100		W	Crow, US
11	BCW_6953	MJZN01000000	37	98/85	99/100	99/99		99/100		W	Faeces, US
12	BCW_6959	MJZT01000000	2	98/85	99/100	99/99		99/100		W	Faeces, US

LOS Group 2; Mix LOS Classes

No.	*C. jejuni* Strain	Accession no.	Contig no.	21-25	26	26'	27	28	29-34	Class	Host, Country
1	CVM 41975	JAKT01000000	2			99/99		98/100		EHOP	Human, US
2	BCW_6883	MJYS01000000	5	95/85		99/99		99/100		EHOP	Crow, US
3	BCW_6885	MJYT01000000	58	96/85		99/99		98/100		EHOP	Crow, US
4	BCW_3781	MEIB01000000	59	95/85		99/99		99/100		EHOP	Crow, US
5	BCW_3784	MEIC01000000	25	96/85		98/99		98/100		EHOP	Crow, US
6	W9	NFMI01000000	1, 6	99/97						EHOP	Environment water, Canada

Draft sequences: LOS Group 3; Classes D, F, K, N, Q, I, J & S

Class D/F

No.	*C. jejuni* Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	OXC6583	CUTY01000000			96/94	98/100	97/100							99/100	F	Unknown, UK
2	OXC6319	CUKC01000000			96/94	98/100	97/100							99/100	F	Pigeon, Sweden
3	OXC6254	CUJH01000000	2		98/94	99/100	98/100							99/100	F	Faeces, UK
4	OXC6582	CUTW01000000	1	100/100	100/100	100/100	100/100							99/100	D	Faeces, UK
5	CDPHFDLB-F15M00665P1	MOUL01000000	1		98/94	99/100	98/100							99/100	F	Faeces, UK
6	CDPHFDLB-F15M00667P1	MOUJ01000000	3		98/94	99/100	98/100							99/100	F	Faeces, UK
7	CDPHFDLB-F15M00668P1	MOUH01000000	1		98/94	99/100	98/100							99/100	F	Cow Milk, US
8	CDPHFDLB-F15M00669P1	MOUF01000000	1		98/94	99/100	98/100							99/100	F	Cow Milk, US
9	CDPHFDLB-F15M00562-b9b1a	MOVR01000000	1		98/94	99/100	98/100							99/100	F	Cow Milk, US
10	CDPHFDLB-F12M00566-a1	MOVM01000000	1		98/94	99/100	98/100							99/100	F	Cow Milk, US
11	CDPHFDLB-F15M00668P2	MOUG01000000	2		98/94	99/100	98/100							99/100	F	Cow, US
12	RC507	CYRT01000000	1		98/86	99/100	98/100							99/100	F	Cow, US
13	W30	NFND01000000	2		98/94	99/100	98/100							99/100	F	Cow Milk, US
14	OXC6608	CUUS01000000	46, 20		98/77	99/100	99/100							99/100	F	Chicken, UK
15	OXC6432	CUOJ01000000	8		98/94	99/100	98/100							99/100	F	Environment water, Canada
16	OXC6412	CUNP01000000	1		98/94	99/100	99/100							99/100	F	Faeces, UK
17	OXC6491	CUQK01000000	3		98/94	99/100	99/100							99/100	F	Faeces, UK
18	OXC6250	CUHN01000000	1		98/94	99/100	98/100							99/100	F	Faeces, UK
19	OXC6450	CUQF01000000	2		98/94	99/100	98/100							99/100	F	Faeces, UK
20	OXC6401	CUND01000000	1		98/94	99/100	98/100							99/100	F	Faeces, UK
21	OXC6362	CULO01000000	2		98/94	99/100	98/100							99/100	F	Faeces, UK
22	OXC6469	CUPM01000000	1		98/94	99/100	98/100							99/100	F	Faeces, UK
23	OXC6404	CUNG01000000	1		98/94	99/100	98/100							99/100	F	Faeces, UK
24	OXC6375	CUMD01000000	2		98/94	99/100	98/100							99/100	F	Faeces, UK
25	OXC6441	CUOU01000000	1		98/94	99/100	98/100							99/100	F	Faeces, UK
26	OXC6368	CULW01000000	2		98/94	99/100	98/100							99/100	F	Faeces, UK
27	11601MD	LKCR01000000	1		98/94	99/100	99/100							99/100	F	Faeces, UK
28	Faeces	CZHX01000000	1		98/94	99/100	99/100							99/100	F	Faeces, UK
29	NC05-27	BCNK01000000	19		98/94	99/100								99/100	F	Turkey, US

Class K

No.	*C. jejuni* Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	7065_7	CUOO01000000	1	96/100	93/100	95/98							99/100		K	Faeces, UK
2	7213_3	CUTB01000000	1	96/100	93/100	95/98							99/100		K	Faeces, UK
3	7092_1	CURB01000000	1	96/100	93/100	95/98							99/100		K	Faeces, UK
4	7038_3	CUIM01000000	1	96/100	94/100	95/98							99/100		K	Faeces, UK
5	7065_7	CUMJ01000000	1	96/100	94/100	95/98							99/100		K	Faeces, UK
6	7065_7	CULP01000000	1	96/100	93/100	95/98							99/100		K	Faeces, UK
7	7213_3	CUVJ01000000	1	96/100	93/100	95/98							99/100		K	Faeces, UK
8	Meat	CZHP01000000	32			95/98							99/100		K	Chicken, Spain

No.	C. jejuni Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
9	CVM 41927	JAJG01000000	10, 15										99/94		K	Human, US
10	BCW_6476	MJYN01000000	39	94/100	92/100	95/98							93/100		K	Crow, US
11	BCW_6887	MKEV01000000	26		93/100	95/98							99/100		K	Crow, US
12	RC51	CYRU01000000	80	96/100		95/98							99/100		K	Chicken, UK
13	BJ-CJD39	LISI01000000	1	96/100	93/100	95/98							99/100		K	Human, China

Class N

No.	C. jejuni Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	BCW_5172	MKHY01000000	8					88/83						95/99	N	Human, US

Class Q

No.	C. jejuni Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	2871	LLWN01000000	1		91/94	91/100	93/100					99/100		79/80	Q	Poultry, Malaysia

Class I

No.	C. jejuni Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	LMG 23263	AIOD01000000	26, 69	99/100	99/85						99/100				I	Chicken, US
2	OXC6374	CUMB01000000	1	99/100	99/100	98/100	96/100		100/100	100/100	99/100				I	Faeces, UK
3	OXC6480	CUQA01000000	1	96/100	98/100	98/100	96/100		98/100	99/100	97/99				I	Faeces, UK

Class J

No.	C. jejuni Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	OXC6518	CURO01000000	1		99/92	99/100	96/90		98/98		96/100				J	Faeces, UK
2	BCW_6457	MJXF01000000	14, 62						97/100		95/99				J	Crow, US
3	BCW_4744	MKBI01000000	29		98/94	99/100	96/100		98/98		97/100				J	Crow, US
4	W13	NFNU01000000	94, 99						97/86		99/100				J	Environment water, Canada

Class S

No.	C. jejuni Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	OXC6287	CUIU01000000	1		98/94	99/100	96/100		98/100	99/100	96/100				S	Faeces, UK
2	NW	AGTE01000000	2, 4						98/100	99/100	96/100				S	Human, US
3	OXC6418	CUNW01000000	1		98/94	99/100	96/100		98/100	99/100	96/100				S	Faeces, UK
4	OXC6517	CURL01000000	2		98/94	99/100	96/100		98/100	99/100	96/100				S	Faeces, UK
5	OXC6451	CUQQ01000000	2		98/94	99/100	96/100		98/100	99/100	96/100				S	Faeces, UK
6	OXC6529	CUSA01000000	1, 21		98/94	99/100	96/100		98/100	99/100	96/100				S	Faeces, UK

LOS Group 3; Mix LOS Classes

No.	C. jejuni Strain	Accession no.	Contig no.	17	18	19	20	38	40	41	42-45	46	49-50	16	Class	Host, Country
1	VA48	NACK01000000	2, 5						99/79	99/100	95/99				I/S	Water, Sweden
2	BCW_6451	MJXA01000000	7	99/100	99/100	98/100	96/100		99/100		97/100				I/J	Faeces, UK
3	BCW_6452	MJXB01000000	6	99/100	99/100	98/100	96/100		99/100		97/100				I/J	Faeces, UK
4	BCW_6954	MJZO01000000	26, 27	99/100	99/100	98/100	98/100		98/98		95/100				I/J	Faeces, UK

Draft sequences: LOS Group 4; Classes G & L

Class G

No.	*C. jejuni* Strain	Accession no.	Contig no.	35	36	37	38	47	48	16	Class	Host, Country
1	OXC6358	CULK01000000	1	99/100	99/100	99/100	100/100			99/100	G	Faeces, UK
2	OXC6498	CUQT01000000	1	98/100	99/100	99/100	99/100			99/100	G	Faeces, UK
3	OXC6614	CUVA01000000	1	99/100	99/100	99/100	100/100			99/100	G	Faeces, UK
4	OXC6360	CULM01000000	1	98/100	99/100	99/100	99/100			99/100	G	Faeces, UK
5	OXC6322	CUKF01000000	1	98/100	99/100	99/100	99/100			99/100	G	Faeces, UK
6	OXC6410	CUNN01000000	1	99/100	99/100	99/100	99/100			99/100	G	Faeces, UK
7	OXC6486	CUQE01000000	2	98/100	99/100	99/100	99/100			99/100	G	Faeces, UK
8	OXC6256	CUKD01000000	5, 20	98/100	99/100	99/90	99/100			99/100	G	Faeces, UK
9	JL-CJHLIU1-1	LISQ01000000	2	98/100	99/100	99/100	99/100			99/100	G	Chicken, China
10	BCW_3794	MJVL01000000	3	97/100	99/100	98/100	98/100			99/100	G	Crow, US

Class L

No.	*C. jejuni* Strain	Accession no.	Contig no.	35	36	37	38	47	48	16	Class	Host, Country
1	BCW_3800	MJVP01000000	64	98/100	98/100	89/100		99/100	98/100	92/93	L	Crow, US
2	BCW_3802	MJVQ01000000	56	98/100	98/100	89/100		99/100	98/100	92/93	L	Crow, US
3	BCW_3810	MJVU01000000	57	98/100	98/100	89/100		99/100	99/100	92/93	L	Crow, US
4	BCW_4231	MJXZ01000000	65	98/100	98/100	89/100		99/100	99/100	92/93	L	Crow, US
5	BCW_6881	MJYQ01000000	89	99/100	97/100	89/100		99/100	98/100	92/93	L	Crow, US
6	OXC6631	CUVR01000000	1	97/100	96/100	89/100		99/100	99/99	91/93	L	Faeces, UK

Table 2: LOS types of *C. coli* complete (n=22) and draft sequences (n=542)

Complete sequences:

LOS Class I

No.	Strain	Accession #	4	5	6	7	8	Gene*	Class	Host
1	YH502	CP018900.1	99/100	99/100	96/100	99/100	99/100	96/100	I	Retail chicken US

LOS Class II

No.	Strain	Accession #	4	5	6	7	8	9	10	11	12	Gene*	Class	Host
1	JL-CDD-LMH	NZ_KZ253957.1	100/100	99/100	99/100	99/100	100/100	99/99	99/100	99/100	99/100	100/100	II	Unknown, China
2	BP3183	CP017871.1	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	95/100	II	Chicken, US
3	WA333	CP017873.1	100/100	100/100	99/100	99/100	100/100	99/100	100/100	99/100	100/100	100/100	II	Chicken, US
4	JV20	GL405235.1	100/100	100/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	100/100	II	Unknown, US

LOS Class III

No.	Strain	Accession #	4	5	6	7	8	9	10	11	Gene*	Class	host
1	BG2108	CP017878.1	99/100	98/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Chicken, US
2	RM2228	AAFL01000002	99/100	99/100	99/100	99/100	100/100	99/100	99/100	100/100	100/100	III	Unknown, US
3	YF2105	CP017865.1	99/100	98/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Chicken, US

LOS Class V

No.	Strain	Accession#	4	5	6	7	8	9	10	11/11'	12	13	14	15	Gene*	Class	Host
1	RM1875	CP007183.1	99/100	99/100	100/100	99/100	99/100	99/100	96/100	99/100	100/100	93/99	95/100	98/99	99/100	V	Unknown, US
2	ZV1224	CP017875.1	99/100	99/100			99/100	100/100	100/100	99/100	99/100	98/100	96/100	99/99		V with a pseudo gene	Pork, US

LOS Class VI

No.	Strain	Accession#	4	5	6	7	8	9	10	11	12	31	32	14	15	Class	Host
1	RM5611	CP007179.1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Unknown, US
2	HC2-48	CP013034.1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Beef, US
3	CF2-75	CP013036.1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Beef, US

LOS Class VII

No.	Strain	Accession#	4	5	6	7	8	9	10	11	33	34	35	36	13	14	15	Class	Host
1	OR12	NZ_CP019977.1	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, UK
2	CFSAN032805	CP023545.1	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, US
3	CO2-160	CP013032.1	99/100	99/100	100/100	99/100	89/99	94/99	87/94							94/98	99/100	VII	Beef, US
4	K7	NZ_KI639691.1	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Unknown, UK

LOS Class VIII

No.	Strain	Accession #	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Gene*	Class	Host
1	CVM N29710	NC_022347.1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
2	15-537360	NC_022660.1	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	94/100	VIII	Human, UK
3	FB1	CP011015.1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
4	YH501	CP015528.1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
5	CVM 41957	NZ_JAJZ01000000	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, US

Draft sequences:

LOS Class I

No.	Strain	Accession #	Contig #	4	5	6	7	Gene*	8	Class	Host
1	86119	AIMU01000000	31	99/100	99/100	99/100	99/100		100/100	I	Chicken US
2	OXC6372	CULY01000000	2	99/100	99/100	99/100	99/100	99/100	99/100	I	Faeces UK
3	OXC6276	CUII01000000	1	99/100	99/100	99/100	100/100	99/100	99/100	I	Faeces UK
4	OXC6378	CUMG01000000	1	99/100	99/100	99/100	99/100	99/100	99/100	I	Faeces UK
5	OXC6253	CUIX01000000	1	99/100	99/100	95/100	96/100		99/100	I	Faeces UK
6	CVM N44721	LBEL01000000	6	99/100	99/100	95/100	96/100	96/100	98/100	I	Breast chicken, US
7	CVM N46876	LBEJ01000000	4	99/100	99/100	96/100	96/100	96/100	98/100	I	Breast chicken, US
8	CVM N45714F	LBDV01000000	2	100/100	99/100	96/100	99/100		97/100	I	Turkey, US
9	CVM 41945	JAJP01000000	8	99/100	99/100	99/100	100/100		99/100	I	Humans, US
10	CVM 41970	JAJH01000000	11			99/100	99/100	96/100	97/100	I	Humans, US
11	CVM N26697	JOUO01000000	2	99/100	99/100	99/100	100/100	96/100	98/100	I	Chicken, US
12	OXC6258	CUKZ01000000	3	99/100	99/100	95/100	99/100	100/100	98/100	I	FAECES, UK
13	CVM N18323	JOUJ01000000	1	99/100	99/100	97/100	99/100	96/100	99/100	I	Chicken, US
14	CVM N26070	JOUN01000000	3	99/100	99/100	97/100	99/100	96/100	99/100	I	Turkey, US
15	H103060185	FBAG01000000	27	99/100	99/100	99/100	100/100	96/100	97/100	I	Environmental WATER, UK
16	P604D	FBLZ01000000	16	99/100	99/100	99/100	99/100	96/100	97/100	I	Soil, UK
17	SS_2357	FBEC01000000	13	99/100	99/100	99/100	100/100	97/100	100/100	I	Chicken, UK
18	H093100604	FBQF01000000	19	99/100	99/100	99/100	99/100	96/100	97/100	I	Environmental WATER, UK
19	H043880651	FBNR01000000	23	99/100	99/100	99/100	100/100	96/100	95/100	I	Human, UK
20	H063800417	FBQI01000000	11	99/100	98/100	99/100	99/100	98/100	97/100	I	Environmental WATER, UK
21	H102520382	FAZD01000000	38	99/100	99/100	99/100	99/100	97/100	100/100	I	Environmental WATER, UK
22	H140200373	FBLY01000000	15	99/100	99/100	99/100	100/100	98/100	97/100	I	Human, UK
23	UNOR383B	FBJO01000000	19	99/100	99/100	99/100	100/100	98/100	97/100	I	Chicken, UK
24	UNF5421c	FBMG01000000	25	99/100	99/100	99/100	99/100	96/100	97/100	I	Chicken, UK
25	H094280625	FBQL01000000	28	99/100	99/100	99/100	100/100	97/100	100/100	I	Environmental WATER, UK
26	H102380405	FAYB01000000	30	99/100	99/100	99/100	99/100		99/100	I	Environmental WATER, UK
27	H132580232	FBND01000000	77	99/100	99/100	95/100	96/100	100/100	100/100	I	Environmental WATER, UK
28	H124620276b	FBPL01000000	15	100/100	100/100	99/100	99/100	96/100	99/100	I	Human, UK
29	UNOR8693b	FBMI01000000	14	99/100	99/100	99/100	100/100	98/100	97/100	I	Chicken, UK
30	SS_2289	FBFR01000000	16			99/100	99/100		100/100	I	Chicken, UK
31	H142080277	FBPH01000000	21	99/100	99/100	99/100	100/100	96/100	97/100	I	Human, UK
32	11601	LKCS01000000	23	100/100	99/100	96/100	99/100	96/100	97/100	I	Turkey, US
33	CVM N23392	JOUM01000000	7			99/100	99/100	100/100	100/100	I	Chicken, US
34	BCW_6860	MJYP01000000	36	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
35	BCW_6914	MJZH01000000	34	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
36	BCW_6948	MJZK01000000	32	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
37	BCW_6950	MJZL01000000	28	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
38	BCW_6958	MJZS01000000	29	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
39	BCW_7437	MJZY01000000	32	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
40	BCW_5818	MKAO01000000	12	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
41	BCW_6946	MKEX01000000	18	99/100	99/100	99/100	99/100	96/100	97/100	I	Monkey, US
42	OXC6476	CUPT01000000	2	99/100	99/100	99/100	99/100	96/100	98/100	I	Faeces, UK
43	RC282	CYRA01000000	77			99/100	99/100	100/100	99/100	I	Supermarket, UK
44	SH-CCD11C671	LISD01000000	4	93/100	99/100	99/100	100/100	96/100	99/100	I	Human, china
45	SH-CCHF12C088	LISE01000000	5	93/100	99/100	99/100	100/100	96/100	99/100	I	Chicken, china
46	SH-CCD12C100	LISF01000000	1	93/100	99/100	99/100	99/100	96/100	99/100	I	Human, china
47	BCW_4453	MJWB01000000	34	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
48	BCW_4457	MJWC01000000	35	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
49	BCW_5916	MJWD01000000	32	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
50	BCW_5914	MJYL01000000	36	99/100	99/100	99/100	99/100	96/100	97/100	I	Faeces, US
51	6067	LKCQ01000000	14	100/100	100/100	96/100	99/100	96/100	97/100	I	Turkey house water, US
52	BFR-CA-9557	CP011777.1		100/100	100/100	99/100	100/100	96/100	99/100	I	Chicken, Germany
53	RC182	CYQT01000000	57	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
54	RC096	CYQI01000000	56	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
55	RC264	CYQW01000000	106			99/100	99/100		99/100	I	Supermarket, UK
56	RC285	CYRE01000000	27	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
57	RC126	CYQM01000000	11			99/93	99/100	100//100	99/100	I	Supermarket, UK
58	RC387	CYRK01000000	7	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
59	RC127	CYQN01000000	85	99/100	99/100	98/100	99/100	99/100	99/100	I	Supermarket, UK

No.	Strain	Accession #	Contig#	4	5	6	7	8	9	Class	Host
60	RC037	CYQD01000000	27			99/94	100/100	96/100	97/100	I	Supermarket, UK
61	RC289	CYRG01000000	88	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
62	RC415	CYRO01000000	3,81			99/86	99/100	100/100		I	Supermarket, UK
63	RC038	CYQE01000000	37	99/100	99/100	99/100	100/100	96/100	97/100	I	Supermarket, UK
64	RC428	CYRP01000000	6	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
65	RC116	CYQL01000000	82, 91	99/100	99/100	99/100	99/100	100/100	98/90	I	Supermarket, UK
66	RC043	CYQF01000000	3	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
67	RC281	CYQZ01000000	38			99/100	99/100	100/100	99/100	I	Supermarket, UK
68	RC284	CYRD01000000	73	99/100	99/100	99/100	99/100	100/100	99/100	I	Supermarket, UK
69	SH-CCD12C136	JXTU01000000	4	93/100	99/100	99/100	100/100	96/100	99/100	I	Human, China

LOS Class II

No.	Strain	Accession #	Contig#	4	5	6	7	8	9	10	11	12	Gene*	Class	Host
1	H8	AINU01000000	4, 27	100/100	100/100	99/100	99/100	100/100	99/100	100/100	99/100	99/100	92/100	II	Human, Switzerland
2	OXC6523	CURT01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	91/100	II	Faeces, UK
3	OXC6442	CUOV01000000	1	100/100	100/100	99/100	99/100	100/100	99/100	99/100	99/100	99/100	99/100	II	Faeces, UK
4	OXC6443	CUOW01000000	1	100/100	100/100	99/100	99/100	100/100	99/100	99/100	99/100	99/100	99/100	II	Faeces, UK
5	OXC6537	CUSJ01000000	1	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Faeces, UK
6	COL B1-266	LKIV01000000	274	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100		II	Animal, Colombia
7	CVM N7464	JOVA01000000	1	100/84	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	93/100	II	Chicken, US
8	CVM N6388	JOUY01000000	4,15	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	II	Chicken, US
9	CVM N7454	JOUZ01000000	10	100/100	100/100	99/100	99/100	100/100	99/100	100/100	99/100			II	Chicken, US
10	CVM N9036	JOVE01000000	6, 9, 36	100/100	100/100	99/100	99/100	100/100	100/100	100/100	93/100	100/100	100/100	II	Pork, US
11	UNAJL222	FBIG01000000	25, 43	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	II	Pig, UK
12	H092660305	FBNL01000000	36	99/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Human, UK
13	CCN349	FBIX01000000	28	99/100	100/100	99/100	99/100	100/100	99/100	100/100	99/100	100/100	91/99	II	Chicken, UK
14	UNNQFC9	FBLR01000000	19	99/100	100/100	99/100	99/100	100/100	99/100	99/100	100/100	100/100	95/99	II	Duck, UK
15	H060260417	FAXS01000000	11	100/100	100/100	99/100	99/100	100/100	99/100	99/100	100/100	100/90		II	Human, UK
16	UNOR581A	FBHW01000000	29	99/100	100/100	99/100	99/100	100/100	99/100	99/100	100/100	100/100	95/99	II	Chicken, UK
17	H044660164	FBKS01000000	10	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	II	Human, UK
18	H063900401	FBKM01000000	26	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	II	Human, UK
19	H072820535	FBBC01000000	15	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Human, UK
20	SS_2356	FBED01000000	11	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	91/99	II	Chicken, UK
21	H102740168	FBOG01000000	25	99/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	91/100	II	Environmental water, UK
22	UNES9	FBHB01000000	26	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Chicken, UK
23	H063540531	FBNJ01000000	16	99/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	91/100	II	Human, UK
24	H102240159	FBQQ01000000	233	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Environmental water, UK
25	1535	FBGC01000000	7	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	II	Soil, UK
26	EC0349	FBJR01000000	23,27	99/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	99/100	91/99	II	Dairy, UK
27	H060280132	FBNZ01000000	24	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	92/100	II	Human, UK
28	H051160594	FBPK01000000	67	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Human, UK
29	H072820536	FBNS01000000	18	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Human, UK
30	M1483PM	LQXL01000000	23	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Chicken, Colombia
31	M1486PM	LQXK01000000	19	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Retail store, Colombia
32	C15	NFPY01000000	99, 133	100/100	100/100	99/99	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Faeces, Canada
33	5	NFQH01000000	62, 72	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Faeces, Canada
34	3	NFQJ01000000	43, 50	100/100	100/100	99/97	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Retail Chicken, Canada
35	OXC6460	CUPB01000000	2	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	II	Faeces, UK
36	OXC6386	CUMP01000000	1	100/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	99/100	II	Faeces, UK
37	OXC6559	CUSX01000000	1	100/100	100/100	99/100	99/100	100/100	99/100	99/100	100/100	100/100	100/100	II	Faeces, UK
38	SH-CCF11C627	LISC01000000	1	100/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	93/100	II	Chicken, China
39	BCW_5137	MKCC01000000	23	100/100	100/100	99/100	99/100	100/100	100/100	100/100	100/100	100/100	92/100	II	Human, US
40	ICDCCC-SHCCH11C314	JXAD01000000	1	100/100	100/100	100/100	99/100	99/100	100/100	100/100	100/100	100/100	93/100	II	Chicken, China
41	SH-CCH11C605	LISB01000000	1	100/100	100/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	93/100	II	Chicken, China
42	SH-CCH11C390	LISA01000000	1,6	100/100	100/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	93/100	II	Chicken, China
43	SH-CCH11C334	LIRZ01000000	1	100/100	100/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	93/100	II	Chicken, China

LOS Class III

No.	Strain	Accession #	Contig #	4	5	6	7	8	9	10	11	Gene*	Class	host
1	CVM41915	JAJM01000000	55	99/100	99/100	99/100	99/100	100/100	100/100	99/100	99/100	100/100	III	Human, US
2	OXC6263	CUHU01000000	1	99/100	99/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	III	Faeces, UK
3	OXC6447	CUOY01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Faeces, UK
4	OXC6513	CURI01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Faeces, UK
5	OXC6297	CUJD01000000	2	99/100	99/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	III	Faeces, UK
6	OXC6337	CUKW01000000	1	99/100	100/100	100/100	99/100	100/100	99/100	100/100	100/100	100/100	III	Faeces, UK
7	OXC6380	CUMH01000000	2	99/100	98/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Faeces, UK
8	OXC6472	CUPQ01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Faeces, UK
9	OXC6471	CUPO01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Faeces, UK
10	OXC6428	CUOH01000000	1	99/100	100/100	100/100	99/100	100/100	97/100	100/100	100/100	100/100	III	Faeces, UK
11	OXC6308	CUJQ01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Faeces, UK
12	OXC6267	CUHY01000000	1	99/100	100/100	100/100	100/100	100/100	97/100	100/100	100/100	100/100	III	Faeces, UK
13	CVM 41898	JAJK01000000	2, 66, 86, 85, 102	99/100	100/100		99/100	100/100	100/100	99/100	100/100	100/100	III	Human, US
14	CVM N14784	JOVU01000000	37	99/100	98/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Chicken, US
15	CVM N23169	JOUL01000000	15	99/100	100/100	99/100	99/100	100/100	100/100	99/100	99/100	100/100	III	Chicken, US
16	CVM N26699	JOUP01000000	5	99/100	98/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Chicken, US
17	CVM N462	JOUU01000000	11	99/100	98/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Chicken, US
18	CVM N9077	JOVF01000000	11	99/100	100/100	99/100	100/100	100/100	100/100	99/100	99/100	100/100	III	Chicken, US
19	CVM N3508	JOUS01000000	16	99/100	99/100	99/100	99/100	100/100	99/100	99/100	100/100	100/100	III	Chicken, US
20	CVM N9093	JOVG01000000	7, 75, 90	99/100	98/100	100/100	100/100	100/100		100/100	100/100	100/100	III	Chicken, US
21	CVM N6401	JOVZ01000000	11	99/100	99/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	III	Chicken, US
22	CCN153	FBHE01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Chicken, UK
23	P604B	FBLL01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Soil, UK
24	EC3298	FBJM01000000	13	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
25	EC3529	FAZB01000000	8	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
26	EC3357	FBJD01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
27	UNOR13691b	FBMF01000000	5	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Chicken, UK
28	EC3505	FBBL01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
29	EC3619	FAZR01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
30	EC6049	FBFE01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
31	CCN26	FBMP01000000	9	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
32	UNQMCIIS18a	FAYE01000000	5	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
33	CCN181	FBJE01000000	29	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
34	EC3879	FAZZ01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
35	EC6304	FBCC01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
36	EC4194	FAZJ01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
37	EC3501	FAYH01000000	22	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
38	EC3693	FBAH01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
39	EC3615	FAYJ01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
40	EC5259	FBBO01000000	8	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
41	EC4473	FBCJ01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
42	EC3623	FBDC01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
43	EC3365	FBGZ01000000	12	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
44	EC3533	FBAE01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
45	EC6299	FBDF01000000	23	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
46	EC5850	FBBX01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
47	EC6124	FBCE01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
48	H133040289	FBBI01000000	46	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Environmental water, UK
49	CCN265	FBJH01000000	10	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry farm , UK
50	EC3782	FBAD01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
51	EC5923	FBBY01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
52	H081940749	FBNA01000000	12	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Environmental water, UK
53	UNOR10622c	FBMD01000000	49	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	III	Chicken, UK
54	EC3478	FBHJ01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
55	EC4593	FBDH01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
56	EC4910	FBFH01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
57	EC3537	FBBA01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
58	EC3849	FAXR01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
59	CCN123	FBIO01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry, UK
60	EC3940	FBBK01000000	7	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Farm environment, UK

61	P474A	FBKG01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Soil, UK
62	EC3146	FBGF01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Farm environment, UK
63	EC5721	FBFG01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
64	BRIS1041X	FBIH01000000	15	99/100	99/100	100/100	100/100	100/100	99/100	99/100	99/100	100/100	III	Soil, UK
65	EC3627	FBAA01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
66	H103480422	FBQB01000000	13	99/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	III	Environmental Water, UK
67	UNF383D	FBGP01000000	49	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Chicken, UK
68	EC3511	FBHI01000000	15	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Farm environment, UK
69	EC3575	FBAR01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Farm environment, UK
70	EC3525	FAYA01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
71	EC4297	FBEV01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
72	EC3952	FBCP01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Farm environment, UK
73	EC3521	FBAN01000000	15	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
74	H110420358	FAYG01000000	40	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	93/100	III	Environmental Water, UK
75	EC3389	FAZG01000000	5	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
76	H132760749	FAYP01000000	5	99/100	99/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Environmental Water, UK
77	EC4238	FBFF01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Environmental Water, UK
78	CCN288	FBGL01000000	22	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Poultry farm water, UK
79	EC6168	FBBM01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
80	EC3786	FBBH01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
81	EC4768	FBDG01000000	22	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
82	EC3631	FBAQ01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
83	EC3731	FAZA01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
84	EC4098	FBEZ01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
85	P546D	FBKR01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Soil, UK
86	H091320788	FBPN01000000	7	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Environmental Water, UK
87	CCN154	FBHM01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Poultry Environment, UK
88	H140660843	FBJG01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Human, UK
89	EC4722	FBDA01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Environmental Water, UK
90	CCN71	FBIV01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry Environment, UK
91	EC6158	FBBN01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
92	CCN292	FBJS01000000	11	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Poultry Environment, Water, UK
93	EC5240a	FBBG01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
94	EC3607	FAYZ01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
95	H065100499	FBKW01000000	23	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Environmental Water, UK
96	EC4102	FBDR01000000	15	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
97	EC3390	FBHC01000000	11	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
98	H132580239	FBQD01000000	25	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
99	EC5246a	FBBW01000000	5	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
100	H132580228	FBAS01000000	67	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Environmental Water, UK
101	EC3756	FAZK01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Farm environment, UK
102	EC3348	FBBD01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
103	EC5183	FBDO01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
104	H073900238	FBOT01000000	44	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Environmental Water, UK
105	EC6122	FBFM01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
106	EC5240b	FBDI01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
107	CCN293	FBIY01000000	9	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Poultry Environment, Water, UK
108	EC3361	FBIC01000000	13	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy farm, Water, UK
109	EC5679	FBBR01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
110	EC4709	FBCD01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Farm environment, UK
111	EC4214	FAXQ01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Dairy Farm, Faeces, UK
112	H084040382b	FBPU01000000	8	99/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Human, UK
113	H132940658	FBPY01000000	23	99/100	100/100	99/100	99/100	100/100	100/100	100/100	99/100	100/100	III	Human, UK
114	EC4250	FBCS01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
115	EC5520	FBCO01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
116	EC4393	FBAY01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
117	EC3956	FAZP01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
118	EC5726	FAYM01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
119	NCTC12570	FBHN01000000	15	-	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Unknown
120	H120880380	FAYN01000000	44	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
121	P474C	FBKF01000000	9, 12	-	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Soil, UK
122	EC4258	FBCM01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
123	EC5992	FBDL01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK

124	EC5693	FBFN01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	III	Dairy Farm, Faeces, UK
125	H130500174	FBQH01000000	39	99/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
126	CCN56	FBIU01000000	15	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry Farm, Faeces, UK
127	SWAN392	FBLQ01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Duck, UK
128	EC3721	FBDP01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
129	EC4690	FBDB01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
130	CCN60	FBGI01000000	5	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry Farm, Faeces, UK
131	EC4287	FBCK01000000	6	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
132	EC4946	FBCR01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
133	EC5479	FAYI01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
134	EC5841	FBBP01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
135	EC3689	FBDN01000000	22	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
136	EC6173	FBCV01000000	26	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
137	H140460193	FBMV01000000	9	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
138	BRISLC31-1	FBGO01000000	29	99/100	99/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	III	Dog, UK
139	EC4060	FAYU01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
140	EC3370	FAZT01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
141	EC5905	FBCF01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
142	EC4675	FBCY01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
143	EC3385	FBIS01000000	15	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
144	P568B	FAXX01000000	13	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Soil, UK
145	H040680225	FAYW01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
146	NCTC11438	FBMN01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
147	CCN59	FBJF01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry Farm, Faeces, UK
148	EC3727	FAZQ01000000	5	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
149	EC4242	FBCW01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
150	EC3381	FBIN01000000	24	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
151	H053280346	FBOY01000000	15	99/100	99/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	III	Human, UK
152	EC5531	FBBZ01000000	22	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
153	EC3397	FBCQ01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
154	EC3774	FBAJ01000000	7	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
155	EC3373	FBGU01000000	7	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
156	P635D	FBKB01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Soil, UK
157	EC3611	FBCL01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
158	H063800423	FBQU01000000	38	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	III	Environmental Water, UK
159	H092260569a	FBNX01000000	10	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Environmental Water, UK
160	EC3400	FBAB01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
161	H073180384	FBJW01000000	15	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
162	EC4356	FBCI01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
163	EC3444	FBBE01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
164	P546A	FBNB01000000	23	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Soil, UK
165	CCN119	FBIL01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry Environmental, UK
166	EC3490	FBHP01000000	23	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
167	EC4530	FBDD01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
168	EC3344	FBJK01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
169	EC4462	FBCH01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
170	EC5338	FBFJ01000000	17	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
171	EC5604	FBBV01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
172	H044040580	FAZN01000000	48	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
173	H111620356	FAYY01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Environmental Water, UK
174	EC4868	FBCB01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
175	H062180535	FAZV01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
176	P635A	FBKH01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Soil, UK
177	EC3705	FBCZ01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Farm environment, UK
178	EC5072	FBFL01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
179	H114640463a	FBLE01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
180	EC3326	FBAC01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
181	H053780444	FBBB01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Human, UK
182	EC4448	FBAZ01000000	6	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy Farm, Faeces, UK
183	EC4978	FBDJ01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
184	EC3735	FAXT01000000	7	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Dairy farm, Water, UK
185	H132340486	FAZC01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Environmental Water, UK
186	CCN64	FBJU01000000	24	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry Environmental, UK
187	CCN72	FBIQ01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	III	Poultry Environmental, UK

No.	Strain	Accession #	Contig#													Class	Host
188	H072680465	FBOF01000000	10	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Environmental Water, UK
189	EC3322	FBJQ01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
190	EC3725	FBAV01000000	16	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
191	EC6047	FBCA01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
192	EC5983	FBFC01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
193	EC6016	FBBF01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Farm environment, UK
194	H132760142	FBOU01000000	73	99/100	99/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100				III	Environmental Water, UK
195	H073700405	FBNF01000000	27	99/100	99/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100				III	Environmental Water, UK
196	EC3356	FBJC01000000	7	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Environmental Water, UK
197	EC4951	FBAX01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
198	EC4383	FBCU01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
199	EC4277	FBDE01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
200	EC5246	FBDM01000000	3	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
201	CCN124	FBJT01000000	9	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Poultry Environmental, UK
202	EC6203	FBBT01000000	10	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
203	EC3466	FBJJ01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
204	EC4808	FBFD01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
205	EC5806	FBEY01000000	15	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
206	EC4685	FBDK01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
207	EC3748	FAYT01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Farm environment, UK
208	EC4267	FBFB01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
209	EC3369	FAYQ01000000	2	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
210	EC3713	FBAU01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
211	EC3928	FBCX01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Farm environment, UK
212	EC3875	FAZU01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Farm environment, UK
213	EC5899	FBEO01000000	20	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
214	CCN55	FBMM01000000	21	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Poultry Environmental, UK
215	EC5947	FBCN01000000	3	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
216	EC4956	FBFK01000000	14	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy Farm, Faeces, UK
217	EC3498	FBGS01000000	11	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
218	EC4730	FBAO01000000	18	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
219	EC3338	FAYS01000000	19	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Dairy farm, Water, UK
220	H120880379	FAZX01000000	35	99/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100				III	Human, UK
221	20A_420	MCFT01000000	1	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100				III	Human, UK
222	OXC6504	CUQY01000000	1	99/100	100/100	100/100	99/100	100/100	97/100	100/100	100/100	100/100				III	Faeces, UK
223	OXC6474	CUPR01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Faeces, UK
224	OXC6312	CUJV01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Faeces, UK
225	U2	NFOA01000000	4	99/100	98/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Retail Chicken, Canada
226	ZJ2013CCHD50	JXTV01000000	1	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100				III	Chicken, China

LOS Class IV

No.	Strain	Accession #	Contig#	4	5	6	7	8	9	10	11	12	13	14	15	Class	Host
1	LMG9860	AINS01000000	6, 47	99/100	99/100	100/100	100/100	100/100	99/100	95/82	99/100	99/100	95/99	96/100	99/100	IV	Human, Canada
2	H56	AINW01000000	6	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	97/100	96/100	IV	Human, Switzerland
3	OXC6425	CUOB01000000	1	99/100	100/100	100/100	100/100	100/100	99/100	99/100	99/100	99/100	95/99	95/100	99/100	IV	Faeces, UK
4	CVM 41963	JAJR01000000	16				100/100	100/100	99/100	99/100	99/100	99/100	95/99	99/100	99/100	IV	Human, US
5	CVM N18725	JOUK01000000	7	100/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	99/100	99/100	96/100	99/100	IV	Chicken, US
6	CVM N287	JOVY01000000	5	99/100	99/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	95/99	98/100	99/100	IV	Pork, US
7	H044220458	FBAP01000000	39	99/100	99/100	100/100	100/100	100/100	99/100	96/100	99/100	99/100	100/100	96/100	99/100	IV	Human, UK
8	SS_2326	FBER01000000	13	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
9	SS_2238	FBEP01000000	35	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
10	SS_2226	FBEF01000000	44	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
11	H121240507	FAZF01000000	27	99/100	99/100	100/100	100/100	99/100	99/100	99/100	99/100	99/100	95/99	96/100	99/100	IV	Human, UK
12	SS_2296	FBEQ01000000	8	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
13	SS_2318	FBEE01000000	14	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
14	H043940494	FBNH01000000	24	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	95/100	98/100	IV	Human, UK
15	SS_2291	FBEN01000000	11	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
16	SS_2230	FBEI01000000	10	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
17	SS_2351	FBDV01000000	19	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
18	SS_2315	FBEG01000000	39	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK
19	UNOR5482c	FBME01000000	23	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	95/100	99/100	IV	Chicken, UK
20	H121760164	FBQE01000000	23	99/100	99/100	100/100	100/100	100/100	99/100	99/100	99/100	99/100	95/99	96/100	99/100	IV	Human, UK
21	SS_2279	FBEW01000000	13	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100	IV	Chicken, UK

No.	Strain	Accession#	Contig#	4	5	6	7	8	9	10	11/11'	12	13	14	15	Gene*	Class	Host
22	SS_2325	FBEK01000000	23	99/100	99/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	96/100	99/100		IV	Chicken, UK
23	CCN264	FBMQ01000000	11	99/100	100/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	95/100	98/100		IV	Poultry Environmental, UK
24	BCW_7432	MJZU01000000	34	99/100	100/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	95/100	98/100		IV	Faeces, US
25	BCW_7435	MJZX01000000	19	99/100	100/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	95/100	98/100		IV	Faeces, US
26	BCW_6447	MJWL01000000	14	99/100	100/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	95/100	98/100		IV	Faeces, US
27	BCW_6448	MJWM01000000	14	99/100	100/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	95/99	95/100	98/100		IV	Faeces, US

LOS Class V

No.	Strain	Accession#	Contig#	4	5	6	7	8	9	10	11/11'	12	13	14	15	Gene*	Class	Host
1	SS_2286	FBDZ01000000	21	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	99/99		V	Chicken, UK
2	SS_2278	FBEL01000000	27	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	98/100		V	Chicken, UK
3	H132680116	FBOL01000000	34	99/100	99/100	100/100	99/100	99/100	99/100	96/100	99/100	100/100	93/99	95/100	98/99	99/100	V	Environmental water, UK
4	SS_2285	FBES01000000	35	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	98/100		V	Chicken, UK
5	SS_2266	FBEJ01000000	40	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	99/99		V	Chicken, UK
6	H121240502	FBPC01000000	27	100/100	99/100	100/100	99/100	99/100	99/100	96/100	99/100	100/100	93/99	95/100	99/100	100/100	V	Human, UK
7	SS_2349	FBDY01000000	35	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	98/100		V	Chicken, UK
8	SS_2249	FBFO01000000	5	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	98/100		V	Chicken, UK
9	SS_2284	FBDX01000000	49	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	99/99		V	Chicken, UK
10	SS_2276	FBEX01000000	40	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	99/99		V	Chicken, UK
11	H094560717	FBOM01000000	5	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	96/99		V	Environmental water, UK
12	SS_2352	FBEH01000000	10	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	98/100		V	Chicken, UK
13	p604A	FBPT01000000	18	99/100	99/100	100/100	99/100	99/100	98/100	100/100	99/100	100/100	99/100	99/100	96/99		V	Soil, UK
14	H114640463b	FBPS01000000	13	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	96/100		V	Human, UK
15	NCTC11353	FBNV01000000	29	99/100	99/100	100/100	99/100	99/100	99/100	100/100	99/100	100/100	99/100	99/100	96/100		V	Pig, UK
16	H075200514	FBLS01000000	19	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	96/100		V	Environmental water, UK
17	SS_2271	FBEU01000000	8	99/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	100/100	92/99	95/100	99/99		V	Chicken, UK
18	H061980521a	FBLK01000000	15	99/100	99/100	99/100	99/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	96/100		V	Environmental water, UK
19	P588B	FBKK01000000	17	99/100	99/100	100/100	99/100	99/100	99/100	100/100	99/100	100/100	99/100	99/100	96/99		V	Soil, UK

LOS Class VI

No.	Strain	Accession#	Contig#	4	5	6	7	8	9	10	11	12	31	32	14	15	Class	Host
1	84-2	AIMS01000000	17	99/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	VI	Swine, UK
2	1091	AIMV01000000	2, 61	100/100	100/100	100/100	100/100	94/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Bovine, US
3	LMG 9854	AINL01000000	54	99/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VI	Human, Canada
4	CVM N44505F	LBDX01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Cow, US
5	CVM N51226F	LBDL01000000	3, 4	100/100	100/100	100/100			100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Cow, US
6	CVM N13165	JAJT01000000	12	99/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	98/100	100/100	99/100	98/100	96/100	VI	Chicken, US
7	CVM N20344	JAJU01000000	27, 38, 45	100/100	100/100	100/100			100/100	100/100	100/100	100/100		100/100	100/100	100/100	VI	Chicken, US
8	CVM N20402	JAJV01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Chicken, US
9	UNAJC222	FBHF01000000	8	99/100	99/100	99/100	100/100	99/100	100/100	100/100	99/100	98/100	100/100	100/100	97/100	99/100	VI	Pig, UK
10	H130600457	FAYX01000000	18	99/100	99/100	98/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	97/100	99/100	VI	Human, UK
11	P474D	FBOC01000000	22	99/100	99/100	99/100	99/100	91/100	99/100	95/100	99/100	98/100	99/100	99/100	96/100	98/100	VI	Soil, UK
12	NCTC11437	FBHL01000000	8	99/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	98/100	100/100	99/100	97/100	96/100	VI	Human, UK
13	H043920292	FBPD01000000	9	99/100	99/100	98/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	97/100	99/100	VI	Human, UK
14	UNLL3.1	FBIK01000000	4	99/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	98/100	100/100	99/100	97/100	98/100	VI	Pig, UK
15	UNQMCIIS16	FBMK01000000	14	99/100	99/100	100/100	100/100	99/100	100/100	99/100	99/100	98/100	100/100	99/100	97/100	96/100	VI	Human, UK
16	BRISPIG3	FBHR01000000	14	99/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	98/100	100/100	99/100	97/100	96/100	VI	Pig, UK
17	H125280575	FBKU01000000	19	99/100	99/100	100/100	99/100	99/100	98/90	94/94	99/100	98/100	100/100	100/100	97/100	96/100	VI	Human, UK
18	20G12	LWIH01000000	17, 130, 132,133, 160, 169	99/100	99/76	73/100			99/100	100/100	96/96	99/100	98/100	100/100	100/100	96/100	VI	Milk, US
19	FNWR7B4_44	MCFS01000000	9	99/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	98/100	100/100	100/100	97/100	99/100	VI	Unknown
20	BCW_6913	MJZG01000000	22	99/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	98/100	100/100	99/100	98/100	96/100	VI	Faeces, US
21	BCW_7433	MJZV01000000	36	99/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	98/100	100/100	99/100	98/100	96/100	VI	Faeces, US
22	Tx40	MDCN01000000	8	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VI	Cattle faeces, US

LOS Class VII

No.	Strain	Accession#	Contig#	4	5	6	7	8	9	10	11	33	34	35	36	13	14	15	Class	Host
1	OXC6309	CUJR01000000	1	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Faeces, UK
2	OXC6385	CUMN01000000	1	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	98/100	99/100	VII	Faeces, UK
3	OXC6376	CUMC01000000	2	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Faeces, UK
4	OXC6601	CUUO01000000	1	99/100	99/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	100/100	99/100	100/100	100/100	97/100	98/100	VII	Faeces, UK
5	OXC6551	CUVK01000000	1	100/100	99/100	100/100	99/100	90/99	94/99	98/94	100/100	100/100	99/100	99/100	100/100	100/100	97/100	99/100	VII	Faeces, UK
6	OXC6587	CUUB01000000	1	99/100	99/100	100/100	99/100	99/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Faeces, UK
7	OXC6426	CUOF01000000	1	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Faeces, UK
8	OXC6371	CULZ01000000	1	99/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Faeces, UK
9	OXC6434	CUON01000000	1	99/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	98/100	99/100	VII	Faeces, UK
10	CVMN41652	LBEQ01000000	4	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, US
11	CVMN49243	LBEH01000000	4	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, US
12	CVMN51619	LBEE01000000	2	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, US
13	CVMN8133	JOVB01000000	8	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VII	Turkey, US
14	CVMN20320	JOVX01000000	2	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, US
15	H063900532	FBPQ01000000	18	99/100	99/100	99/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	99/100	99/100	VII	Environmental water, UK
16	SS_2294	FBDW01000000	7	99/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Human, UK
17	H103600372	FBOB01000000	21	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Human, UK
18	H043200357	FAYD01000000	15	99/100	99/100	99/100	99/100	99/100	100/100	99/100	100/100	100/100	100/100	99/100	100/100	100/100	98/100	99/100	VII	Human, UK
19	H121060205	FBOW01000000	9	99/100	99/100	100/100	99/100	99/100	100/100	99/100	100/100	100/100	99/100	99/100	99/100	100/100	98/100	99/100	VII	Human, UK
20	UNOR4451c	FBMH01000000	60	99/100	99/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, UK
21	UNOR532A	FBIJ01000000	32	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, UK
22	H132600169	FBAF01000000	28	99/100	99/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	97/100	99/100	VII	Environmental water, UK
23	SWAN331	FBGR01000000	39	93/100	93/100	98/100	99/100	99/100	96/100	99/100	99/100	99/100	99/100	96/100	91/99	98/100	96/100	93/100	VII	Duck, UK
24	SS_2295	FBDS01000000	8	99/100	99/100	100/100	99/100	100/100	99/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	97/100	99/100	VII	Chicken, UK
25	SWAN195-1	FBGK01000000	46	93/100	93/100	98/100	99/100	99/100	96/100	99/100	99/100	99/100	99/100	96/100	91/99	98/100	96/100	93/100	VII	Duck, UK
26	UNOR7592c	FBMC01000000	19	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Chicken, UK
27	H084040382a	FBJY01000000	22	99/100	99/100	99/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	100/100	99/100	99/100	VII	Human, UK
28	H105280404	FAZS01000000	22	99/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Environmental water, UK
29	H081820599a	FAZL01000000	35	99/100	99/100	100/100	99/100	100/100	99/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	97/100	99/100	VII	Environmental water, UK
30	BCW_5918	MJVY01000000	38	99/100	99/100	100/100	99/100	100/100	99/100	100/100	100/94	100/100	99/100	99/100	100/100	99/100	97/100	99/100	VII	Faeces, US
31	BCW_4454	MJVZ01000000	39	99/100	99/100	100/100	99/100	100/100	99/100	100/100	100/94	100/100	99/100	99/100	100/100	99/100	97/100	99/100	VII	Faeces, US
32	BCW_6450	MJWA01000000	24	99/100	99/100	100/100	99/100	100/100	99/100	100/100	100/94	100/100	99/100	99/100	100/100	99/100	97/100	99/100	VII	Faeces, US
33	BCW_6949	MJZJ01000000	41	99/100	99/100	100/100	99/100	89/99	98/99	99/100	100/100	100/100	99/100	99/100	100/100	100/100	95/100	97/100	VII	Faeces, US
34	BCW_6951	MJZM01000000	9	99/100	99/100	100/100	99/100	100/100	99/100	100/100	100/94	100/100	99/100	99/100	100/100	99/100	97/100	99/100	VII	Faeces, US
35	BCW_6957	MJZR01000000	21	86/97	99/100	100/100	99/100	100/100	100/100	99/100	99/100	99/100	99/100	99/100	100/100	99/100	98/100	99/100	VII	Faeces, US
36	CAM962	BDRY01000000	7	99/100	99/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	99/100	99/100	100/100	100/100	99/100	99/100	VII	Faeces, Japan
37	BCW_4455	MJWE01000000	10	86/97	99/100	100/100	99/100	100/100	100/100	99/100	99/100	99/100	99/100	100/100	100/100	100/100	98/100	99/100	VII	Faeces, US
38	CVM 41971	JAJS01000000	23, 47, 38	99/100	99/100	100/100	99/100		100/100	99/100	99/100	100/100	99/100	99/100	100/100	100/100	98/100	99/100	VII	Human, US

LOS Class VIII

No.	Strain	Accession #	Contig#	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Gene*	Class	Host
1	CVM N29716	ANMS01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
2	OXC6424	CUOD01000000	1	100/100	100/100	99/100	100/100	100/100	99/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	VIII	Faeces, UK
3	OXC6630	CUVQ01000000	1	98/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	VIII	Faeces, UK
4	OXC6568	CUTH01000000	1	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100		VIII	Faeces, UK
5	OXC6343	CULD01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	94/100	VIII	Faeces, UK
6	OXC6577	CUTR01000000	1	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100		VIII	Faeces, UK
7	OXC6576	CUTQ01000000	2	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	99/100	99/100	91/100	VIII	Faeces, UK
8	OXC6400	CUNC01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100		VIII	Faeces, UK
9	CVM N45963	LBEK01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
10	CVM N44406F	LBDY01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Turkey, US
11	CVM N44984F	LBDW01000000	7	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
12	CVM N47608F	LBDS01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
13	CVM N48647F	LBDR01000000	3	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US

#	Strain	Accession	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Avg	Clade	Source
14	CVM N49369F	LBDQ01000000	35,59	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
15	CVM N51183F	LBDN01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
16	CVM N39665	LBEV01000000	10	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
17	CVM N39671	LBEU01000000	8	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
18	CVM N39677	LBET01000000	2,8	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
19	CVM N40944	LBES01000000	6, 15	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
20	CVM N40946	LBER01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
21	CVM N41661	LBEP01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
22	CVM N47960	LBEI01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
23	CVM N51712	LBEB01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
24	CVM N51987	LBEA01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
25	CVM N44396F	LBDZ01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
26	CVM N46596F	LBDT01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
27	CVM N50039F	LBDP01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
28	CVM N51201F	LBDM01000000	3, 4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, US
29	CVM 41917	JAJN01000000	2	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	94/100	VIII	Human, US
30	CVM 41932	JAJW01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, US
31	CVM 41939	JAJX01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, US
32	CVM 41955	JAJY01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, US
33	CVM 41958	JAKA01000000	2	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, US
34	CVM 41965	JAJI01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, US
35	CVM 41976	JAKB01000000	13	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, US
36	CVM 41986	JAJJ01000000	21	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/99	VIII	Human, US
37	CCN178	FBHU01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VII	Poultry Farm environment, UK
38	H131800148	FAYF01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	94/100	VIII	Human, UK
39	CCN397	FBGV01000000	15	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VII	Poultry Farm environment, UK
40	H054900335	FBQT01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
41	P495D	FBLV01000000	8	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VII	Soil, UK
42	H095340114b	FBKC01000000	12	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	VIII	Human, UK
43	H093960099	FBPM01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	VIII	Human, UK
44	P515A	FBAM01000000	17	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
45	CCN289	FBMU01000000	1	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VII	Poultry farm water, UK
46	H051080182	FBOQ01000000	16	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
47	H093580323	FBON01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	VIII	Environmental Water, UK
48	CCN257	FBHS01000000	5	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	94/100	VIII	Poultry farm, faeces, UK
49	H042120298	FBPV01000000	30	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	94/100	VIII	Human, UK
50	H043940500	FBQK01000000	5	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	97/100	VIII	Human, UK
51	H11820480	FBPF01000000	8	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	VIII	Human, UK
52	CCN19	FBJI01000000	20	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Farm Environment, UK
53	SWAN269	FBLP01000000	18	88/100	98/100	93/99	93/100	95/100	95/100	92/100	94/100	95/100	95/100	95/100	96/100	91/100	91/100		VIII	Duck, UK
54	CCN355	FBIR01000000	15	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	94/100	VIII	Poultry farm, faeces, UK
55	H123080386	FBKI01000000	10, 12	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
56	P515B	FBKE01000000	5	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
57	CCN177	FBHT01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VIII	Farm Environment, UK
58	H133020651a	FAYK01000000	12	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
59	H140420240	FBMS01000000	8	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
60	P474B	FBKX01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
61	H095340114a	FAZE01000000	15	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	VIII	Human, UK
62	H083960630	FBNN01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	VIII	Human, UK
63	CCN205	FBHO01000000	19	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Poultry Farm Water, UK
64	P546C	FBLN01000000	5	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	94/100	VIII	Soil, UK
65	SWAN350	FBLU01000000	12	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Duck, UK
66	CCN357	FBJL01000000	14	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	VIII	Poultry Farm faeces, UK
67	H132480140	FBQA01000000	15	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Environmental Water, UK
68	SS_2322	FBFP01000000	6	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	91/100	VIII	Chicken, UK
69	H133020651b	FBLB01000000	13	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
70	P495B	FAXU01000000	11	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
71	H140420134	FBII01000000	18	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	94/100	VIII	Human, UK

No	Strain	Accession	#	1	2	3	4	5	6	7	8	9	10	11	12	13	14		Group	Source
72	H092260570	FBQR01000000	18	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Environmental Water, UK
73	P515D	FAZH01000000	15	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
74	P588D	FBLA01000000	4	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
75	CCN245	FBGW01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VIII	Poultry Farm faeces, UK
76	H134460277	FBPG01000000	11	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	91/100	VIII	Human, UK
77	SWAN195-3	FBLI01000000	4	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	VIII	Duck, UK
78	P494D	FBMB01000000	4	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
79	H090520713	FBOZ01000000	9	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	84/99	VIII	Environmental Water, UK
80	CCN246	FBHD01000000	14	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	VIII	Poultry Farm faeces, UK
81	NCTC12568	FBNI01000000	24	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	VIII	Pig, UK
82	H054000444	FBOO01000000	28	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	90/100	VIII	Environmental Water, UK
83	P494B	FBAK01000000	15	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Soil, UK
84	H102240161	FAYC01000000	13	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	99/100	VIII	Environmental Water, UK
85	H090660740	FBPI01000000	22	100/100		100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	99/100	100/100	VIII	Environmental Water, UK
86	H054000445	FBPB01000000	15	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Environmental Water, UK
87	NCTC12567	FBGX01000000	18	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Human, UK
88	SWAN249	FBGQ01000000	23	88/100	98/100	93/99	93/100	95/100	95/100	92/100	94/100	95/100	95/100	95/100	96/100	91/100	91/100		VIII	Duck, UK
89	H07068142	FBNW01000000	9	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/85	100/100	VIII	Human, UK
90	SS_2329	FBFS01000000	10	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	91/100	VIII	Chicken, UK
91	P495c	FBQW01000000	13	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	99/100	99/100	VIII	Soil, UK
92	CCN20	FBJP01000000	6	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Farm Environment, UK
93	UNCIC2	FBIW01000000	11	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Chicken, UK
94	VA6	MPIQ01000000	2	88/100	98/100	93/99	93/100	96/100	95/100	92/`100	94/100	95/100	95/100	90/97	89/98	91/100	91/100		VIII	Raw water, Sweden
95	OXC6296	CUJE01000000	1	99/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	91/100	VIII	Faeces, UK
96	OXC6597	CUUK01000000	1	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	91/100	VIII	Faeces, UK
97	RC105	CYQJ01000000	15, 18	100/100	100/100		99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	VIII	Supermarket, UK
98	RC148	CYQO01000000	4	100/100	100/100	100/100	99/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	99/100	100/100	VIII	Supermarket, UK

Appendix-III

Presentation of Abstracts

(Associated to unpublished manuscripts)

❖ **Hameed, A**., Machado, L., Woodacre, A., Marsden, G. Induction of inflammasome-dependent signalling in the human monocytic cell line THP-1 by *Campylobacter* lipooligosaccharides. A poster presented to: ASM Microbe 2019, Moscone Center, California, US, 20 – 24 June 2019.

❖ **Hameed, A**., Machado, L., Woodacre, A., Marsden, G. Interleukin-1β induction in the human monocytic cell line THP-1 by lipooligosaccharides of *Campylobacter* species. A poster presented to: 5th Midlands Molecular Biology Meeting (M4), University of Warwick, UK, 13 -14 Sep 2018.

❖ **Hameed, A**., Machado, L., Woodacre, A., Marsden, G. Analysis of the Genetic Diversity of the *Campylobacter* Lipooligosaccharide Biosynthesis Locus using *In Silico* and Molecular Typing. A poster presented to: 47th World Congress on Microbiology, London, UK, 10 -11 Sep 2018.

Unpublished Manuscripts

❖ **Hameed A**. Human immunity against *Campylobacter* infection. **Immune Network** (A review article; accepted; under process).

❖ **Hameed A**, Machado LR, Woodacre A, Marsden GL. An updated classification system and review of the lipooligosaccharide biosynthesis gene locus in *Campylobacter jejuni*. (A review article; submitted on 30th of October 2019).

❖ **Hameed A**, Woodacre A, Allen S, Machado LR, Marsden GL. Analysis of the genetic diversity of the *Campylobacter* lipooligosaccharide biosynthesis locus using *in silico* and molecular typing (A research article; in preparation for submission).

❖ **Hameed A**, Woodacre A, Allen S, Marsden GL, Machado LR. Induction of NLRP-3 inflammasome-dependent signalling in the human monocytic cell line THP-1 by *Campylobacter* lipooligosaccharides (A research article; in preparation for submission).