Comparative analysis of New Zealand *Campylobacter* isolates using MLST, PFGE and *flaA* PCR RFLP genotyping

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ABSTRACT

Campylobacter jejuni and *Campylobacter coli* are the most commonly identified sources of campylobacteriosis in New Zealand, yet little is known about the distribution of genotypes within the respective population structures. Using multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and *flaA* genotyping, the current study identified the distribution of genotypes within New Zealand *C. jejuni* and *C. coli* isolates from an outbreak situation, as well as isolates present in the ESR *Campylobacter* collection. Although the most commonly identified MLST genotypes were similar to international genotypes, a number of internationally rare, or unique to New Zealand genotypes were observed.

One rare dominant genotype, ST-474, arising from a point source outbreak, was found to cause a large proportion of human campylobacteriosis cases in New Zealand. A unique cluster of New Zealand genotypes were isolated only from river water, identifying a potentially water adapted *C. jejuni* strain. Frequent homologous recombination and horizontal gene transfer events were identified within the seven housekeeping genes characterised in the New Zealand sample and the MLST *C. jejuni*/*C. coli* database. The identified genetic instability within the current study questions the legitimacy of bacterial species boundaries, especially when examining closely related species such as *C. jejuni* and *C. coli.*

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ABBREVIATIONS USED

AFLP	Amplified Fragment Length Polymorphism
aspA	Aspartase gene
Bp	Base pair
CBA	Colombia Blood Agar
CC	Clonal Complex
CHEF	Contour-clamped Homogeneous Electric Field Electrophoresis
CL	Clonal Lineage
CSC	Christchurch Science Centre
DDH	DNA-DNA Hybridisation
DHB	District Health Board
DLV	Double Locus Variant
d_N/d_S	Ratio of non-synonymous to synonymous substitutions
DNA	Deoxyribonucleic Acid
ERL	Enteric Reference Laboratory
ESR	Environmental, Science & Research
EtBr	Ethidium Bromide
EtOH	Ethanol
FAGE	Field Alteration Gel Electrophoresis
flaA	Flagellin A gene
FlaA	Flagellin subunit A
***flaA* SVR**	flagellin A Short Variable Region
FlaB	Flagellin subunit B
GBS	Guillain-Barre Syndrome
gDNA	Genomic DNA
glnA	glutamine synthetase gene
gltA	citrate synthase gene
glyA	serine hydroxy methyl transferase gene
H2S TSI	Hydrogen Sulphide Triple Sugar Iron
HGT	Horizontal Gene Transfer
HR	Homologous Recombination
I_A	Index of Association
Kb	Kilobase
KSC	Kenepuru Science Centre
mCCDA	Modified Charcoal-Cefoperazone-Deoxycholate Agar
ME	Minimum Evolution
MLEE	Multi-locus Enzyme Electrophoresis
MLST	Multi-locus Sequence Typing
MRP	Macro Restriction Profile

MSc	Masters of Science
MW	Molecular Weight
NaCl	Sodium Chloride
Nt	Nucleotide
O	O – antigen
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
Pgm	phospho glucomutase gene
ReA	Reactive Arthritis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
SDW	Sterile Distilled Water
SLV	Single Locus Variant
Sm	*SmaI* MRP
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
tetO	tetracycline resistance gene
Tkt	transketolase gene
U/A	Unassigned
uncA	ATP synthase alpha subunit gene
UV	Ultra violet
V	Version
$\mathbf{V_e}$	Expected Variance
$\mathbf{V_o}$	Observed Variance

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CHAPTER ONE: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

In 2006 there were 14,119 notifiable cases of bacterial gastroenteritis in New Zealand, the overwhelming majority of cases caused by *C. jejuni* (12). The true incidence of campylobacteriosis may be higher than these figures indicate, one study suggesting that for every reported case there are 38 unreported cases (143). The reported rates of campylobacteriosis have been increasing in New Zealand since 1980 when the disease became notifiable (122). New Zealand has two to three times the reported rate of campylobacteriosis cases compared to any other developed country (128). Devane *et al* (2005) suggested the high rate of incidence of campylobacteriosis in New Zealand may be due to better public health intelligence reporting and the unknown ecology of *C. jejuni* so the transmission can not be effectively controlled (46).

The annual economic cost of campylobacteriosis to New Zealand in 2000 was estimated to be $61.7 million (192), which can only have increased with the current rate of incidence. The authors acknowledged that food borne gastroenteritis caused by *C. jejuni* and other enteric bacteria were self limiting and did not generate high health care costs. Most of the costs incurred when a person has bacterial gastroenteritis are non-medical costs i.e. loss of working hours (192). It is important for both public health intelligence and reduction of economic costs of campylobacteriosis that sources of *Campylobacter* are identified and active steps taken to eliminate or reduce the possibility of infection (128, 192).

Although *Campylobacter* infections are notifiable in New Zealand, isolates are not characterised further than the genus level due to the volume of reported cases (personal communication, C. Pope). If species identification does occur, phenotypic subtyping tests such as the hippurate hydrolysis (154) are employed, rarely are *Campylobacter* isolates characterised using genotypic subtyping schemes such as multiplex identification PCR (personal communication, C. Pope).

The majority of studies characterising *Campylobacter* in New Zealand used indirect methods of DNA analysis such as pulsed-field gel electrophoresis (PFGE) and Penner serotyping (38, 66, 174, 185, 186). Although PFGE and Penner serotyping are considered the gold standard methods for characterisation of outbreak isolates (105, 194), these methods have limited applications for describing *Campylobacter* populations. With the development of direct methods to characterise the genetic diversity of bacterial isolates such as multi-locus sequence typing (MLST), it is now possible to identify genotypes associated with different host reservoirs (37, 138) or vectors (166).

1.2 Literature Review

1.2 *Campylobacter*

A member of the *Campylobacteraceae* family, *Campylobacter* is closely related to the *Helicobacter* and *Arcobacter* genera (168, 218). *Campylobacter* are commensal bacteria of avian and certain mammalian gastrointestinal systems, and pathogens of the human oral cavity and intestine (236). Escherich is believed to have characterised the first enteropathogen of the *Campylobacter* genus in 1884, when he isolated and visualised a spiral shaped bacteria from human diarrhoeic faecal samples (218). Unable to grow and therefore characterise the spiral shaped bacteria, *Campylobacter* was not characterised until Smith *et al* (199) and Jones *et al* (116) described a *Vibrio*-like bacteria associated with dysentery in cattle. The *Vibrio*-like bacteria was characterised and named *Vibrio jejuni* and shown to be the causative agent of "Winter dysentery" in cattle (116). The first significant outbreak of campylobacteriosis occurred in 1938 (218), the aetiological agent was identified as 'bovine-*Vibrio*' (218). In 1944 Doyle *et al* identified another *Vibrio*-like bacteria (*Vibrio coli*) from dysenteric pigs, another possible agent of the 1938 outbreak (54).

1.2.1 Taxonomy of *Campylobacter*

Based on DNA hybridisation studies, sequencing and protein analysis, biochemical characteristics and growth conditions, the *Campylobacter* genus is recognised as containing 18 different species and six sub-species (76, 106, 131, 168, 218, 238). Classification of the species within the *Campylobacter* genus by the "gold standard" (204) of DNA-DNA hybridisation has been augmented by

AFLP fingerprinting (57), MLEE using 11 housekeeping enzymes (144), microarray analysis using SNPs in the *hsp60* gene (238), MLST using seven housekeeping genes (136) and sequencing of the 16S rRNA (76, 106), 23S rRNA (106), *cpn60* (106) and *rpoB* genes (125). Several studies have noted that due to high levels of rRNA sequence conservation between bacterial species, classification using sequencing of the 16S rRNA gene alone may lead to misclassification of isolates (85, 112). For this reason more than one molecular technique should be employed to accurately classify *Campylobacter* species, especially with highly related species such as *C. jejuni* and *C. coli*.

1.2.2 Physiological characteristics of *C. jejuni* and *C. coli*

C. jejuni and C. coli are small (approximately 1.5 – 6.0 μm by 0.2 – 0.5 μm), Gram-negative, uni or bi-polar flagellate, non-spore forming, curved rods (106, 124). Originally named and placed in the *Vibrio* genus due to their spiral or "seagull" shape (54, 116) *Campylobacter* are easily identifiable by microscopic examination. Under stress the morphology of *Campylobacter* may alter to a more cocci or "doughnut" shape, hampering identification by microscopy (124).

Table 1.1: Physiological characteristics and growth conditions of *C. jejuni* and *C. coli*

Species	Temperature	1	2	3	4	5	6	7	8	9
C. jejuni	37C-42C	✓	✓	✓	✓	✓	✓	×	✓	×
C. coli	37C-42C	×	✓	✓	✓	✓	✓	×	✓	×

1 = Hippurate hydrolysis, 2 = Catalase positive, 3 = Nitrate reduction, 4 = H_2S TSI, 5 = Urease negative, 6 = Indoxyl acetate positive, 7 = growth in 1% NaCl, 8 = growth on McConkey agar, 9 = growth in 1% glycine; Adapted from Koneman *et al* 1997 (124)

C. jejuni and C. coli are relatively biochemically inert and have fastidious growth conditions (Table 1.1) (124). Physiologically *C. jejuni* and *C. coli* are highly similar, the only major phenotypic difference being hippurate hydrolysis, although approximately 5% of *C. jejuni* are hippurate negative and may be misclassified if this is the only method of classification (154).

1.2.3 Pathogenesis of *C. jejuni* and *C. coli*

The disease mechanisms of *C. jejuni* and *C. coli* are identical, for this reason both species will be referred to as *Campylobacter*. Campylobacteriosis is considered a gastrointestinal disease in the first instance, on rare occasions the bacteria may escape the gastrointestinal tract and cause septicaemia and/or wound infections (197). Campylobacteriosis, often considered a mild, non-serious, self-resolving illness, serious sequelae occur rarely, usually secondary to the original gastrointestinal disease often but not exclusively, as a result of the patients own immune system overreacting (120, 197).

1.2.4 Clinical symptoms and sequelae

Campylobacteriosis can be initiated by consumption of as few as 500 *Campylobacter* cells (179). *Campylobacter* infections are often characterised by an extended incubation period of up to eight days, where the patient may be unaware of infection (197). The range of symptoms experienced by the patient can include: abdominal cramping, diarrhoea (secretory or inflammatory), fever, headaches, and general myalgia, symptoms may be experienced for up to seven days (7, 197, 236). The diarrhoea experienced by the patient may be watery, bloody and contain white blood cells dependant on the type of diarrhoea (120,

197). Medical intervention is unusual, unless the infection is severe or the patient immunocompromised (124), treatment involves erythromycin and rehydration of the patient (6). Rarely, treatment of campylobacteriosis may be compromised if the isolate is resistant to erythromycin, other antibiotics may be required. If untreated the diarrhoea resolves, although the patient can excrete *Campylobacter* cells for up to a month (120, 197).

The range of clinical symptoms correlate with both the patient having a competent immune system and whether the patient has had campylobacteriosis previously (197). The more immunocompromised a patient is the greater likelihood of severe clinical symptoms occurring, conversely if the patient has had campylobacteriosis previously the clinical symptoms will be less severe (197). The experience of patients in developed countries and those in developing countries can be quite different (165).

Table 1.2: Sequelae associated with *Campylobacter* infections

Sequelae	**Reference**
Bacteraemia	Walder 1982 (220), Ezpeleta 1992 (68), Jackson 1997 (109), Schuster 1997 (190)
*Coli*tis	Willoughby 1979 (229), Kollitz 1981 (123), Guandalini 1983 (88), Siegal 2005 (195)
Guillain-Barre syndrome	Issacson 1998 (108), Nachamkin 2001 (157), Tsang 2002 (215), Tam 2003 (58), Howitz 2007 (99)
Hemolytic-uremic syndrome	Sillero 1999 (196), Fumarola 1985 (79), Chamovitz (33)
Fisher syndrome	Nachamikin 2000 (156), Servan 1995 (193)
Myocarditis	Pena 2007 (170), Cox 2001 (41), Cunningham 2003 (43)
Pancreatitis	Ezpeleta 1992 (68), Coton 2000 (40)
Reactive arthritis	Hannu 2002 (94), Eastmond 1981 (59), Pope 2007 (175)
Toxic Megacolon	Larvol 1994 (129), Jackson 1999 (110), Schneider 2000 (188)

Sequelae (Table 1.2) experienced by patients after the initial *Campylobacter* infections are rare; although rare, the sequelae are serious and can be fatal (197). The sequelae can be divided into two groups: complications associated with the gastroenteritis and those complications arising secondary to the gastroenteritis. The later group is more frequently reported in the literature than the former. GBS has been reported as 1 in 1000 – 3000 cases of campylobacteriosis (84). ReA is thought to be around 4 in every 100, 000 cases of campylobacteriosis (95). Myocarditis associated with a *Campylobacter* infection has been reported, the majority of cases resulting in fatalities (30, 41, 43, 170). However, direct causality between campylobacteriosis and myocarditis has not been identified to date.

1.2.5 Host range of *C. jejuni* and *C. coli*

Campylobacter have been isolated from a wide variety of avian and mammalian animal reservoirs (Table 1.3). Primarily, *Campylobacter* has been identified in poultry such as chickens (29) and ducks (185), ruminant animals such as cattle (37, 228) and sheep (37, 206), it is also found in pigs (113).

Table 1.3: Animal reservoirs and vectors for *Campylobacter*

Source			**Reference***
Animal Reservoirs			
C. jejuni	***C. coli***		
✓		Badger	Petersen 2001 (172)
✓	✓	Cats	Baker 1999 (17)
✓	✓	Cattle	Colles 2003 (37), Milnes 2007 (152), Savill 2003 (185)
✓	✓	Chickens	Bull 2006 (29), Petersen 2001 (172), Bates 2004 (21)
✓		Common buzzard	Petersen 2001 (172)
✓		Deer	Lillehaug 2005
✓	✓	Dogs	Fox 1988, Baker 1999 (17)
✓		Ducks	Lillehaug 2005, Baker 2002 (18)
✓		Fox	Petersen 2001 (172)
✓		Gorillas	Nizeyi 2001 (163)

✓		Hare	Petersen 2001 (172)
✓	✓	House Crows	Ganapathy 2007(81)
✓		Magpie	Petersen 2001 (172)
✓		Mice	Adhikari 2002 (4)
✓		Pheasant	Petersen 2001 (172)
✓	✓	Pigs	Gibbons 2006 , Jensen 2006 (113), Workman 2005
✓		Pigeons	Lillehaug 2005 , Petersen 2001 (172)
✓		Protozoa	Snelling 2006 (200)
✓		Raven	Petersen 2001 (172)
✓		Rook	Petersen 2001 (172)
✓		Seagulls	Petersen 2001 (172), Quessy 1992 (177), Broman 2002 (27)
✓		Seal	Petersen 2001 (172)
✓	✓	Sheep	Colles 2003 (37), Savill 2003 (185), Stanley 2003 (206)
✓		Sparrow Hawks	Petersen 2001 (172)
✓		Sparrows	Adhikari 2002 (4), Craven 2000 (42)
✓		Squirrel	Peterson 2001 (172)
✓	✓	Turkey	Colles 2003 (37), Borck 2005 (23)
✓		Vervet Monkeys	Workman 2005 (232)
Insect Vectors			
C. jejuni	***C. coli***		
✓		Houseflies	Adhikari 2002 (4), Nicols 2005 (141, 213)
✓		Darkling Beetles	Bates 2004 (21)
✓		Litter Beetles	Skov 2004 (198)
Environmental Vectors			
C. jejuni	***C. coli***		
✓		Soil	Brandl 2003 (25) , Jensen 2006 (113)
✓	✓	Sand	Studer 1999
✓	✓	Water	Ogden 2007 (166), Hanninen 2003 (77), Abulreesh 2006 (2)
Plants Vectors			
C. jejuni	***C. coli***		
✓		Spinach and Radish	Brandl 2004 (25)
Food product Vectors			
C. jejuni	***C. coli***		
✓	✓	Beef /Veal	Zhao 2001 (239), Wong 2006 (231)
✓	✓	Pork	Zhao 2001 (239), Wong 2006 (231)
✓	✓	Poultry meat	Saito 2005 (184), Praakle-Amin 2006 (176), Atanssova 1999 , Wong 2006 (231), Zhao 2001(239), Workman 2005 (232)
✓		Tuna Salad	Roels 1998 (180)
✓		Butter	Zhao 2000 (239)
✓	✓	Sheep Liver	Cornelius 2005 (26)
✓		Milk	Philips 1995 (109), Hudson 1999 (63) Peterson 2003 (173)
✓		Vegetables	Doyle 1986 (55), Chai 2007 (32)
✓	✓	Lamb/mutton meat	Wong 2006 (231)
	✓	Eggs	Adesiyun 2005 (3)

* These references are a sample of the many studies that have been undertaken examining host reservoirs and vectors for *C. coli* and *C. jejuni.*

1.2.6 Vector range of *Campylobacter*

1.2.6.1 Aquatic vectors

C. jejuni and *C. coli* have been isolated from fresh water systems including rivers (98, 186, 219), lakes (98) and streams (219). The bacteria can survive in fresh water for up to 160 days at 4°C (181), suggesting that water needs to be considered a transmission route of *C. jejuni* and *C. coli.* One New Zealand study identified surface water, reticulated drinking water, roof water and shallow ground water as reservoirs of *Campylobacter* (186). *C. coli* and *C. jejuni* have been identified as the aetiological agents of large outbreaks of campylobacteriosis in France and Canada, due to contamination of water systems and failure of the chlorination systems (35, 80). Although *Campylobacter* appears to survive in water, growth and colonisation of an aquatic environment by the bacteria has not been proven. Uptake and carriage of *Campylobacter* cells by protozoa has also been identified as a possible transmission route and unusual vector (200).

1.2.6.2 Food product vectors

Undercooked chicken meat or chicken products are believed to be the primary vehicle for most human campylobacteriosis infections (39), although many other poultry and meat sources have been identified as sources of Campylobacter (Table 1.3). Any cooking utensils not properly disinfected and cleaned may also become vehicles for transmitting raw meat associated *Campylobacter* cells. Improperly prepared animal offal, especially the livers of ruminant animals have also been observed to contain high numbers of *C. jejuni* cells (38). The observed prevalence in these organs may reflect a potential transmission route for *Campylobacter*.

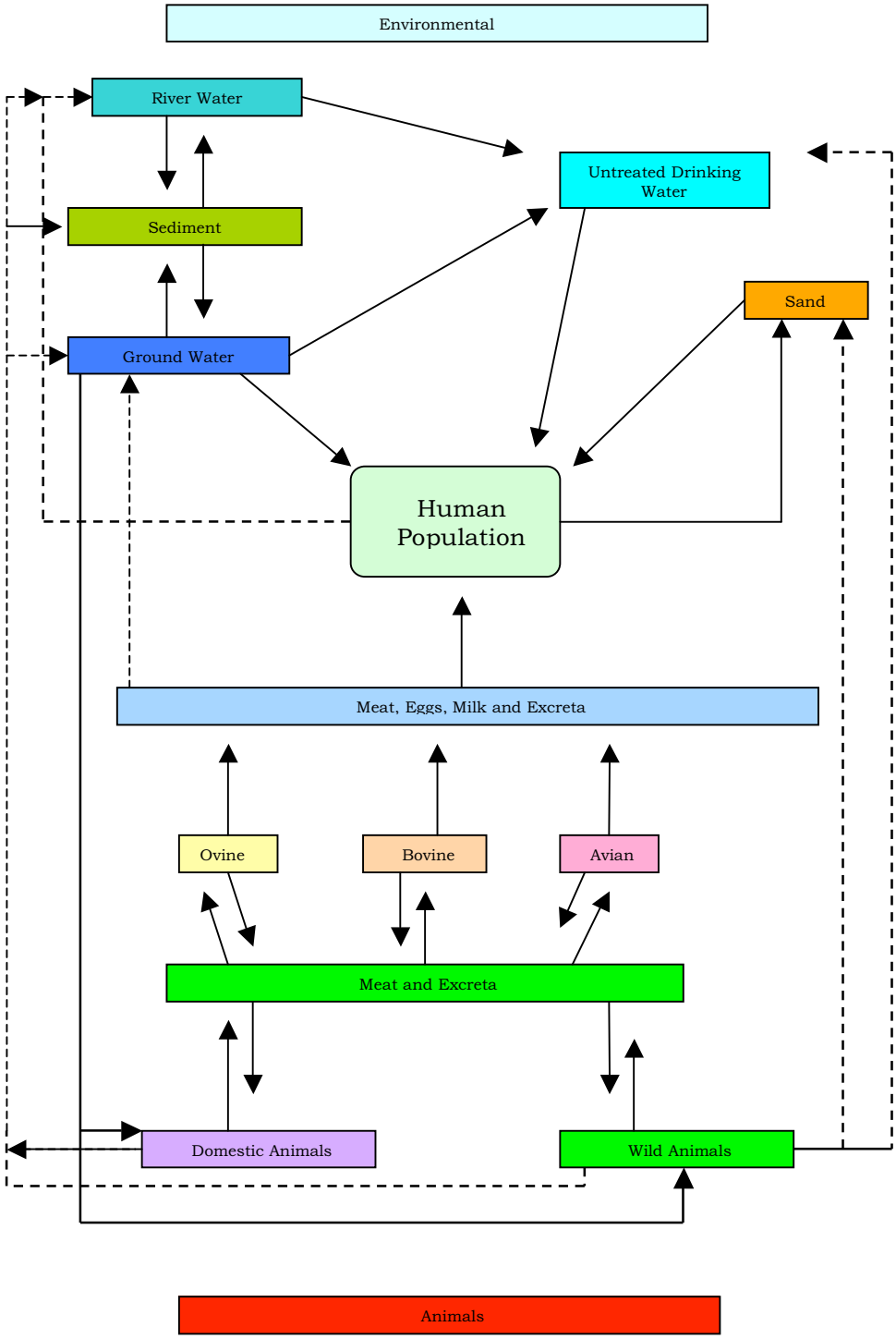
Regular identification and characterisation of *C. coli* in pig faeces (113, 137, 152), suggests that pork and pork-associated products would be a possible vehicle for *C. coli* cells. This observation is not borne out in the published literature possibly due to the increased vigilance of cooking associated with these products. Ruminant animal faeces have also been observed to carry high levels of *C. coli*, which can survive for days after defecation (161). Beef and lamb product (post processing compared to poultry products) often carry lower levels of the bacteria due to correct handling procedures during the slaughter process (134).

C. jejuni has also been isolated from seafood such as crabs (178) and seafood products such as tuna salad (180), most likely to have resulted as a by product of contamination as opposed to the seafood being a reservoir of the bacteria (111). Contamination of raw vegetables has been identified as a route for transmission of *Campylobacter* (32, 64). The internal uptake of *C. jejuni* in spinach and radish plants and survival in this novel environment has also been observed (25, 97), this mechanism in vegetables may be the same route observed in the 2006 *E. coli* O157:H7 outbreak in the United States of America, which was associated with washed spinach (10).

1.2.6.3 Insect vectors for *Campylobacter*

Insects, including the housefly (4), darkling beetles (21), litter beetles (198) and the lesser mealworm (208) are probable vehicles for the transmission of *C. jejuni* (Table 1.3). Darkling beetles and houseflies are believed to a significant role in the introduction and spread of *C. jejuni* in broiler houses and farms (4, 21).

Figure 1.1: Transmission routes of *C. jejuni* and *C. coli*



The transmission routes of both *C. jejuni* and *C. coli* are outlined in Figure 1.1. The routes *Campylobacter* cells move between animals, humans and the environment are identified. Adapted from Baker *et al* 2002 (18).

1.2.8 Epidemiology of *C. jejuni* and *C. coli*

1.2.8.1 Incidence of campylobacteriosis in the developed countries

Campylobacteriosis outbreaks in developed countries are infrequent; most cases appear to be sporadic (77). Reported outbreaks have been associated with water, chicken, raw milk, contaminated vegetables and cooked foods (Table 1.4). The consumption of contaminated water was the most frequently identified cause of large campylobacteriosis outbreaks; chicken and raw milk were the next most commonly identified sources (111).

Table 1.4: Major sources of campylobacteriosis outbreaks in developed countries

Source	Country	Reference
Water	Finland, Austria, Sweden, France, Wales	O'Rielly 2007 (164), Gallay 2006 (80), Kuusi 2005 (127), Hanninen 2003 (93)
Chicken	Japan, Australia, Denmark, United Kingdom, Austria, Wales	Yoda 2006 (235), Black 2006 (22), Mazick 2006 (140), Pearson 2000 (169), Allerberger 2003 (5), Evans 1998 (63), Deming 1987 (45)
Milk	UK, USA, Finland, Wales, Hungary	Stuart 1997 (209), Wright 1983 (233), Schildt 2006 (187), Peterson 2003 (173), Evans 1996 (65), Djuretic 1997 (52)

Contamination of raw and cooked foods with *Campylobacter* cells was identified in five case reports; the case reports identified tuna salad (180), sweet potatoes (230), lettuce (13), cucumber (117) and custard (114) as vectors for *Campylobacter*.

1.2.8.2 Incidence of campylobacteriosis in New Zealand

C. jejuni and C. coli are ubiquitous and endemic in New Zealand and are the most frequent causes of campylobacteriosis in New Zealand (18). The rate of reported cases of campylobacteriosis has steadily increased since infection with *Campylobacter* became a notifiable disease in 1980, under section A of the 1956

Health Act; hospitalisations due to campylobacteriosis have also increased in the past 10 years (19). One estimate suggests that the current rate of reported campylobacteriosis cases, 341 per 100,000 is under reporting the actual rate of incidence by 7.6 (20). The current rates of reported cases of campylobacteriosis in New Zealand are two to three times that of any other developed country (Table 1.5). Alternative theories have been suggested for the difference in notification rates between New Zealand and other developed countries including the efficacy of the infectious disease reporting system in New Zealand (18) and increased poultry consumption (19).

Table 1.5: Rate of campylobacteriosis in developed countries compared to New Zealand

Country	Rate	Reference
New Zealand	341.0	Anonymous 2006 (11)
Australia	116.5	Micken 2007 (146)
England & Wales	78.4	Louis 2005 (133)
The Netherlands	37.1	Van Hees 2007 (217)
USA	15.0	CDC 2007 (14)

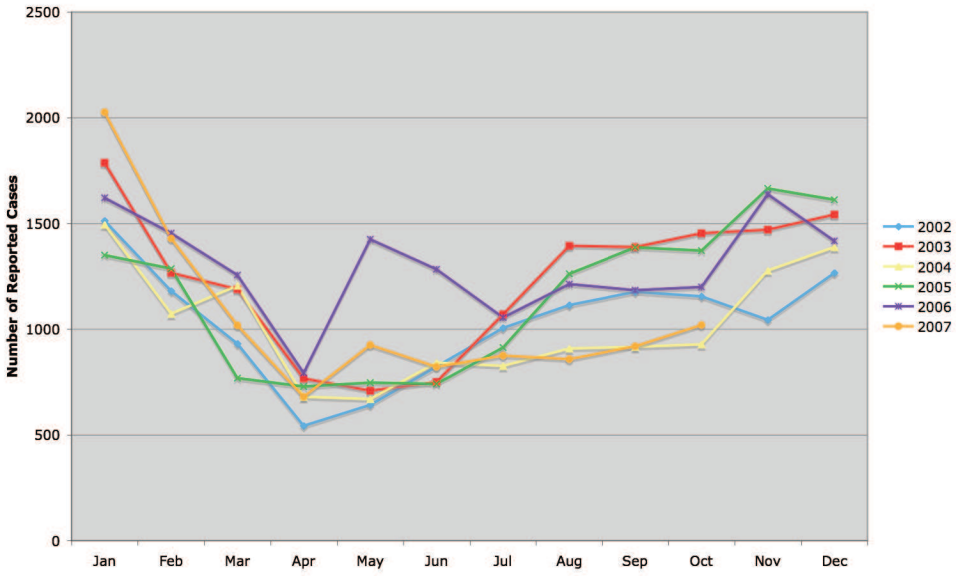
The seasonality of campylobacteriosis in New Zealand is similar to that of other developed countries, with increased incidence in autumn and spring, unlike other countries the number of campylobacterosis cases reported in New Zealand for January (summer) has seen a marked increase since 2005 (Figure 1.2) (77).

1.2.8.3 Outbreaks of campylobacteriosis in New Zealand

There are five New Zealand campylobacteriosis outbreaks reported over the past 20 years in the published literature; the outbreaks were associated with pre-cooked

sausages, raw milk, water and poultry (26, 60, 86, 207). The number of reports presented as case studies is not representative of actual *Campylobacter* outbreaks occurring in recent history. Evidence for a more accurate representation of the status of outbreaks occurring of *Campylobacter* occurring in New Zealand can be found at the NZPHSR website. In 2006, there were 38 outbreaks comprised of 260 cases spread over wide geographic locations, yet there were few reported cases of campylobacteriosis in New Zealand (12). The level of both reported cases and cases published in the literature suggests that there are either a large number of sporadic cases or there is inadequate investigation of outbreaks in New Zealand (142).

Figure 1.2: Incidence of campylobacteriosis in New Zealand 2002 – 2007



Notification rates taken from the monthly health reports for 2002 – 2007 found at http://www.surv.esr.cri.nz/surveillance/NZPHSR.php

The incidence of campylobacteriosis occurring in New Zealand from 2002 to 2007 is shown monthly. Note the spike in reported cases in May 2006 and 2007.

1.3 Molecular subtyping of *Campylobacter*

The role of molecular subtyping in the epidemiological setting is to provide reliable, reproducible and efficient evidence of bacterial isolate relationships (74, 105). Depending on the subtyping method, both the phylogenetic relationships and transmission routes of the bacteria may be identified. To this end subtyping methods for use in the laboratory need to be quick, reliable, reproducible, discriminatory and cost effective (162). This section describes more common subtyping methods that are used to characterise *Campylobacter*: Penner serotyping, multi-locus enzyme electrophoresis (MLEE), multi-locus sequence typing (MLST), *flaA* polymerase chain reaction – restriction fragment length polymorphism (*flaA* PCR RFLP) and pulsed-field gel electrophoresis (PFGE). Although serotyping, MLEE and genotypic methods are described, they are not in day-to-day use in most laboratories in New Zealand. Most *Campylobacter* isolates are characterised only to the genus level, with growth conditions and microscopic examination only if there is a large outbreak or the *Campylobacter* is derived from an unusual source and further identification is required (personal communication, C. Pope, C. Nicol).

1.3.1 Phenotypic methods

Phenotypic methods are based on biochemical attributes and/or protein production of the bacteria. Phenotypic methods used to subtype *Campylobacter* include hippurate hydrolysis, biochemical tests and resistotyping (124). Resistotyping is the typing of *Campylobacter* isolates by the use of antibiotic discs, either *Campylobacter* cells are either resistant or sensitive to Cephalothin and Nalidixic acid (124). Although these methods are valid for speciating *Campylobacter*

isolates, they fail to provide any information on the relationships between the isolates.

1.3.1.1 Serotyping

Two serotyping methods were developed for serotyping of *Campylobacter* isolates, the Lior and Penner serotyping methods. The Lior serotyping method was developed in 1981 based on slide agglutination (132), but has since fallen out of favour as a typing method. The Penner serotyping method was described in 1980 (171) and has become recognised as the "gold standard" for serotyping *Campylobacter* (141, 194). Penner serotyping has been used in many epidemiological studies, and when compared to other methods of serotyping it was found to require less time than other methods (141).

Up to 36% *C. jejuni* isolates can be untypable (150), while others cross react with multiple antigens for example O: 23 and O: 36, due to similarities in the core oligosaccharide structures (155). Despite the drawbacks, Penner Serotyping has been accepted as a tool for characterising *Campylobacter* when used in conjunction with other methods such as MLST or PFGE (49, 67, 142).

1.3.1.2 Multi-locus enzyme electrophoresis

Milkman first described the use of Multi Locus Enzyme Electrophoresis (MLEE) to characterize *E. coli* isolates (147). MLEE characterises bacterial isolates via the distance housekeeping gene products travel on a starch gel (62). Differences in electrophoretic mobility of gene products define alleles at each loci, allowing an allelic profile of the isolate to be established (62). MLEE has been shown to discriminate between isolates within the same species (138) and between different

species of the same genus (144). However, MLEE results were not transportable between laboratories and the method relied on skilled operators to get consistent results (62). MLEE has fallen out of favour and is not used as it has been superceded by MLST, a faster and more efficient method than MLEE (62).

1.3.2 Genotyping methods

Genotyping methods described in this section, MLST, PFGE and *flaA* PCR RFLP, overcame some of the difficulties observed with the use of phenotypic methods, in particular reproducibility and discrimination between closely related isolates.

1.3.2.1 Multi-locus sequence typing

MLST, initially designed for *Neiserria*, has been used for the characterisation of many bacterial populations including multiple *Campylobacter* species (50, 149, 153, 212, 237). First suggested by Maiden *et al* 1998, is a subtyping method that assumes that housekeeping genes are not under selective pressure (136). Genetic variation observed in these genes should be less than genes associated with selective pressures such as the human immune system (136). Multiple unlinked housekeeping genes were chosen for the subtyping scheme with the expectation that if recombination occurred at one locus then the increased number of gene loci would lessen the likelihood incorrect assumptions about bacterial relationships would be made (136). MLST overcame the inherent difficulties associated with MLEE, providing accurate, reproducible and importantly portable evidence of bacterial relationships (136). The discriminatory ability of MLST is less than PFGE therefore MLST is a tool to describe bacterial populations, rather than to use as a singular subtyping tool in an outbreak investigations (69).

1.3.2.2 MLST method for *Campylobacter*

In 2001 Dingle *et al* proposed a MLST system for *C. jejuni* (50) which has since been updated to include *C. coli* with both species using the same gene fragments. The gene loci suggested for *C. jejuni* were: *aspA* (aspartase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxy methyl transferase), *tkt* (transketolase), *pgm* (phospho glucomutase) and *uncA* (ATP synthase alpha subunit) (50, 100). The unlinked gene loci chosen were distributed throughout the *C. jejuni* chromosome and provided a suitable level of variation within the alleles (50).

Dingle *et al* (2001) based their primer design on the previously published *C. jejuni* genome (50). The primers used amplified a 450-550 bp gene fragment dependant on the gene loci (50). The gene fragments from each of the seven loci are sequenced and the derived alleles given a number based on when they were identified (50). The seven gene loci form a seven-integer sequence (ST), which is also given a number based on when it was identified. The first allele identified at the *aspA* loci would be *aspA*1 and the first sequence type (ST) would be ST-1 and so on (50). Dingle *et al* (2001) then used the BURST algorithm (115) to identify clonal complexes (CC) (50); isolates were identified as belonging to a clonal complex if each isolate had at least four out of the seven alleles in common. The sequence data Dingle *et al* (2001) generated was analysed using the Burst program, which presented the data in an UPMGA phylogenetic tree (50). Their results suggested that *C. jejuni* had a weakly clonal population structure (50). The MLST scheme has since been extended to characterise a wider range of

Campylobacter species including *C. lari*, *C. upsaliensis*, *C. fetus* and *C. helviticus* (149).

1.3.2.3 MLST data analysis

1.3.2.3.1 Burst and eBurst

The BURST algorithm was designed by Feil *et al* (2001) to analyse MLST data (101). BURST clusters isolates in to CC based on the number of gene loci shared in each ST, allowing for identification of the most probable founder ST (101). The algorithm then draws conclusions about the relatedness of individual STs, describing the ST as a single locus variant (SLV), double locus variant (DLV) or triple locus variant (TLV) in relation to the most probable founder ST (101). The main drawback of the BURST algorithm was that it graphically represented the relationships between isolates on a dendrogram using UPMGA as way to identify clusters of related bacterial isolates (72). Dendrograms allowed no information as to how isolates that were clustered into CCs, may have evolved (72). For this reason a new algorithm was devised in 2004 entitled eBURST (72). eBURST clusters genetically similar (based on STs) isolates together in 'nonoverlapping' groups (72). The algorithm then parsimoniously decides how they may have evolved and identifies the most probable founder of the group (72). Similar to BURST, eBURST identifies SLVs, DLVs and TLVs. eBurst is able to gauge the relative age of a CC by how many SLV's it has and if there are any subgroups related to it (203).

1.3.2.3.2 START

The START program designed by Jolley *et al* (2001) uses the BURST program to analyse MLST data (115). The program analyses the sequence data for phylogenetic relationships between STs, allelic frequency and variation, recombination, linkage disequilibrium and calculates the ratio of non-synonymous to synonymous mutations in each allele.

1.3.2.4 *flaA* PCR RFLP

The *Campylobacter* flagellum has been identified as a virulence factor which enables movement towards, and colonisation of, the mucous layer (236). The flagellum composed of two subunits, FlaA and FlaB, is coded for by two, 1.7kb flanking genes (*flaA* and *flaB*) (89). The two flagellin genes exhibit approximately 93% homology dependant on the strain (89). The *flaA* gene has been observed to be necessary for motility and colonisation (227). The role of *flaB* has been observed to act as a reservoir for recombination (8), and to be up regulated under abnormal environmental conditions where decreased motility is required (223).

Nachamkin *et al* (1993) identified both conserved and variable regions within the *C. jejuni flaA* gene locus (158); the variable and conserved regions seen within the locus allowed a typing scheme for *C. jejuni* and *C. coli* to be developed (226). The subtyping method was based on the amplification of the *flaA* gene and subsequent digestion of the PCR product by a restriction enzyme, *DdeI* (158). The restriction profile produced from one isolate could then be compared to other isolates sourced from different patients, host animals or environmental sources

(158). Although *flaA* PCR-RFLP is reproducible, and most isolates are typeable, the inherent genetic instability observed at this locus suggests that *flaA* PCR-RFLP should be used as a confirmatory technique as opposed to a stand-alone typing technique (96, 224).

1.3.2.5 PFGE and Macrorestriction analysis

1.3.2.5.1 FAGE/PFGE

Field alteration gel electrophoresis (FAGE) or pulsed-field gel electrophoresis (PFGE) (191) was developed in 1983 by Schwartz *et al* to overcome the inability of conventional agarose gel electrophoresis to separate and resolve of large DNA molecules (<30 kb) (216). Prior to the development of PFGE researchers found larger DNA molecules hard to visualise on a gel (34). Unable to travel in a straight path, due to the size of the DNA, the DNA would elongate and compact allowing it to move through the gaps in the gel matrix (61, 214); this however caused the DNA to travel in snake like paths through the gel in an effort to find pores of the correct size (214). PFGE overcame this by pulsing the electrical current from different points around the gel at given time intervals, allowing the previously cut DNA sample to migrate in a straight line (34, 214).

The most common PFGE method used is contour-clamped homogeneous electric field electrophoresis (CHEF) (34, 61). CHEF was developed by Chu *et al* (1986) to resolve large DNA molecules (34). The CHEF-PFGE current field is arranged in a hexagonal shape, with electrodes delivering pulses from an angle of 120˚ (61).The current pulsed at 120˚ angles pushes larger DNA molecules through the gel matrix in a uniform manner, allowing better resolution (61) than other electric fields generated using with 60-90˚ angles (34).

PFGE is generally recognised as the gold standard for tracking outbreaks of pathogenic bacteria, as it is highly discriminatory (162). PFGE has been shown by many studies to be an effective tool in molecular epidemiology (for example: (1, 74, 127, 183)). PFGE is a labour intensive method of indirect genotyping the whole genome of bacterial isolates (73). The main drawback of PFGE is that it requires skilled operators to get consistent, reproducible results. In the early 1990s inter-laboratory PFGE gels were often be unable to be compared due to different methodologies being used between laboratories (211); which hampered molecular epidemiological investigations into the spread of pathogenic bacteria between countries (211).

Following an *E. coli* outbreak in 1993 CDC researchers recognised the need for an effective way of comparing results between laboratories (211); the Pulsenet standard PFGE method was created to allow inter-laboratory molecular epidemiological investigations, particularly concerning food borne bacterial diseases (211).

1.4 Aims of the thesis

The aims of my MSc thesis were initially to investigate the distribution of MLST genotypes in the New Zealand *Campylobacter* population as represented by those isolates stored in the ESR Ltd (Kenepuru Science Centre), and to investigate whether PFGE macro-restriction profiles (MRPs) could be used to identify MLST STs or CCs. As the project developed further opportunities to investigate New Zealand *Campylobacter* isolates became available and therefore, were incorporated into the study.

The aims of the current thesis were:

1. Describe the distribution of MLST STs and CCs in the current New Zealand *C. jejuni* and *C. coli* library stored at ESR Ltd (KSC).

2. Investigate whether PFGE MRPs could be used to identify MLST STs and CCs

3. Investigate the validity of MLST as a regular subtyping tool in an outbreak situation compared to other subtyping methods such as PFGE, Penner serotyping and *flaA* PCR RFLP.

4. Identify the effect that HR and HGT events might have on subtyping schemes for *Campylobacter*.

CHAPTER 2: METHODS AND MATERIALS

2.1 Bacteria and growth conditions

2.1.1 *Campylobacter* strains

Three hundred and sixty eight *C. jejuni* and 93 *C. coli* strains were characterised in this study. Two hundred and sixty one *C. jejuni* and 35 *C. coli* isolates were selected from previously characterised *SmaI* MRPs. Selected MRPs represented both unique and common MRPs, and included isolates derived from animal reservoirs, water vectors and human clinical samples. *C. coli* (n = 53) isolates held at ESR Ltd Kenepuru and Massey University had not been characterised by PFGE. A representative outbreak survey sample of one hundred and seven *C. jejuni* and five *C. coli* isolates was selected by medical laboratories and sent to ESR Ltd Kenepuru for characterisation. *Campylobacter* isolates characterised in the current study were supplied by ESR Ltd (Kenepuru & Christchurch Science Centres), Massey University and New Zealand Medical Laboratories.

2.1.2 Growth media and conditions

Campylobacter isolates were grown on 5% Columbia blood agar plates (CBA) (Fort Richard Laboratories Ltd). Contaminated and mixed *Campylobacter* samples were restreaked for single colonies and reselected. If a *Campylobacter* sample was too contaminated to be extracted via purity streaking, the sample was grown on mCCDA plates. *Campylobacter* isolates grown on mCCDA plates were restreaked on CBA plates and single colonies selected. All *Campylobacter* isolates were grown in microaerophilic conditions for 48 hours at either 37°C or

43□C, depending on methods used to further characterise the isolates.

2.2 Materials

2.2.1 Enzymes

The following enzymes used in this study, *DdeI* (Invitrogen, Auckland, New Zealand), *KpnI* (Invitrogen), *SmaI* (Roche, Auckland, New Zealand) and *XbaI* (Roche) were obtained from Invitrogen and Roche.

2.2.2 Primers

Primers used in this study were synthesised by Invitrogen. *C. jejuni* and *C. coli* MLST PCR and sequence primers (Tables 2.1, 2.2 and 2.3) were downloaded from the pubMLST web site http://pubmlst.org/campylobacter/mlst-info/c*jejuni*/c*jejuni*-info.shtml. *C. coli* MLST sequencing primers used were the same as the PCR primers. Primers (Table 2.4) used in *flaA* PCR were previously published by Nachamkin *et al* (160). *Campylobacter* multiplex identification primers (Table 2.5) were as published by Wang *et al* (221).

Table 2.1: MLST *C. jejuni* primers

Gene	Primer
aspA	Reverse: A2 AAGCGCAATATCAGCCACTC Forward: A1 AAAGCTGCAGCTATGGC
glnA	Reverse: A2 TTGGACGAGCTTCTACTGGC Forward: A1 TAGGAACTTGGCATCATATTACC
gltA	Reverse: A2 CCAAATAAAGTTGTCTTGGACGG Forward: A1 GGGCTTGACTTCTACAGCTACTTG
glyA	Reverse: A2 AAACCTCTGGCAGTAAGGGC Forward: A1 GAGTTAGAGCGTCAATGTGAAGG
pgm	Reverse: A2 AAGAGCTTAATATCTCTGGCTTCTAG Forward: A1 TTGGAACTGATGGAGTTCG
tkt	Reverse: A4 CATAGCGTGTTCTCTGATACC Forward: A1 TTTAAGTGCTGATATGGTGC
uncA	Reverse: A3 AAAGCTGATGAGATCACTTC Forward: A2 GCTAAGCGGAGAATAAGGTGG

Table 2.2: MLST *C. jejuni* sequence primers

Gene	Primer
aspA	S3: CCAACTGCAAGATGCTGTACC S6: TTCATTTGCGGTAATACCATC
glnA	S3: CATGCAATCAATGAAGAAAC S6: TTCCATAAGCTCATATGAAC
gltA	S3: CTTATATTGATGGAGAAAATGG S6: CCAAAGCGCACCAATACCTG
glyA	S3: AGCTAATCAAGGTGTTTATGCGG S4: AGGTGATTATCCGTTCCATCGC
pgm	S5: GGTTTTAGATGTGGCTCATG S2: TCCAGAATAGCGAAATAAGG
tkt	S5: GCTTAGCAGATATTTTAAGTG S6: AAGCCTGCTTGTTCTTTGGC
uncA	S3: AAAGTACAGTGGCACAAGTGG S4: TGCCTCATCTAAATCACTAGC

Table 2.3: MLST *C. coli* primers

Gene	Primer
aspA	S1: CAACTTCAAGATGCAGTACC S2: ATCTGCTAAAGTATGCATTGC
glnA	S1: TTCATGGATGGCAACCTATTG S2: GCTTTGGCATAAAAGTTGCAG
gltA	S1: GATGTAGTGCATCTTTTACTC S2: AAGCGCTCCAATACCTGCTG
glyA	S1: TCAAGGCGTTTATGCTGCAC S2: CCATCACTTACAAGCTTATAC
pgm	S1: TTATAAGGTAGCTCCGACTG S2: GTTCCGAATAGCGAAATAACAC
tkt	S1: AGGCTTGTGTTTTCAGGCGG S2: TGACTTCCTTCAAGCTCTCC
uncA	S1: AAGCACAGTGGCTCAAGTTG S2: CTACTTGCCTCATCCAATCAC

Table 2.4: *flaA* primers

Forward Primer	Nucleotides 1- 26	5’ - GGATTTCGTATTAACACAAATGGTGC-3’
Reverse Primer	Nucleotides 1705 - 1728	5'-CTGTAGTAATCTTAAAACATTTTG-3'

Table 2.5: *Campylobacter* multiplex identification primers

Primer	Primer Sequence
C. jejuni forward	5-ACTTCTTTATTGCTTGCTGC-3’
C. jejuni reverse	5-GCCACAACAAGTAAAGAAGC-3’
C. coli forward	5-GTAAAACCAAAGCTTATCGTG-3’
C. coli reverse	5-TCCAGCAATGTGTGCAATG-3’
23S rRNA forward	5-TATACCGGTAAGGAGTGCTGGAG-3’
23S rRNA reverse	5-ATCAATTAACCTTCGAGCACCG-3’

2.3 Methods

2.3.1 Genomic DNA extraction

Genomic DNA (gDNA) was isolated from freshly grown *Campylobacter* cultures by the boiling method or a Hi Pure PCR template purification kit (Roche).

2.3.1.1 Boiling method

A loop of two or three colonies from a fresh culture were resuspended in 500 µL of SDW in a sterile Eppendorf, vortexed briefly and boiled for ten minutes. The solution was centrifuged for two minutes at 3000 rpm using a Heraeus Fresco centrifuge (BioStrategies), and the supernatant removed to a sterile Eppendorf and stored at -20°C.

2.3.1.2 Hi-Pure template purification kit method

A loopful of fresh *Campylobacter* culture was added to 200 µL of PBS, centrifuged for five minutes at 3000 rpm, the supernatant removed and the pellet resuspended in 200 µL of PBS. Five µL of lysozyme (10mg/mL in Tris-HCl, pH 8.0) was added to the cell suspension and incubated at 37°C for 15 minutes. The suspension was removed from incubation, 200 µL binding buffer and 40 µL reconstituted proteinase K were added, mixed and further incubated at 70°C for ten minutes.

Isopropanol (100 µL) was added to the solution, mixed and removed to the upper reservoir of a filter tube. The filter tube was placed inside a collection tube and spun at 8000 rpm for one minute. The filter tube was placed in a new collection tube and 500µL inhibitor removal buffer added and centrifuged at 8000 rpm for one minute.

The filter tube was placed in a new collection tube and 500 uL wash buffer added, spun at 8000 rpm for one minute and the procedure repeated once. The collection tube and liquid was removed and the filter tube placed in a new collection tube to spun at 13000 rpm for ten seconds. The filter tube was removed to a new sterile Eppendorf, 200 µL of pre-warmed (70°C) elution buffer added, and centrifuged at 8000 rpm for one minute. The gDNA was stored at -20°C.

2.3.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in gel concentrations of 1.0% and 2.0% agarose in 0.5x TBE buffer. Gels were run with either, a 1 kb or 100 bp ladder, dependant on the size of the expected products. Gels were stained with EtBr for 20 minutes, destained with SDW, and photographed using UV light. To check if MLST and *flaA* PCR amplifications PCR products were present, the products were run on a 2.0% and 1.0% gels respectively. Digested *flaA* PCR products were run on a 2.0% gel.

2.3.3 *flaA* PCR RFLP

2.3.3.1 *flaA* PCR amplification

The *flaA* gene (1.7 kb) was amplified for each of the *Campylobacter* isolates as described by Nachamkin *et al* (1996), with modifications (160). The *flaA* gene was amplified (to a total volume of 50 µL) using 25 µL AmpliTAQ Gold mastermix (Applied Biosystems, Auckland, New Zealand), 5 pmoles of each primer, 2 µL template DNA and 21 µL SDW. PCR amplification were performed in 96 well plates using a ABI 9700 thermal cycler (Applied Biosystems,

Auckland, New Zealand) using a cycle of 94°C for 15 min, then 35 cycles of 94°C for 15 seconds, 55ºC for 45 seconds, 72°C for 1 minute 45 seconds, held at 72ºC for 5 minutes and then held at 10°C indefinitely.

2.3.3.2 PCR product digestion

If a product was present 8 µL of PCR product was digested in a solution of 1.5µL of *DdeI* Buffer, 0.5 µL (5 units) of *DdeI* (Invitrogen) and 5µL RNase/DNase free SDW (a total of 15 µL per digestion). The solution was incubated at 36°C for three hours.

2.3.4 MLST

2.3.4.1 MLST PCR amplification

MLST PCR amplifications were carried out in a 96 well plate containing: 1 µL of primer (5pmol), 2 µL bacterial DNA, 12.5µL of AmpliTAQ Gold solution (Applied Biosystems, Auckland, New Zealand) and 9.5 µL SDW per well. MLST PCR amplification was performed using an ABI 9700 thermal cycler. Amplification solutions underwent initial heat activation 94ºC for 15 minutes, then 35 cycles of: denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for one minute and 30 seconds. The samples were held at 72ºC for seven minutes, then 12ºC until removed from the thermal cycler.

MLST PCR amplicons were purified using a PEG solution (Appendix 1). To each well 26 µL of PEG solution was added, incubated at 37ºC for 15 minutes and spun for 30 minutes at 2500 rpm. The plate was then inverted, spun for two minutes at 190 rpm using an Eppendorf 5180 plate centrifuge (Global). Each well received

150 µL of cold ethanol and the plate was centrifuged for 10 minutes at 2500 rpm. The plate removed, inverted and spun for two minutes at 190 rpm. After removal from the centrifuge 12 µL of SDW was added to each well and left for at least 10 minutes to redissolve the purified PCR products, and stored until sequenced.

2.3.4.2 MLST sequence reaction

Sequence reactions were carried out in a 96 well plate containing: 1.0 µL primer (3.2 µM), 2.0 µL MLST PCR product and 7.0 µL of Master mix (Big Dye Ready Reaction Mix 0.5 µL, 5X Sequencing Buffer 2.0 µL and 4.5 µL of SDW) per well. Sequence reactions were 96.0 ºC for 3 minutes, 25 cycles of 96.0 ºC for 15 seconds, 50.0 ºC for 15 seconds and 60.0 ºC for 4 minutes, with a final extension of 60ºC for 10 seconds, sequence products were then held indefinitely at 12ºC.

The sequence reaction product was purified using a mastermix solution of 1 mL 96% EtOH, 40µL Sodium Acetate (3M) pH 5.2, and 200 µL SDW. To each well 62 µL of the mastermix was added and mixed. Sequence plates were left at room temperature for 15 minutes and centrifuged at 2500 rpm for 30 minutes. Following centrifugation the plate was inverted and spun for a further two minutes at 1650 rpm to remove the last of the EtOH/Sodium Acetate mix. To each well 150 µL of 70% EtOH was added and left to stand for one minute. The plate was inverted, spun for two minutes at 190 rpm to remove residual EtOH, and allowed to air dry for two minutes. Sequence reaction product pellets were resuspended in 10 µL of Hi-Di Formamide and boiled using the following programme on the ABI 9700 thermal cycler: Rapid Ramp to 96ºC for five minutes and Rapid Ramp to 15ºC. The plate was placed in a cold block prior to products being sequenced on

an ABI 3130XL automated DNA sequencer (Applied Biosystems).

2.3.4.3 MLST sequence analysis

DNA sequence data for each *Campylobacter* isolate was assembled and analysed using the BioNumerics and Chromas Pro programs (http://www.applied-maths.com/bionumerics/bionumerics.html)(http://www.technelysium.com.au/chromas.html). Once assembled, the sequences were compared to the pubMLST *Campylobacter jejuni* and *Campylobacter coli* database using the BLAST programme, available at (http://pubmlst.org/campylobacter/) and MLST allelic numbers assigned. Sequence types were generated by comparison against the *Campylobacter* database. Novel alleles and sequence types were submitted for allele and ST designation as appropriate. The data was further analyzed using START (http://pubmlst.org) (115), eBurst (http://eburst.mlst.net/) (72, 203) and dnaSP (182) programmes. Individual MLST ST loci were concatenated and aligned using CLUSTALW found in the Mega v 3.1 program (126).

2.3.5 PFGE

2.3.5.1 Plug preparation

Fresh *Campylobacter* cells were harvested, resuspended in 2 mL of PBS (pH 7.0) to a turbidity of between 0.35 and 0.45. Into a sterile Eppendorf, 20 µL of proteinase K (20mg/mL), 400 µL of cell suspension and 400 µL melted 1% SeaKem Gold agarose / 10% SDS was added, gently mixed and transferred to a plug mold. The plugs were allowed to solidify at 5°C for five minutes, transferred to bijoux tubes containing 5 mL cell lysis buffer (50mM EDTA, pH 8.0 and 1.0% Sarcosyl) and 25 µL proteinase K (20mg/mL), and incubated in a 56°C water bath for 30 minutes. The cell lysis buffer was removed by rinsing with SDW, the plugs were washed

with two SDW washes and three TE Buffer washes with incubation at 50ºC for at least 15 minutes between washes. Plugs were stored at 5ºC until required for digestion.

2.3.5.2 Plug digestion

Each 2mm slice of plug was incubated in a sterile Eppendorf with a solution of 1x enzyme buffer for 15 minutes at room temperature. The buffer solution was removed and the enzyme master mix (Table 2.6) added. Plugs were then incubated at 25°C (*SmaI*) or 37°C (*KpnI*) for two and six hours respectively. *S. enterica* serotype Branderup H9812 (NZRM4085) was used as a control, the control plugs were incubated at 25°C with *XbaI* for two hours.

Table 2.6: Restriction enzyme solution for plug digestion

Restriction Enzyme	SDW (µL)	Buffer (µL)	Enzyme (µL)	Total Volume (µL)
KpnI	176	20	4	200
SmaI	179	20	1	200
XbaI	175	20	5	200

2.3.5.3 Gel electrophoresis

Gel plug slices were removed from the enzyme digest solution and inserted into preformed wells in a 1% SeaKem Gold agarose gel in 0.5x TE buffer, and covered with melted 1% SeaKem Gold agarose gel to prevent movement of the gel plug slices out of the gel. The gel was transferred to the CHEF-Mapper chamber and run in 0.5x TBE buffer for 18 hours under standard CDC Pulsenet conditions (Table 2.7).

Table 2.7: Electrophoresis conditions

Restriction Enzymes	Conditions
SmaI	Auto Algorithm 50kb – low MW 400kb – high MW Run time: 18 hours Initial Switch time: 6.76s Final Switch time: 35.38s Temperature: 14ºC
KpnI	Two State Gradient 6.0V Run time: 18 hours Included Angle: -120 Initial switch time: 5.2s Final switch time: 42.3s Temperature: 14ºC

2.3.5.4 Visualisation of MRFPs

Gels were removed from the CHEF-Mapper chamber, stained with EtBr for up to 20 minutes, and destained with SDW. Photography was under UV light.

2.3.6 Restriction profile analysis

Both *flaA* PCR RFLP profiles and PFGE MRPs were photographed and then analysed using BioNumerics v 4.6 program using the Dice coefficient (95% similarity, 0.5% optimization and 1.5% band tolerance). Photographs and related isolate information were entered, band positions were identified and profiles adjusted using the controls *S. enterica* Branderup *XbaI* pattern and the 100 bp ladder. The restriction profiles were then compared to previously completed restricted profiles using the Dice coefficient and the UPMGA algorithm allowing phylogenetic trees to be constructed.

2.3.7 Further analysis

Further analysis of the MLST, PFGE and *flaA* PCR RFLP genotyping data was

carried out using the Wallace coefficient (31) and the Simpson's index of diversity (104).

2.3.7.1 Simpson's Diversity Index

The Simpson's Index of diversity as proposed by Hunter and Gaston (104), was used to identify the levels of discrimination between isolates in this study. The following equation was used to measure the level of discrimination between methods:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

In the equation N represents the total number of different isolates in the sample, S is the total number of different genotypes present in sample, and n_j are the number of isolates that are the jth genotype (104).

2.3.7.2 The Wallace Coefficient

The use of the Wallace coefficient allows the researcher to decide how much additional information about the samples is provided by another molecular technique (31). Comparisons between the typing methods can then be made and the usefulness of a new additional technique examined (31). The coefficient was calculated using a publicly available script for BioNumerics at http://biomath.itqb.unl.pt/ClusterComp .

CHAPTER 3: DISTRIBUTION OF MLST GENOTYPES IN NEW ZEALAND *C. JEJUNI* ISOLATES

3.1 Introduction

Characterisation of *Campylobacter* isolates has traditionally been carried out using phenotypic (Penner serotyping, hippurate hydrolysis) and indirect examination of the individual *Campylobacter* genomes (PFGE) (226). Penner serotyping and PFGE are generally considered the gold standards for characterising outbreaks, however the high discriminatory power of PFGE and conversely the low discriminatory power of Penner serotyping do not make these subtyping methods useful for the description of bacterial population structures, nor the association of pathogenic bacteria to reservoir animals (63, 100, 161, 204).

Prior to the development of MLST, the characterisation of bacterial populations was limited to comparisons of DNA band based methods eg PFGE, flaA PCR-RFLP, AFLP and RAPD (162),. MLST allows comparison of sequence data for accurate, unambigous proof of bacterial relationships and population structure (136). Following the development of a MLST scheme for *C. jejuni* in 2001, studies have used MLST to identify and characterise both temporally and geographically diverse *C. jejuni* populations (37, 56, 142, 201), as well as to describe the association between reservoirs, vectors and human disease (48, 121, 142, 148, 201). Previous to the current study the majority of studies characterising New Zealand *C. jejuni* isolates have been limited to indirect subtyping methods of DNA analysis such as PFGE and phenotypic subtyping methods such as Penner serotyping (4, 38, 67, 83). One study used *flaA* SVR

typing to describe the genotypes of *Campylobacter* isolates found in darkling beetles (21).

The current study is the first use of both direct and indirect methods of DNA analysis collectively to describe the range of genotypes present in the New Zealand *C. jejuni* population. To investigate the distribution of MLST genotypes observed in New Zealand 261 *C. jejuni* isolates previously characterised by PFGE (*SmaI*) and Penner serotyping by ESR laboratory staff, were obtained from the *Campylobacter* collection at the ESR Ltd Kenepuru Science Centre and characterised using the MLST method of Dingle *et al* (50). The *C. jejuni* isolates were obtained from humans, a range of animals, and environmental sources providing a snapshot of the variety of genotypes in New Zealand *C. jejuni*. Understanding the distribution of *C. jejuni* genotypes present in New Zealand is critical if we are to identify the major sources of infection, and implement and monitor intervention strategies to reduce the significant burden of disease posed by this pathogen.

3.2 Methods

C. jejuni isolates previously subtyped using PFGE (*SmaI*) and Penner Serotyping were characterised using MLST (n = 261) and *flaA* RFLP as described in Chapter 2. Isolates were chosen from MRFPs, representing unique and common MRFPs from animal [poultry (n = 72), cattle (n = 37), sheep (n = 47) and pigs (n = 6)], human (n = 61), and environmental [ground water (n = 2), river water (n = 36), sediment (n = 1)] sources. Different sites for *C. jejuni* animal reservoirs were also sampled, including carcass meat, diced/minced meat, offal and faeces. Isolates

were widely distributed throughout the North and South Islands of New Zealand. MLST sequence data was analysed using Chromas Pro and compared to the pubMLST data base (http://pubmlst.org/perl/mlstdbnet/mlstdbnet.pl?file=pub-cj_profiles.xml) using the BLAST program. Individual alleles for each gene locus were assigned numbers and seven integer sequence type (ST) profiles were determined for each *C. jejuni* isolate. STs were clustered together if four or more loci were in common with another ST. The data was further analysed using the eBURST V.3 (http://eburst.mlst.net/) and START (http://pubmlst.org/software/analysis/start/) programs. The eburst program was used to identified the proposed founder of a clonal complex and single locus, and double locus variants of the founding genotype. The d_N/d_S ratio was also calculated for each gene loci using the START program.

3.3 Results

3.3.1 MLST

3.3.1.1 MLST allele distribution

A total of 370 alleles were identified among the 261 isolates including 32 (8.6%) novel alleles. Six alleles appeared frequently in the data set, *aspA*2 (n = 114), *glnA*1 (n = 82), *gltA*10 (n = 41), *glyA*4 (n = 76), *pgm*2 (n = 87), *tkt*1 (n = 67) and *uncA*5 (n = 93) (Figure 3.3). The *aspA* and *pgm* loci accounted for 48% of the novel alleles. PH526 was omitted from MLST analysis due to a large deletion in the *aspA* allele (see Chapter 4).

The *uncA* locus contained the greatest number of variable sites (n = 84); if the *uncA*17 and *uncA*38 genotypes are removed from the data set, then the *pgm* locus

had the greatest number of variable sites (n = 57) and alleles (n = 33) (Table 3.1). The *aspA* locus had the lowest number of variable sites (n = 21) and alleles (n = 19), although the locus had the highest number of new alleles compared to the rest of the loci.

Table 3.1: Allelic diversity in *C. jejuni*

Gene loci	Sequence length (bp)	*C. jejuni* isolates (*n* = 261)		
		No. of alleles* (Novel alleles)	No. of variable sites	d_N/d_S
aspA	477	19 (8)	21	0.0642
glnA	477	28 (5)	37	0.0773
gltA	402	21 (2)	24	0.0222
glyA	507	26 (4)	44	0.0645
pgm	498	33 (7)	57	0.038
tkt	459	21 (2)	34	0.0311
uncA	489	20 (3)	84	0.0114

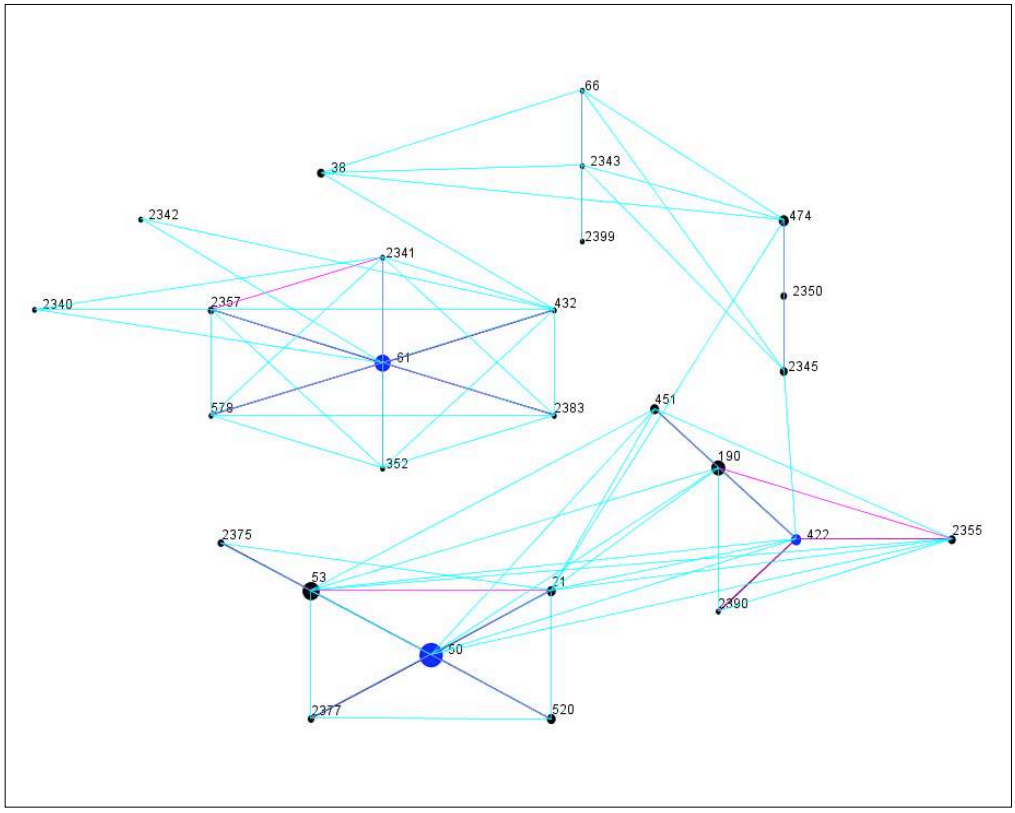
The majority (n = 19, 67%) of novel alleles were found to have one or two point mutations. The exception was a cluster of isolates derived from environmental river water samples with novel genotypes, each novel allele had between 3 and 10 differences when compared to the closest match in the previously described alleles. The d_N/d_S ratios were calculated for each gene locus characterised, with all ratios observed under 1, indicating all allele loci were under negative selection. The *glnA* locus was observed to have the highest ratio of non-synonymous to synonymous mutations (0.0773), and *uncA* had the smallest (0.0114) (Table 3.1).

3.3.1.2 CC and ST distribution

In total 16 clonal complexes (CCs) and 84 sequence types (STs) were identified. One isolate (PH526) contained a large deletion (74 bp) at the *aspA* locus and could not be assigned an allelic number, and was excluded from the MLST data analysis. Seven CCs (CC ST-21, CC ST-45, CC ST-42, ST-48, CC ST-61, CC

ST-257 and CC ST-1034) accounted for 68% of all *C. jejuni* isolates characterised, the majority of STs contained within these CCs had previously been described and are available at http://pubmlst.org/campylobacter. A group of STs (n = 28) in the data set were not able to be assigned to a CC. A total of 47 STs were novel, 25 of the 47 contained new alleles, the remainder were novel combinations of previously characterised alleles.

Figure 3.1: eBURST CL1 diagram

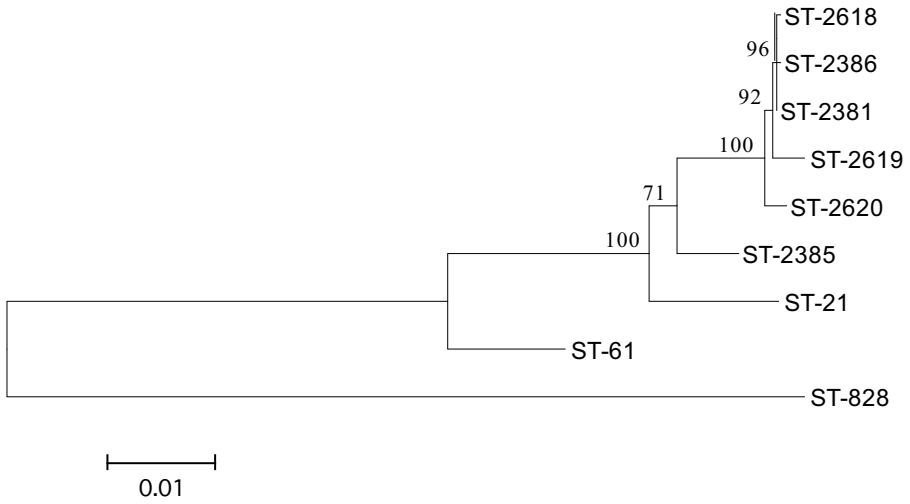


eBurst diagram of CL1 isolates belonging to CC ST-21, CC ST-48, CC ST-61 and CC ST-206. DLVs are indicated by blue lines and SLVs as pink lines. The size of the black or blue circle indicates the number of isolates within that ST.

Analysis of the MLST data using the eBURST program identified 12 clonal lineage groups (CL), representing 95% (n = 248) of all isolates in the data set. CL groups were defined by eBURST according to the number of single locus variants

(SLVs) and/or double locus variants (DLVs) each ST had and those STs with SLVs and DLVs in common were grouped together, those with neither SLVs nor DLVs were classed as singleton STs and not attributed to a CL. Each of the larger CLs contained one or more CCs. The largest CL (CL1) accounted for 44% (n = 116) of all isolates characterised and was dominated by three CCs (CC ST-21, CC ST-48 and CC ST-61) (Figure 3.1). ST-61 was identified by eBURST as the founder of the CL1 group, with the greatest number of SLVs and DLVs.

Figure 3.2: Minimum evolution tree of the new RW cluster

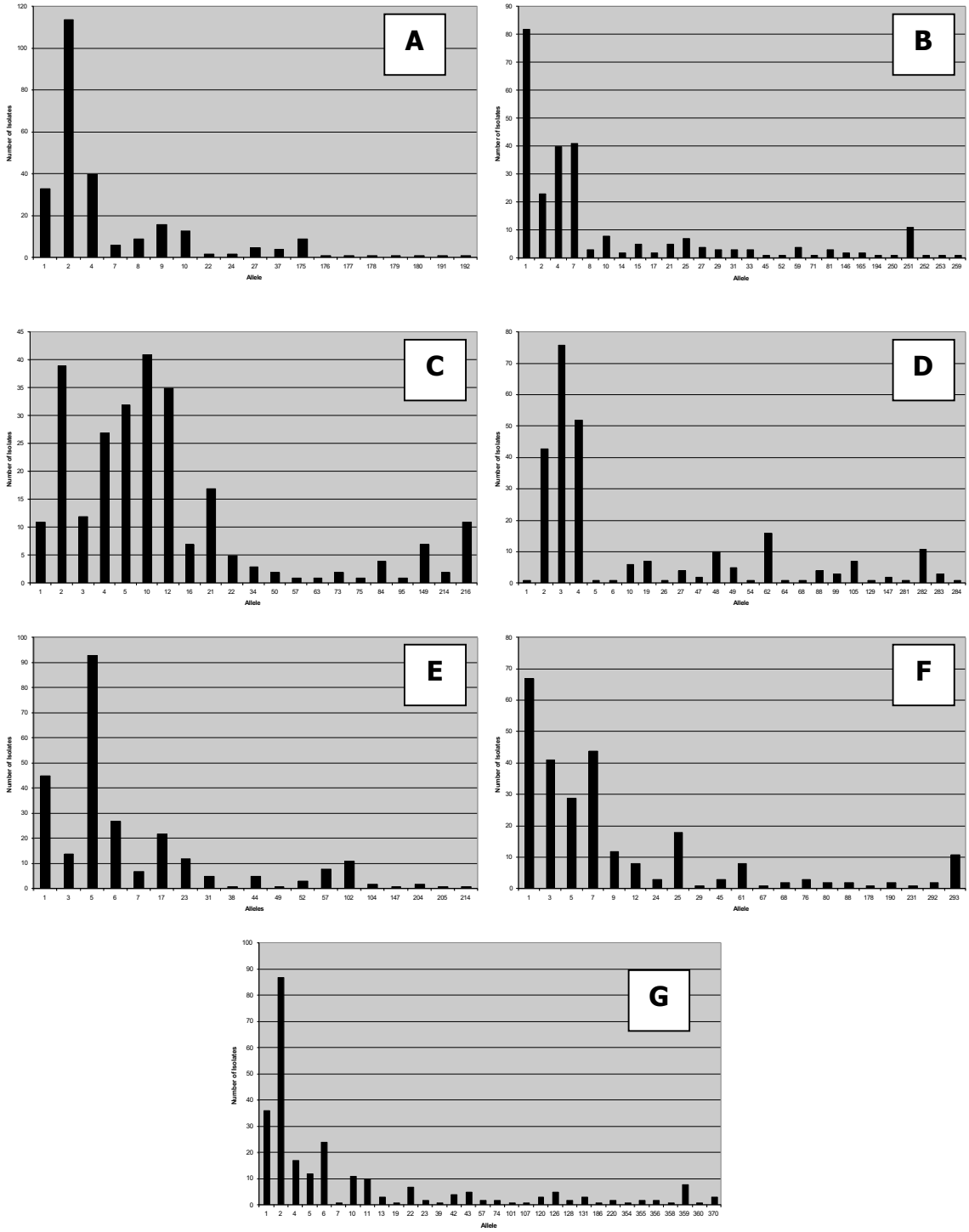


A ME tree (using the Kimura 2-parameter model) was constructed using the concatenated allele sequences of the new RW cluster. The STs clearly group with the *C. jejuni* associated STs (ST-21 and ST-61) and not the *C. coli* associated ST-828.

The other major CLs comprised of more than 3 STs in each CL group were CL2, CL3 and CL4, and the majority of isolates in the current data set were contained within the first four CL groups. The remaining eight CL groups contained 44 isolates assigned to CC ST-52, CC ST-257, CC ST-354, CC ST-403, CC ST-677, CC ST-1034, CC ST-1275 and one CL composed of two STs unassigned to a CC. The singleton group of STs were predominantly composed of isolates unassigned

to a CC, six of which were previously undescribed. The exception to this was CC ST-42 which had no SLVs or DLVs but had a comparatively large number of isolates (n = 12).

Figure 3.3: Allelic diversity



Allelic frequencies were calculated in START and graphed. A – G corresponds to *aspA, glnA, gltA, glyA, pgm, tkt* and *uncA* loci respectively.

3.3.1.3 Allele specificity

The majority of alleles identified in the dataset were found in all sources sampled, for example *aspA*4 and *uncA*5 were found in all sources (Figure 3.3 A & G). At each gene locus unique animal reservoir/environmental source allele distributions were observed, for example *aspA*175 (river water), *aspA*37 (poultry), and *aspA*180 (human) (Table 3.2).

Table 3.2: Unique allele/source associations in the dataset

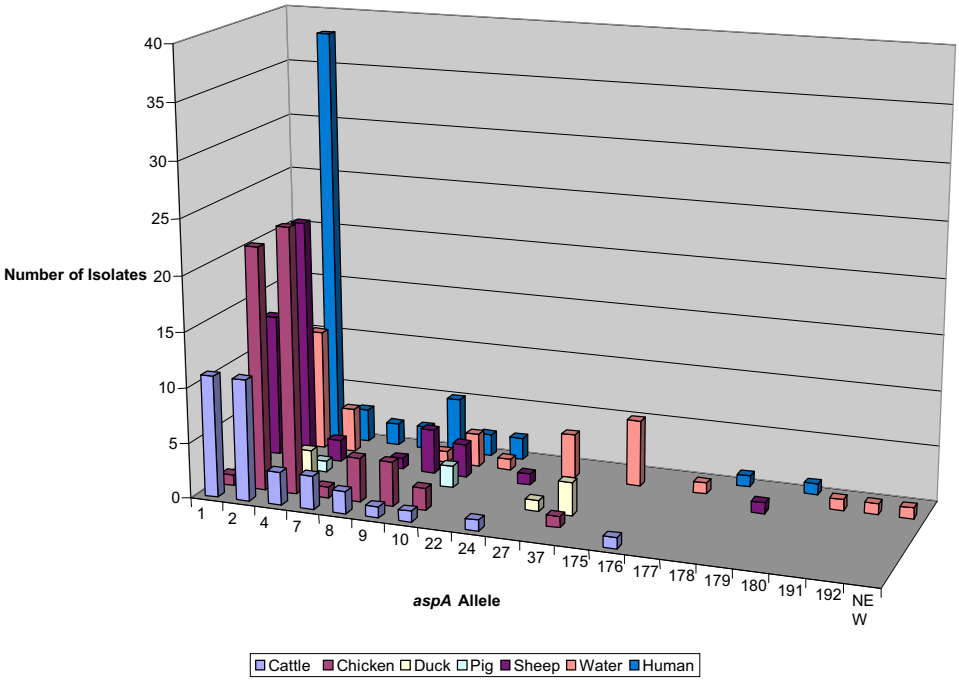
Source	Allele
Cattle	*aspA*176, *glyA*283, *uncA*205
Human	*aspA*180, *gltA*214, *glyA*284
Poultry	*glnA*253, *glnA*252, *gltA*250, *pgm*358, *pgm*355
Sheep	*aspA*179, *tkt*292, *uncA*204
Environmental water	*aspA*175, *aspA*191, *aspA*192, *aspA*177, *pgm*260, *glyA*251, *gltA*216, *pgm*359, *tkt*293, *uncA*102, *uncA*214

CC ST-21 and CC ST-45 were identified in all host reservoirs and environmental sources sampled. CC ST-45 was present in all sources sampled, but the majority of isolates (69.7%) were found in poultry faeces or meat. Isolates with genotypes belonging to CC ST-21 were observed predominantly in poultry, sheep and cattle (Figure 3.5). The majority of human clinical isolates were genotyped as CC ST-21.

Figure 3.4: Allele distribution amongst sources

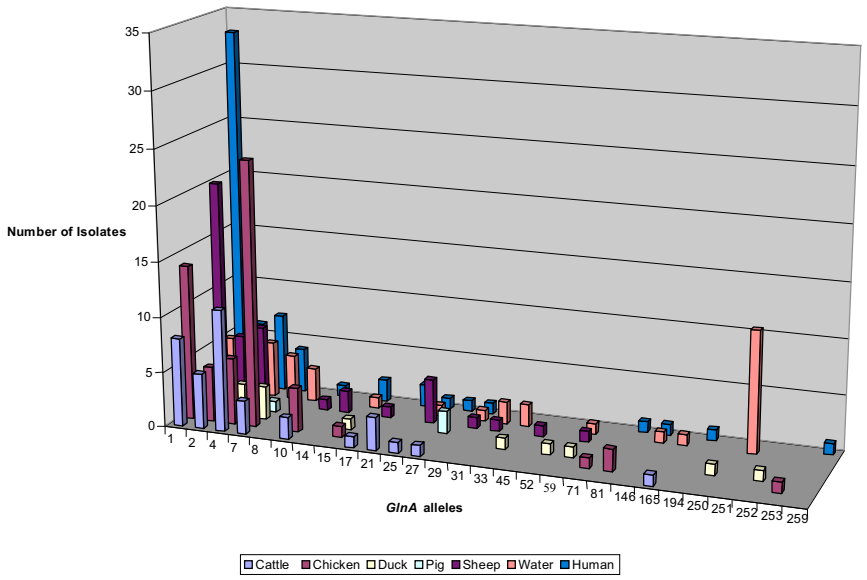
Allele frequencies amongst host or environmental sources were calculated and graphed. Note in rest of chapter duck and chicken isolates are referred to as poultry.

A. *aspA* locus



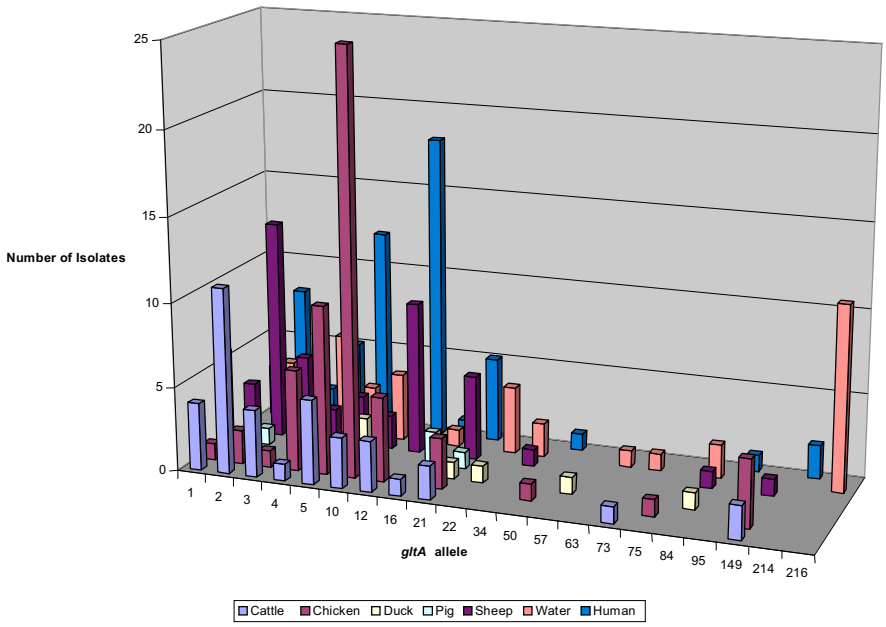
The frequency of *aspA* alleles found in each source was identified. The most frequently identified *aspA* alleles were found in all sources. A number of *aspA* alleles only found in river water isolates were identified.

B. *glnA* locus



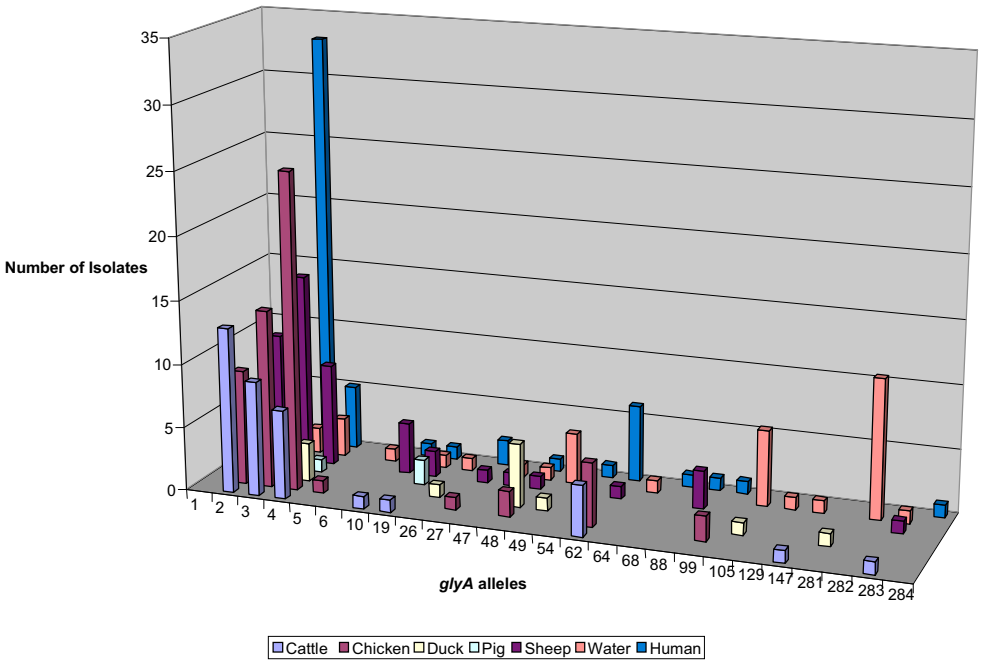
The frequency of *glnA* alleles found in each source was identified. Note the high frequency of the *glnA*1 allele observed in human sources and the *glnA*251 allele in river water sources.

C. *gltA* locus



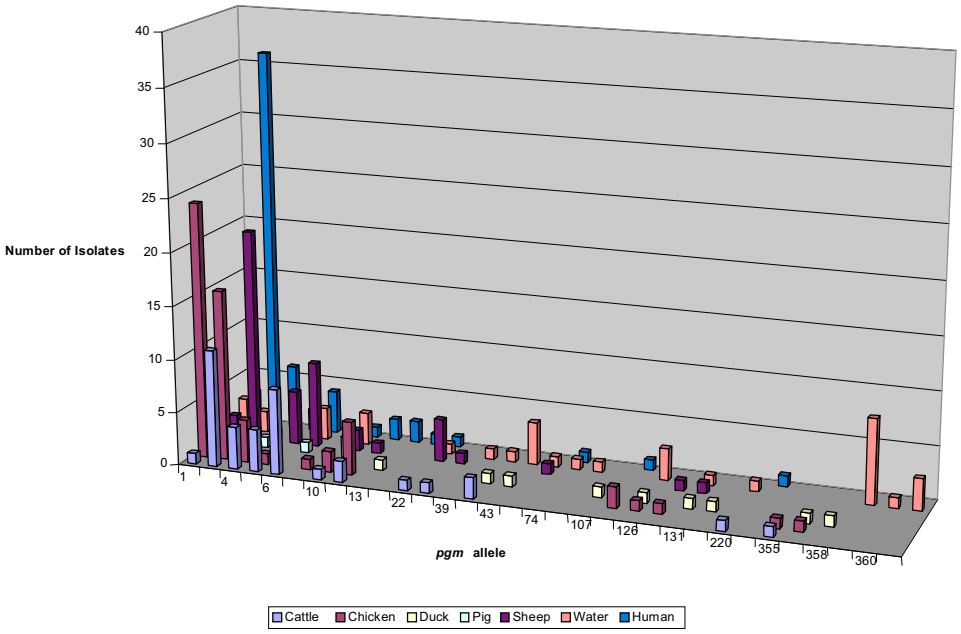
The frequency of *gltA* alleles found in each source was identified. Note the spread of frequencies at the *gltA* locus compared to other loci. The most frequently identified alleles at this locus were *gltA*10 and *gltA*16.

D. *glyA* locus



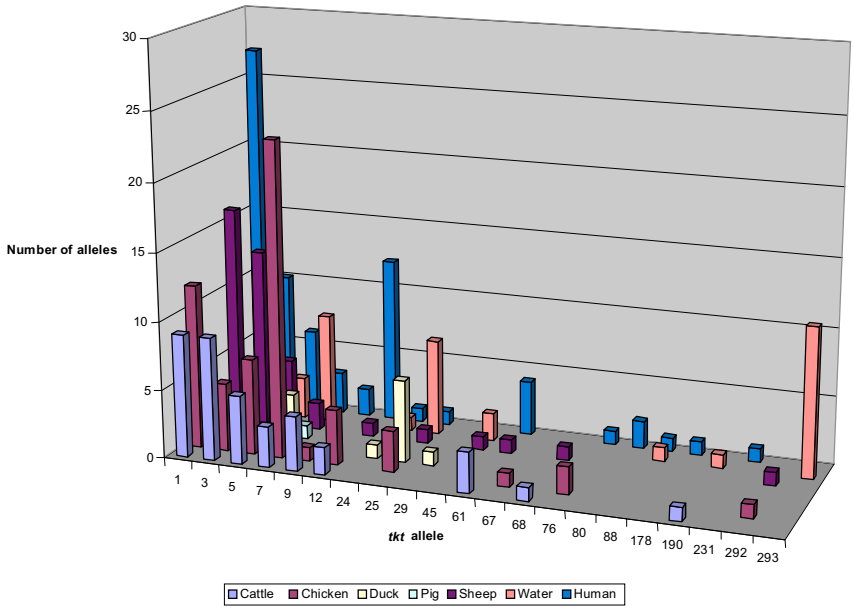
The frequency of *glyA* alleles found in each source was identified. Note the lack of a wide number of alleles at this locus identified in multiple loci compared with other loci.

E. *pgm* locus



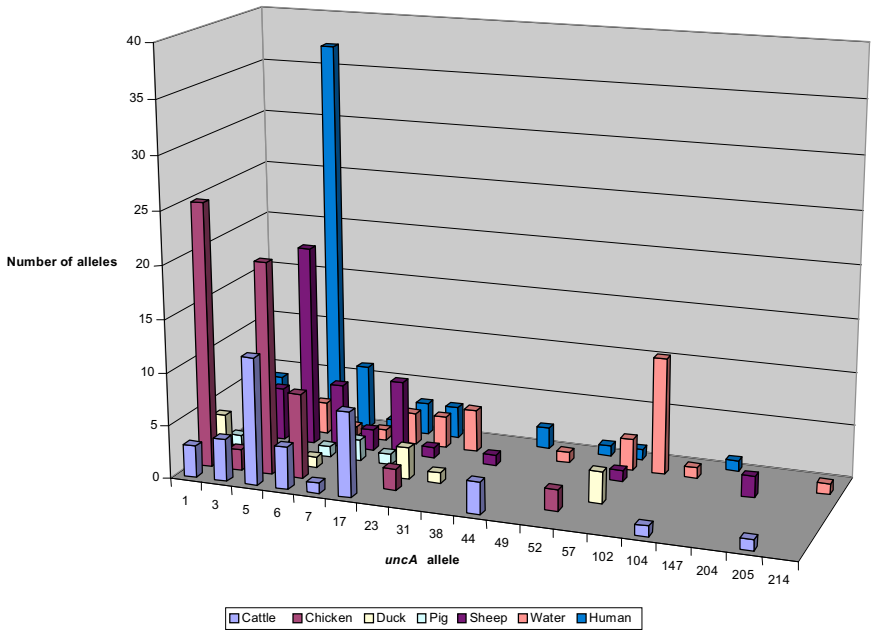
The frequency of *pgm* alleles found in each source was identified. Note the high frequency of the *pgm*2 allele observed in human sources and the *pgm*1 allele in chicken sources.

F. *tkt* locus



The frequency of *tkt* alleles found in each source was identified. Note the frequency of the *tkt*1 allele in human clinical isolates and the *tkt*293 allele in river water sources.

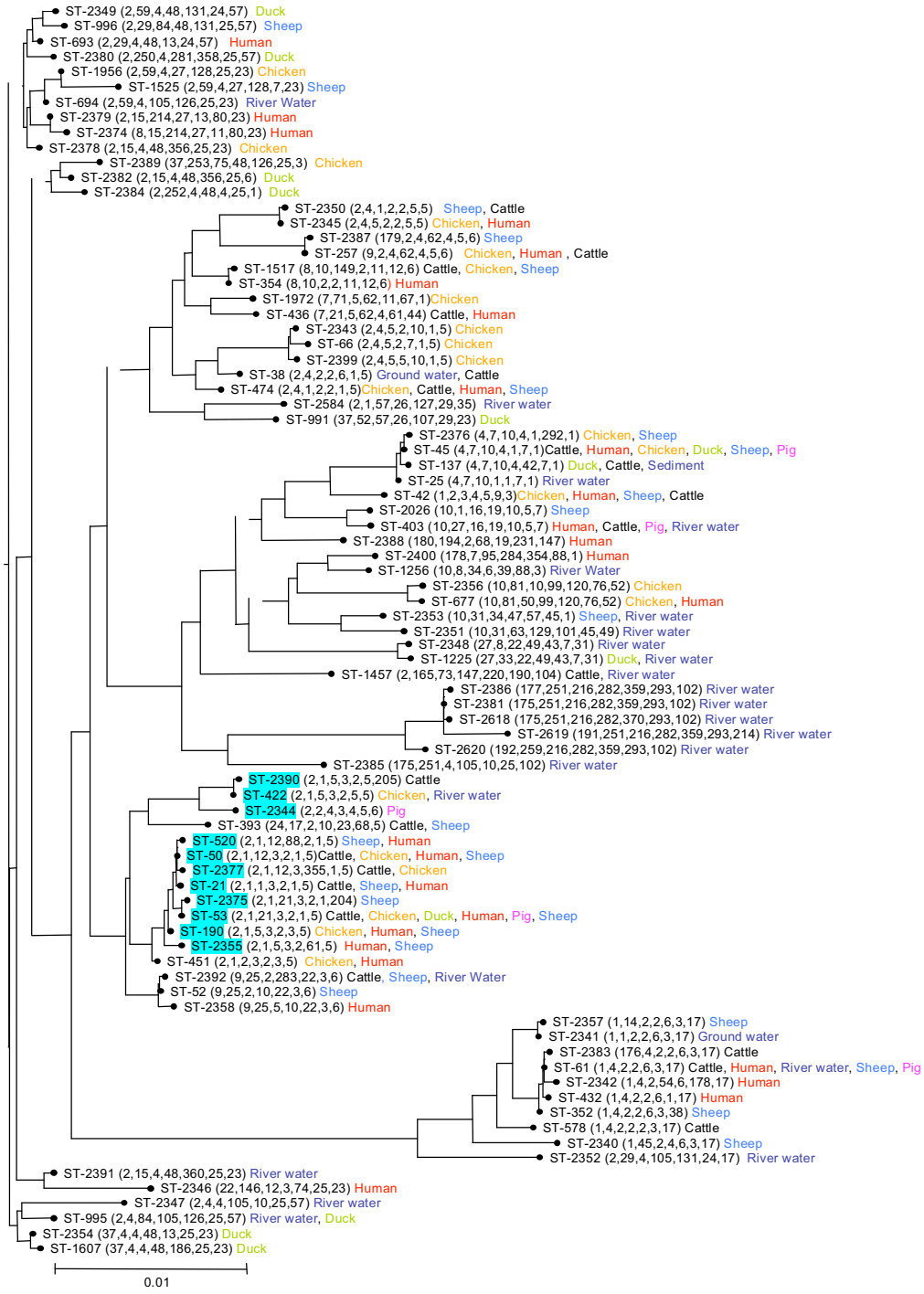
G. *uncA* locus



The frequency of *uncA* alleles found in each source was identified. Note the high frequency of the *uncA*1 allele observed in poultry sources and the *uncA*5 allele in human sources.

Isolates derived from cattle and sheep meat or faeces were predominantly genotyped to CC ST-61 (30%), CC ST-21 (52%) and CC ST-42 (17.3%). CC ST-61 (n = 3, 13.6%) and CC ST-42 (n = 2, 16.6%) were rarely identified in human isolates. The dominant STs identified in human clinical isolates were ST-50 (n = 15, 45.4%), and ST-190 (n = 7, 21.1%). Isolates genotyped to ST-50 were derived from cattle (n = 2), poultry (n = 4), and sheep (n = 6) meat or faecal samples. ST-50 genotypes were not found in pig or environmental water isolates. ST-190 was observed in isolates derived from chicken (n = 3) and sheep (n = 1) meat or faeces, this genotype was not observed in ruminant, pig or environmental samples. The CC ST-21 genotype ST-422 was recovered from isolates derived from river water, chicken, cattle and sheep, but not observed in human clinical samples.

Figure 3.5: Origins of MLST STs



The NJ tree is based on the p-distance of the concatenated sequences of each sequence type present in the New Zealand *C. jejuni* sample. NJ tree was drawn using the START programme, using concatenated sequences derived from http://pubmlst.org/perl/mlstdbnet/mlstdbnet.pl?file=pub-cj_profiles.xml. Blue highlighting indicates STs belonging to CC ST-21.

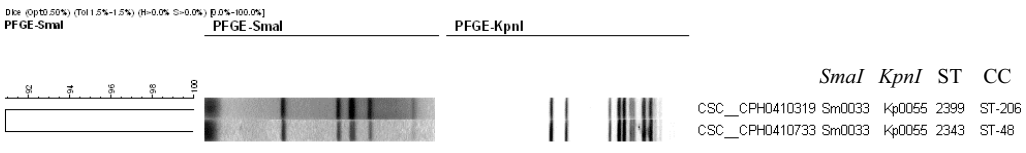
One cluster of isolates with unique genotypes were recovered from the Ashburton

River and found to cluster well away from the majority of the *C. jejuni* STs. These STs comprised six to seven novel alleles dependant on the ST, and one previously identified internationally rare allele (Figure 3.2). The genotypes observed in these isolates had not been previously described and are unique to New Zealand.

3.3.2 ST and CC distribution amongst MRPs and Penner serotypes

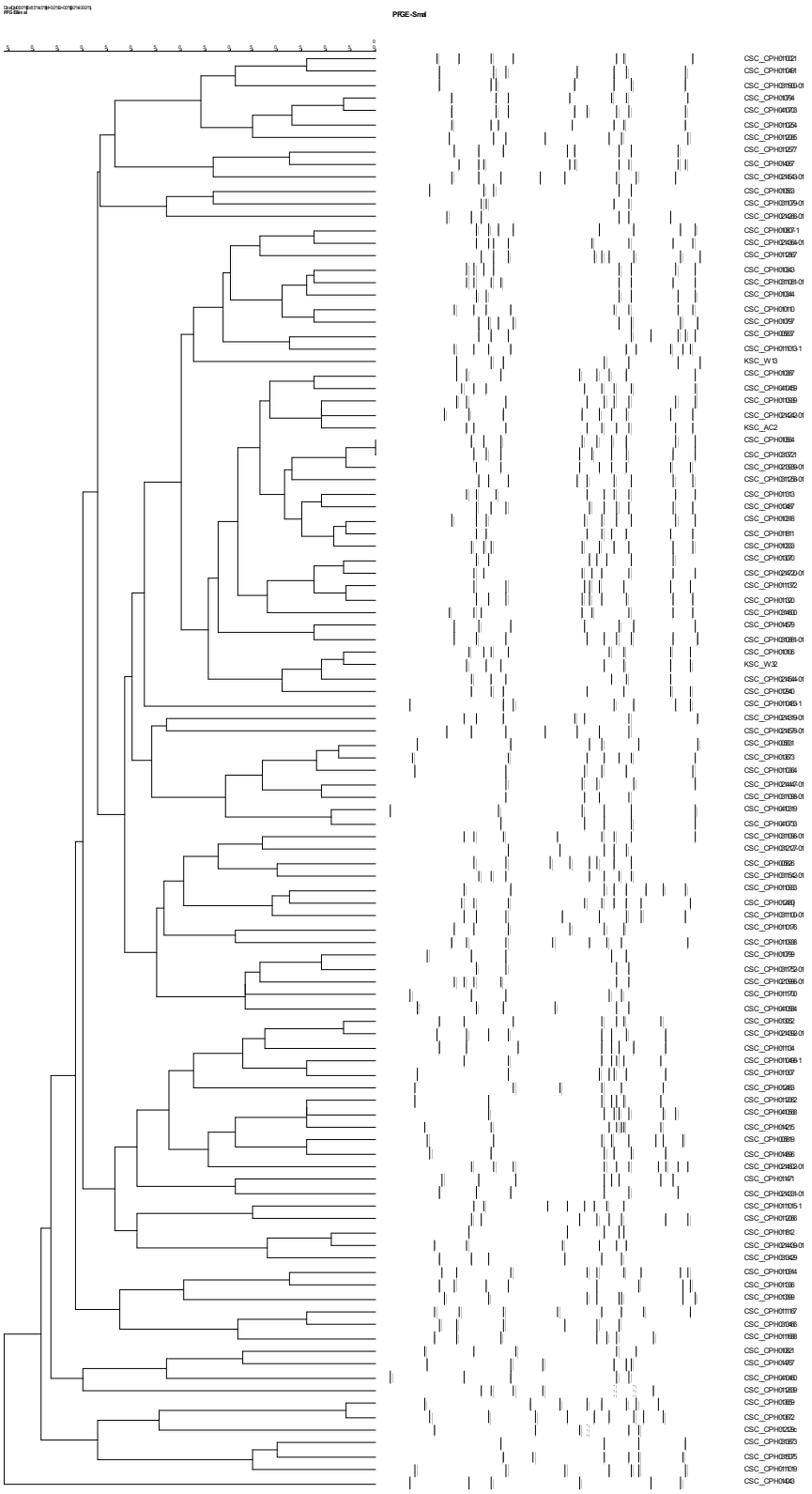
The *SmaI* MRPs (MRPs, n = 124) previously identified on the Pulsenet Aotearoa database were available for all 261 *C. jejuni* isolates (Figure 7), and were composed of between four and nine bands. Analysis of MRPs using the Dice coefficient identified 52 clusters at 95% similarity. The most commonly identified MRPs were Sm1 (isolates, n = 17), Sm35 (n = 15), Sm50 (n = 13) and Sm38 (n =12). The majority of MRPs were associated with one CC, the remaining MRPs were represented by two or more CCs, namely Sm10 (CCs, n = 2), Sm26 (n = 2), Sm31 (n = 2), Sm33 (n = 5), Sm48 (n = 2), Sm80 (n = 2), Sm94 (n =2), Sm151 (n =3) and Sm182 (n =2). Sm1 which was frequently observed in isolates derived from poultry had two STs associated with it (ST-45 and ST-2376).

Figure 3.6: MRPs associated with two different ST genotypes



An UPMGA tree was constructed in BioNumerics using the Dice coefficient (95% similarity, 0.5% optimization, 1.5% band tolerance). The two *C. jejuni* isolates appear to be exactly the same but have two different CCs.

Figure 3.7: *C. jejuni* UPMGA tree



An UPMGA tree (using the Dice coefficient, (95% similarity, 0.5% optimization, 1.5% band tolerance)) was constructed in BioNumerics based on previously characterized PFGE MRPs (69).

Two MRPs previously characterized with two enzymes (*SmaI* and *KpnI*) were genotyped by MLST to two different STs (ST-2399 and ST-2343) belonging to different CCs, although they belonged to the same CL as described by eBURST (Figure 3.6).

3.3.3 The Wallace Coefficient

The Wallace coefficient, a method of assessing the increase in data added by the use of a secondary technique (31), was calculated in BioNumerics to identify whether MRPs could predict either the CC or the ST of a *C. jejuni* isolate. The Wallace coefficient value of 0.9 and 0.49 (Table 3.3) for the ability of a MRP to predict the CC and the ST respectively of an isolate suggests that MRPs are a good predictor of CCs but not STs.

Table 3.3: Wallace coefficient

Subtyping Method	Wallace coefficient
MRPs to Predict CC	0.9
MRPs to Predict ST	0.49

3.4 Discussion

With the development of MLST the ease with which data pertaining to individual isolates and groups of isolates can be shared internationally via electronic databases has increased (136). Although MLST sequence data is highly portable and requires no interpretation, the level of discrimination associated is not high enough for the outbreak identification as a stand-alone subtyping method (69). The *C. jejuni* MLST subtyping scheme requires the amplification and sequencing of seven unlinked, housekeeping gene fragments (477 – 507bp) *aspA*, *glnA*, *gltA*,

glyA, *pgm*, *tkt* and *uncA* (50). The basis of the MLST is the characterization of multiple selectively neutral or nearly neutral genes that give a clearer representation of bacterial interrelatedness than those under positive selection (136). The multiple loci ensuring that if homologous recombination (HR) or horizontal gene transfer (HGT) were to occur then the impact of these events would be lessened and therefore incorrect assumptions about the relationships between bacteria would be less likely to occur (136).

The globalization of MLST data has enabled the characterization of bacterial populations on a much wider scale than previously allowed by indirect DNA analysis methods. MLST has been used to characterize temporally and geographically separate *C. jejuni* isolates in population and outbreak studies (35, 37, 121, 138, 142, 148, 183). Findings suggest that although potentially endemic strains of *C. jejuni* exist, the majority of *C. jejuni* isolates found internationally are of the same CC (50, 138, 201). The current study identified *C. jejuni* MLST CCs and STs that were both unique, and potentially endemic to New Zealand, and common internationally. The most commonly identified CCs in the international database and the literature are CC ST-21, CC ST-45 and ST-61, the results of the current study confirm these observations. Although the current study has characterized a wide range of *C. jejuni* genotypes from multiple sources, the selection of isolates was based on PFGE MRPs, and was therefore not random. The data in the current study allows a snapshot of the range of genotypes from host and environmental sources present in New Zealand over a five year period.

Although the sample was not random, the allelic diversity present in the current

study is similar to that previously described with the *glnA* having the highest d_N/d_S ratio and *uncA* the lowest (121). The *uncA* locus appeared to have a minimal d_N/d_S ratio (0.0114), suggests the locus is under negative or purifying selection contrary to expectations of neutrality. Colles *et al* found that if *uncA*17, which is believed to have come from a HGT event with *C. coli*, was removed from a study sample, the ratio was found to be 0.000 (37), however is more likely that the d_N/d_S ratio was too small for the program used display the true ratio. All gene loci in the *C. jejuni* MLST scheme have previously been observed to be under negative selection not the expected neutral or near neutral selection (37, 121). The results of the current study confirm these observations.

The distribution among animal reservoirs was found to be similar to that previously reported, with the major CCs identified arising from previously recognized sources (37, 121, 138, 148). CC ST-21 was identified in all sources sampled, but was most frequently identified in human clinical isolates. ST-45 was identified in mainly poultry derived samples, similar to previously published findings (138). The finding of ST-45 predominantly in chickens and rarely in human isolates however, does not reflect the isolates in the MLST database, where ST-45 appears in almost equal numbers in human clinical and poultry isolates. The inherent bias in the selection of isolates based on MRPs may have influenced the findings in the current study. ST-61 has been identified in cattle and sheep, but rarely in poultry isolates (see the *C. jejuni*/*C. coli* database http://pubmlst.org) (37), the results of the current study confirm these observations. eBurst analysis identified ST-21 and ST-61 as being part of a single CL group, even though these genotypes were isolated from different animal

reservoirs, whilst CC ST-45 was observed to be part of a stand alone CL group with no links to other CCs.

MRPs identified in the current study were often found to associate with a particular CC and sometimes a particular ST. This observation was unexpected given the high level of discrimination between isolates shown by PFGE and the documented genetic instability of *C. jejuni*. PFGE has previously been identified as a poor predictor of CCs or STs (51), results of the current study would challenge this observation, the calculation of the Wallace coefficient for both the prediction of CCs (0.9) and STs (0.49) suggests that MRPs can predict the CC of a *Campylobacter* isolate but not the ST. Identification of isolates with the same CC for example Sm1 and CC ST-45 over an extended time period suggests that *C. jejuni* is potentially more genetically stable than previously thought. The identification of CCs associated with the same Penner serotypes in diverse geographical locations would support this observation (46, 123, 126).

Although the majority of isolates in many studies have observed the same CCs, potentially endemic strains have also been observed. Potentially endemic strains of *C. jejuni* have been observed in Senegal (121), Curacao (56) and Australia (146). The current study identified a cluster of river water isolates from the Ashburton River in the South Island that were significantly different from the nearest relative in the international MLST database and none of the alleles except *uncA*102 has been identified in any animal reservoir sampled. The recent identification of the founder genotype ST-2381 in a North Island river (unpublished results, N. French) suggests that this genotype and associated

genotypes may represent a widespread and as yet uncharacterized animal reservoir, or that these genotypes represent *C. jejuni* strains that have adapted to an aquatic environment. Another explanation for these findings is that the effective population size of any bacteria is extremely large and the differences observed within the current study are sampling artefacts (personal communication, P. Richie, T.B. Norris).

A number of other unique New Zealand genotypes were identified in isolates derived from poultry and humans. These genotypes did not occur at a high frequency in the data set, but may possibly reflect the diversity present in poultry isolates due to strong selective pressures present in the ecological niche of the chicken gastrointestinal tract (personal communication, C. Pope)

CHAPTER 4: IMPLICATIONS OF GENETIC INSTABILITY ON THE DIVERSITY AND CHARACTERISATION OF *CAMPYLOBACTER* ISOLATES

4.1 Introduction

The taxonomic ordering of living organisms, with each species having unique phenotypic, ecological and genetic characteristics, is relatively easily applied to large, multicellular organisms (36). The concept of species is not so easily applied to prokaryotes, with many closely related bacteria being described as "fuzzy" due to the blurred lines that define each species (91). Bacterial species can have limited phenotypic features differentiating species, and these features may be subject to positive selection and horizontal gene transfer (HGT) from other bacterial species (205). Many bacteria can either be free living or obligate intracellular species organisms, often the same species of bacteria can be found in numerous ecological niches hindering taxonomic characterisation on the basis of ecology (204, 205).

DNA-DNA hybridisation (DDH) is the currently accepted gold standard for taxonomic discrimination between bacterial species (204). DDH discriminates between bacterial species that have less than 70% DNA homology, equating to approximately 96% whole genome nucleotide similarity within a given group of bacteria (82, 205). However due to the inherent time and labour intensive nature of this method of speciation is not used on a regular basis (205).

Campylobacter and other bacteria are often speciated in the laboratory with a single PCR based on a single gene or multiple genes to define each species (112).

Single gene analysis is often used to verify unknown bacteria, however the conserved nature of rRNA and other informative genes limits the usefulness when trying to speciate closely related bacteria (205) such as *C. jejuni*, *C. coli* and *C. lari*. Multiplex PCR is generally used only when the genus of the bacteria is known. Species are often characterised by the presence or absence of genes including the *glyA* in *C. coli* and the *hipO* gene in *C. jejuni* (221).

The use of a single gene or gene product, which may be subject to homologous recombination (HR) (90), or HGT (130) to speciate bacteria may lead to incorrect characterisation of the isolates. Many studies have identified HR and HGT events in multiple loci in *C. jejuni* and *C. coli* (47, 96, 148, 149, 224). The inherent instability of the *C. jejuni,* and to a lesser extent the *C. coli* genomes, hinders the traceability of both related bacterial isolates and source attribution. Although HR and HGT have previously been identified in *Campylobacter* isolates, limited information is available concerning the levels of HR and HGT occurring within housekeeping genes characterised by the *C. jejuni* and *C. coli* MLST schemes (70, 210). The current study aimed to investigate HR and HGT in both housekeeping genes and a surface gene in New Zealand *Campylobacter* isolates, and in the international MLST database using MLST and *flaA* PCR RFLP genotyping, and to explore implications of HR and HGT on subtyping methods. The identification of strong negative selection acting on housekeeping genes, and HR/HGT at all loci examined in the current study has implications for the subtyping of temporally and geographically separate *Campylobacter* isolates.

4.2 Methods

4.2.1 *Campylobacter coli* isolates

Eighty four *C. coli* were obtained from the ESR (Kenepuru and Christchurch Science Centres) and Massey University (Palmerston North) *Campylobacter* collections. The *C. coli* isolates spanned 10 years from 1996 to 2006 and represented several New Zealand locations (Ashburton, Christchurch, Wellington, Auckland and Palmerston North). The bacterial isolates were sourced from cattle (n = 6), pigs (n = 6), poultry (n = 24), sheep (n = 15), water (n = 7) and human clinical samples (n = 30). Fifty *C. coli* isolates were previously characterised using PFGE (*SmaI)*, the remaining 33 isolates were characterised in the current study using previously described methods (Chapter 2). One isolate was unable to be amplified with *flaA* primers.

4.2.2 *Campylobacter jejuni* isolates

Two hundred and seventy eight *C. jejuni* isolates were characterised using MLST and *flaA* PCR RFLP (Chapter 3). *C. jejuni* isolate MLST and *flaA* genotypes used in this chapter were first identified in the FSA study (Chapter 3) and the outbreak survey (Chapter 5). All FSA genotypes were used, however only those genotypes from the outbreak survey that were not present in the FSA study were used. The *C. jejuni* isolates spanned ten years from 1996 to 2006 and were geographically spread over both the North and South islands of New Zealand. The bacterial isolates were sourced from cattle, pigs, poultry, sheep, water and human clinical samples. All 278 *C. jejuni* isolates had been previously characterised by ERL staff using PFGE (*SmaI*) and Penner serotyping, except the 1996/1997 mistyped

C. jejuni isolates. The mistyped *C. jejuni* isolates were correctly speciated as *C. jejuni* following the method of Wang *et al* (221).

4.2.3 MLST and *flaA* PCR RFLP genotyping

Multi-locus sequence typing and *flaA* PCR RFLP were carried out following the method of Dingle *et al* (50) (Chapter 2). Sequence analysis was performed using the BLAST (9) program available from the pubMLST website and sequences compared to published alleles. Sequence types were assigned based on previously known STs in the pubMLST database. Novel alleles and STs were sent to http://pubmlst.org for allele and ST assignment. Bacterial isolates were further characterised using the MEGA v 3.1 (126) program. Genetic variance at individual loci was analysed using the DNAsp (182) and START (115) programs.

4.3 Results

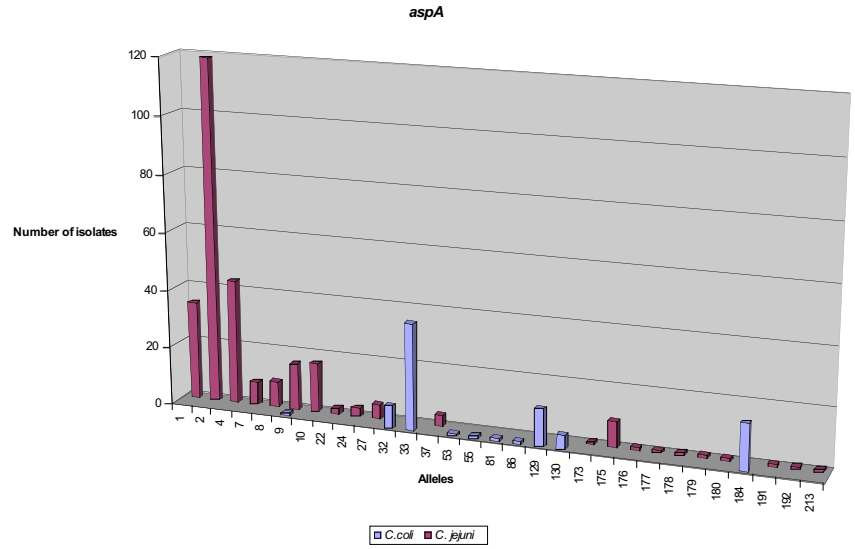
4.3.1 MLST allele distribution

Seven gene fragments (*aspA, glnA, gltA, glyA, pgm, tkt* and *uncA*) were amplified and sequenced for each of the 359 *Campylobacter* isolates, *C. jejuni* (n = 276), *C. coli* (n = 83). Two hundred and twenty nine alleles, consisting of previously described (n = 182, 75.2%), novel (n = 47, 25.8%) alleles were observed in the current study. The frequency of each allele at each gene locus was identified and graphed (Figure 4.1).

Figure 4.1: Frequency of *C. jejuni* and *C. coli* MLST alleles in New Zealand sample

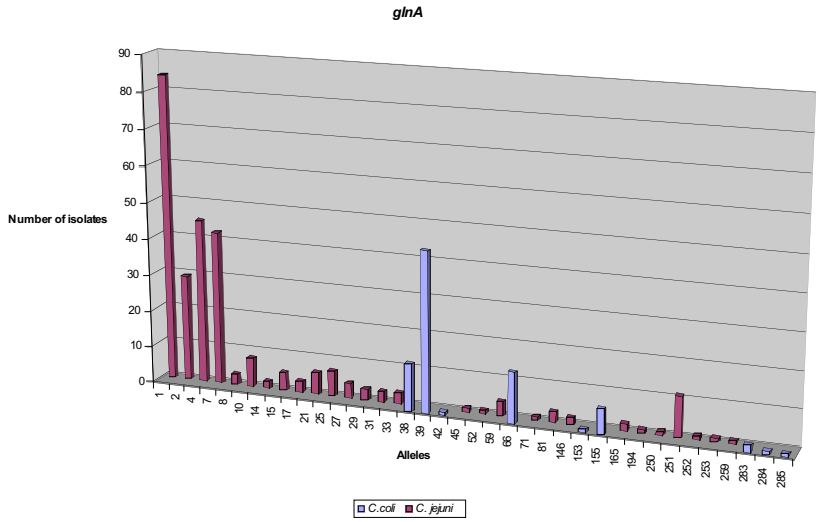
Allele frequencies for each of the seven housekeeping genes were calculated in START and graphed A-G.

A. *aspA* locus



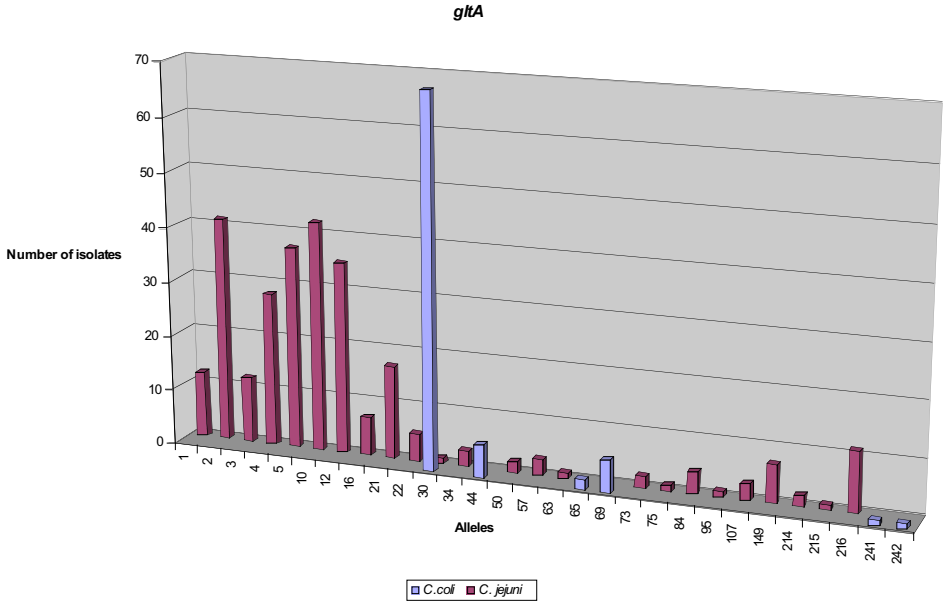
Allelic frequencies were calculated for the *aspA* locus within the New Zealand *Campylobacter* sample using the START program. Note the HGT event at *aspA*9 and *aspA*55. *aspA*55 was identified in a *C. coli* isolate in the New Zealand sample but found to be associated with *C. jejuni* STs. The HGT event at *aspA*55 was not obvious until a ME tree was drawn and the allele was found to cluster with the *C. jejuni* alleles.

B. *glnA* locus



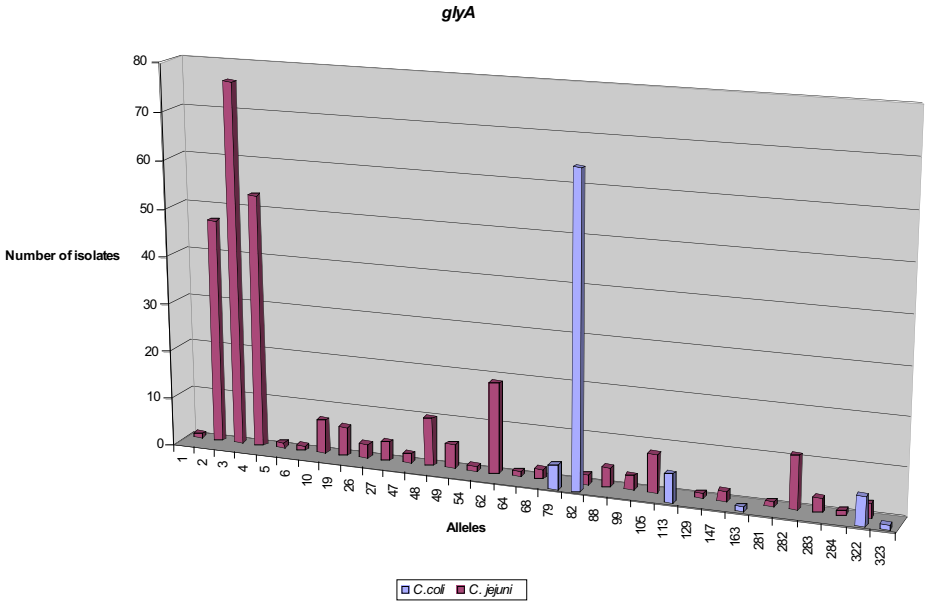
Allelic frequencies were calculated for the *glnA* locus within the New Zealand *Campylobacter* sample using the START program. No HR or HGT events were observed in the New Zealand *Campylobacter* sample.

C. *gltA* locus



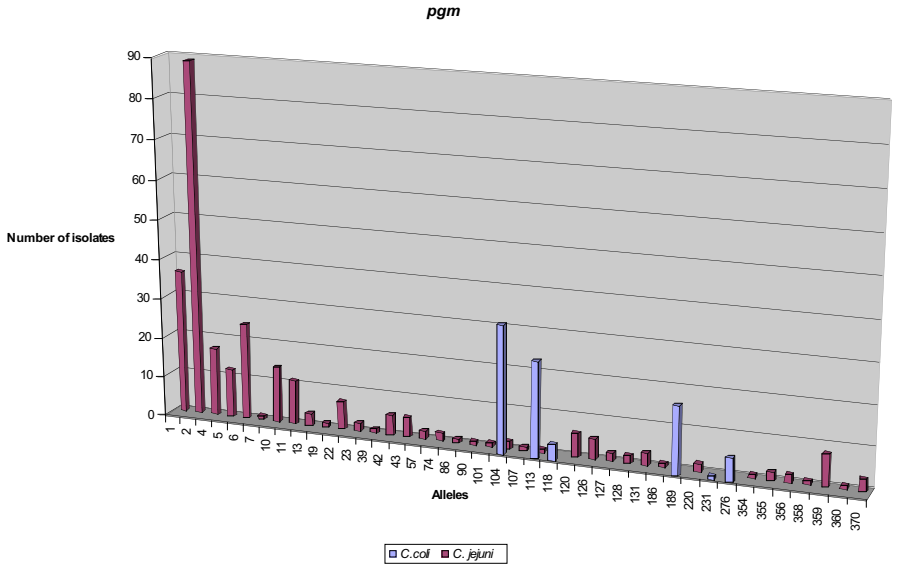
Allelic frequencies were calculated for the *gltA* locus within the New Zealand *Campylobacter* sample using the START program. Note the inter-species HGT event at the *gltA*30 allele.

D. *glyA* locus



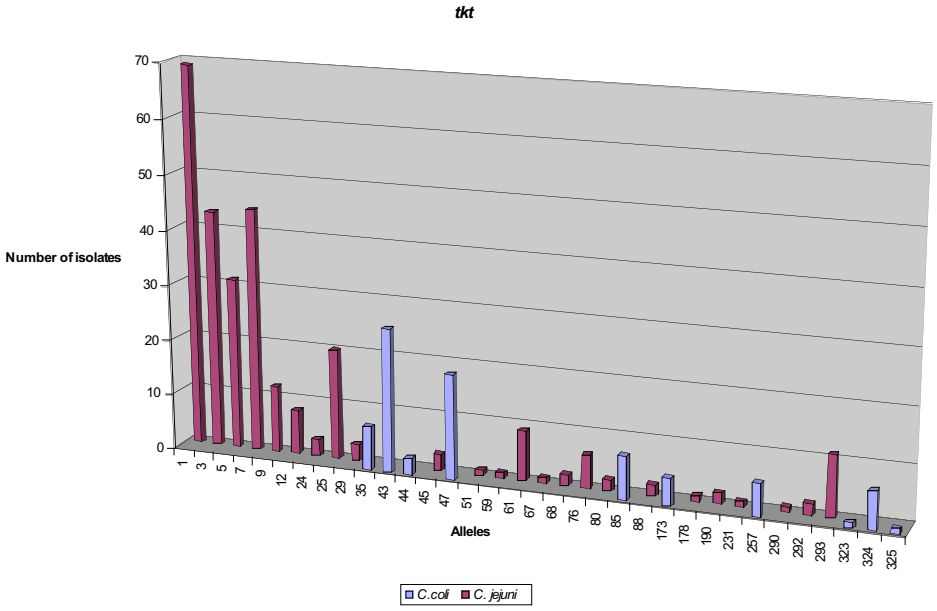
Allelic frequencies were calculated for the *glyA* locus within the New Zealand *Campylobacter* sample using the START program. Note the inter-species HGT events at the *glyA*82 and *glyA*322 alleles

E. *pgm* locus



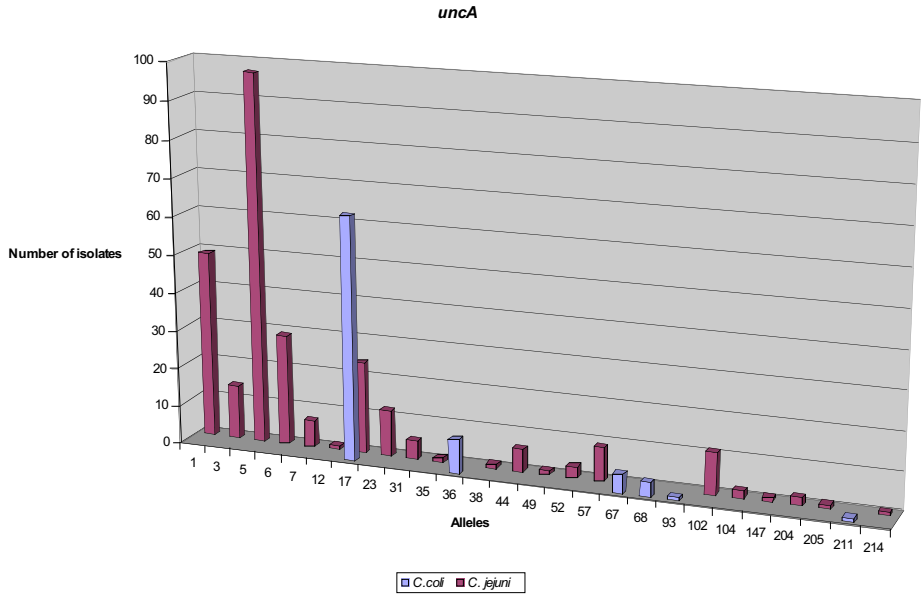
Allelic frequencies were calculated for the *pgm* locus within the New Zealand *Campylobacter* sample using the START program. Note the HGT event at the *pgm*104 allele.

F. *tkt* locus



Allelic frequencies were calculated for the *tkt* locus within the New Zealand *Campylobacter* sample using the START program. No HGT events were present at this locus.

G. *uncA* locus



Allelic frequencies were calculated for the *aspA* locus within the New Zealand *Campylobacter* sample using the START program. Note the HGT event at *uncA17*.

Allelic diversity of New Zealand *C. jejuni* and *C. coli* isolates were compared (Table 4.1) and the frequency of each allele determined. The number of alleles identified at each locus varied between 29 (*gltA*) and 41 (*pgm*), and the number of polymorphic sites differed between gene loci from 41 (*tkt*) to 105 (*glyA*) (Table 4.1). Between three (*uncA*) and 11 (*aspA*) novel alleles were observed at each gene locus (Figure 4.2), with the majority of novel alleles arising from single or double point mutations. Individual neighbour joining trees were constructed using MEGA v 3.1 (126) for each gene locus (Figure 4.2, A - G).

The majority of *C. jejuni* and *C. coli* alleles were resolved into large groups at each gene locus, the only exceptions being *aspA*173, *glnA*259 and *glnA*251. HGT was observed in two *C. coli* isolates, *aspA*55 and *aspA*9. *C. coli* associated alleles, *gltA*30, *glyA*82, *glyA*322, *pgm*104, *pgm*113, *pgm*118 and *uncA*17, were

identified in six *C. jejuni* isolates. The *uncA*17 allele was observed in both *C. jejuni* (n = 11) and *C. coli* STs (n = 17) (Table 4.2).

One allele, *aspA*173 (Chapter 5), was observed to be a result of a HR event most likely between *aspA*33, a *C. coli* associated allele and *aspA*2, a *C. jejuni* associated allele (Figure 4.3), with a maximum chi square test value of 55. A large deletion of 74 nucleotides affecting the reading frame of the *aspA* allele was identified in PH523 (Figure 4.4), the size of the deletion meant the allele could not be used in the MLST data analysis.

Table 4.1: Comparative analysis of allelic diversity in New Zealand and international *C. jejuni* and *C. coli* isolates

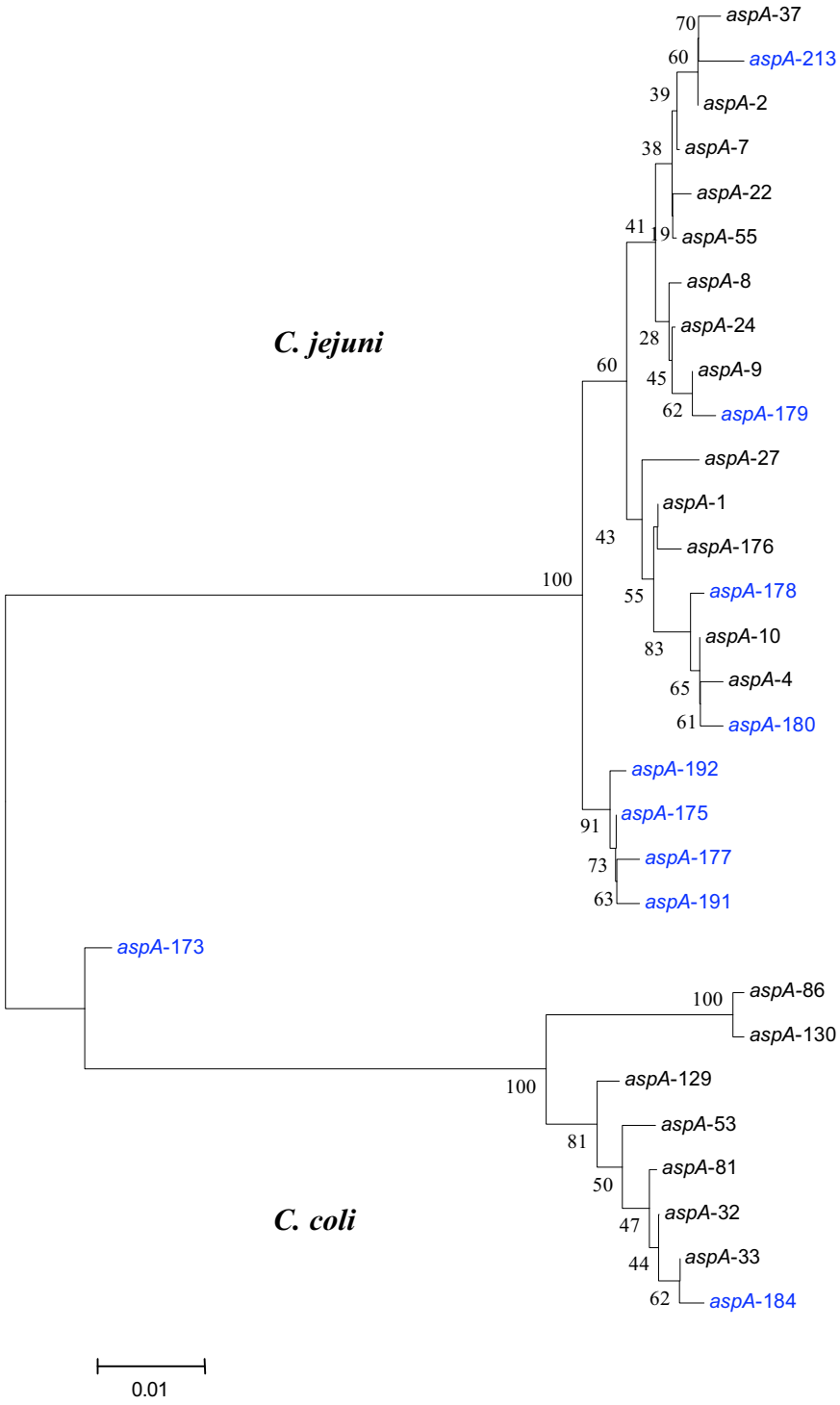
International Database								
Locus	**Alleles**		**S (mean)**		**N (mean)**		**dN/dS**	
	C. jejuni	***C. coli***	***C. jejuni***	***C. coli***	***C. jejuni***	***C. coli***	***C. jejuni***	***C. coli***
aspA	145	74	112.6	113	364.4	364	0.049	0.0386
glnA	198	85	102.8	103	374.2	374	0.0435	0.02
gltA	146	80	89.5	91.5	312.5	310.5	0.0403	0.0348
glyA	206	95	116.7	116.5	390.3	390.5	0.0423	0.0283
pgm	269	100	111.4	111.7	386.6	386.3	0.0499	0.0348
tkt	225	100	99.6	99.1	359.4	359.9	0.0677	0.0811
uncA	160	74	123.7	124.6	365.3	364.4	0.0152	0.007

New Zealand								
Locus	**Alleles**		**S (mean)**		**N (mean)**		**dN/dS**	
	C. jejuni	***C. coli***	***C. jejuni***	***C. coli***	***C. jejuni***	***C. coli***	***C. jejuni***	***C. coli***
aspA	21	10	112.6	113.2	364.4	363.8	0.0649	0.0318
glnA	27	9	102.8	103.3	374.2	373.7	0.0745	0.2741
gltA	24	6	89.4	91.9	312.6	310.1	0.0312	0.0586
glyA	28	6	116.7	116.3	390.3	390.7	0.0448	0.2557
pgm	36	6	111.6	112.3	386.4	385.7	0.0434	0.0561
tkt	24	10	99.6	99.1	359.4	359.9	0.0318	0.0915
uncA	20	6	123.8	125.3	365.2	363.7	0.0116	0

Figure 4.2: Phylogenetic diversity of gene loci sampled

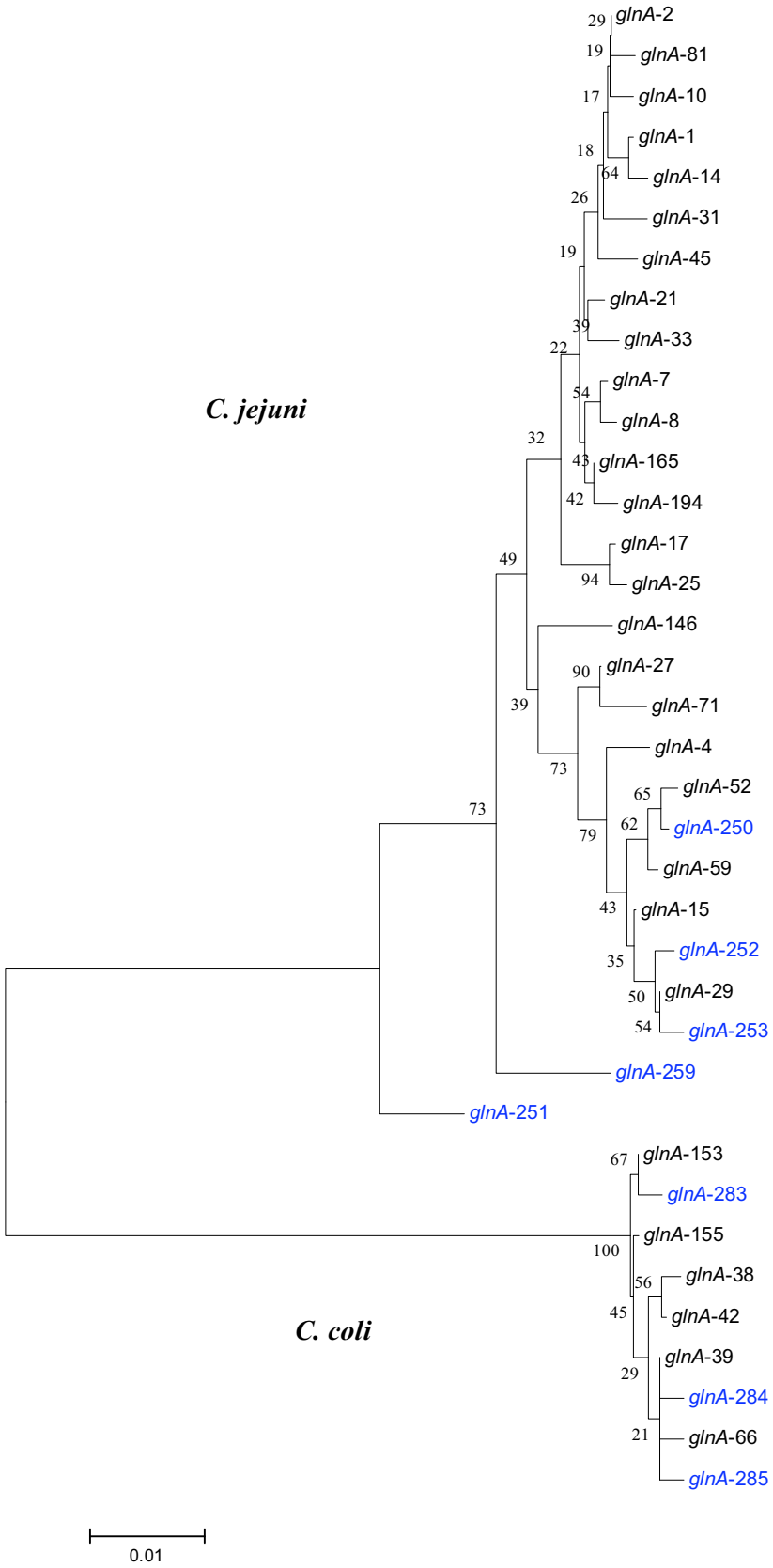
A ME tree, using the Kimura 2-parameter model and a bootstrap value of 1000, was constructed for each of the seven housekeeping gene loci. The ME trees indicated all alleles were closely related and the majority of allele loci produced congruent phylogenetic trees. Blue text indicates novel alleles.

A. *aspA* locus



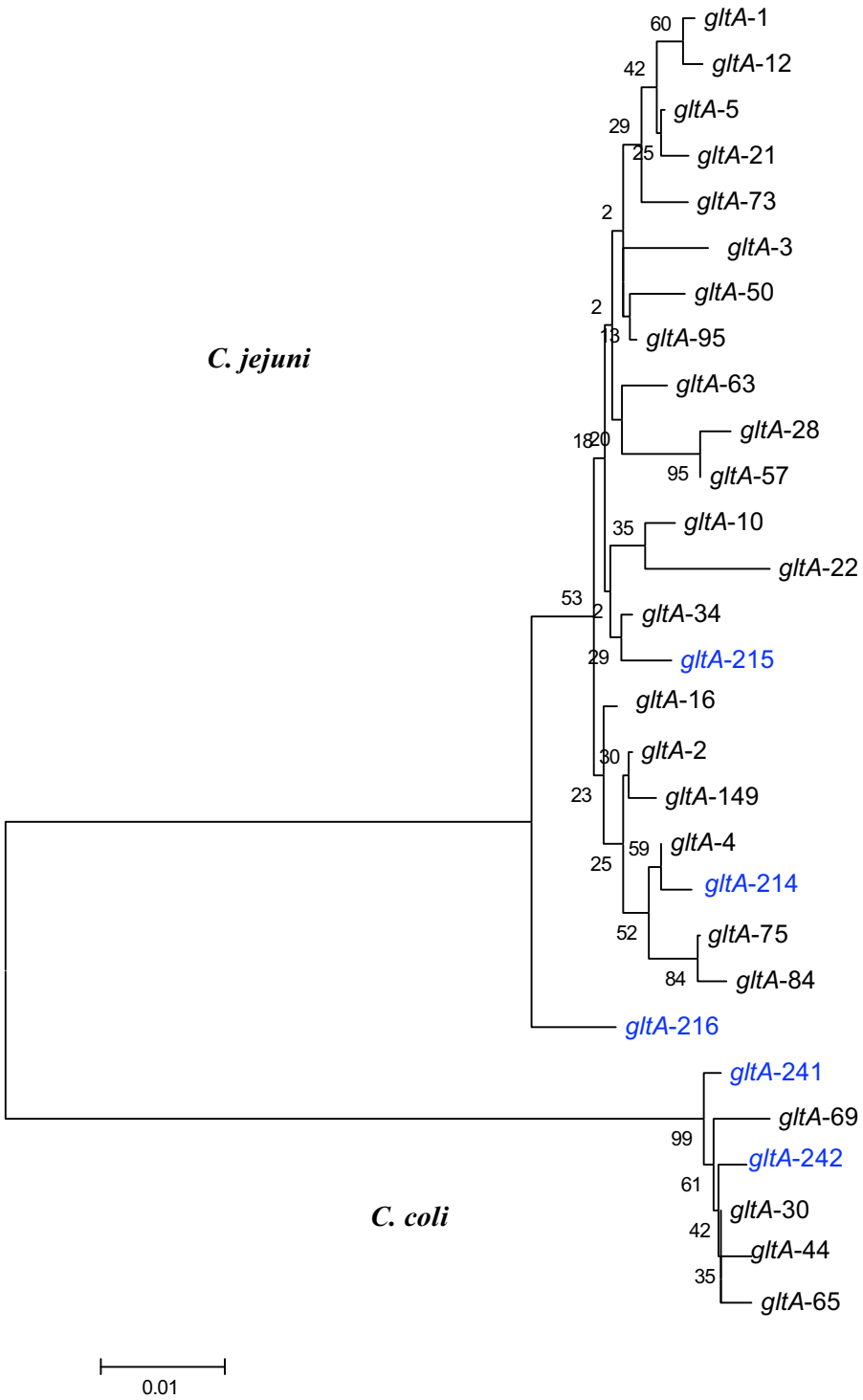
An ME tree (using the Kimura 2-parameter) was constructed for all *aspA* alleles in the New Zealand *Campylobacter* sample. Note the HR event at this locus *aspA*173. Blue text indicates new alleles.

B. *glnA* locus



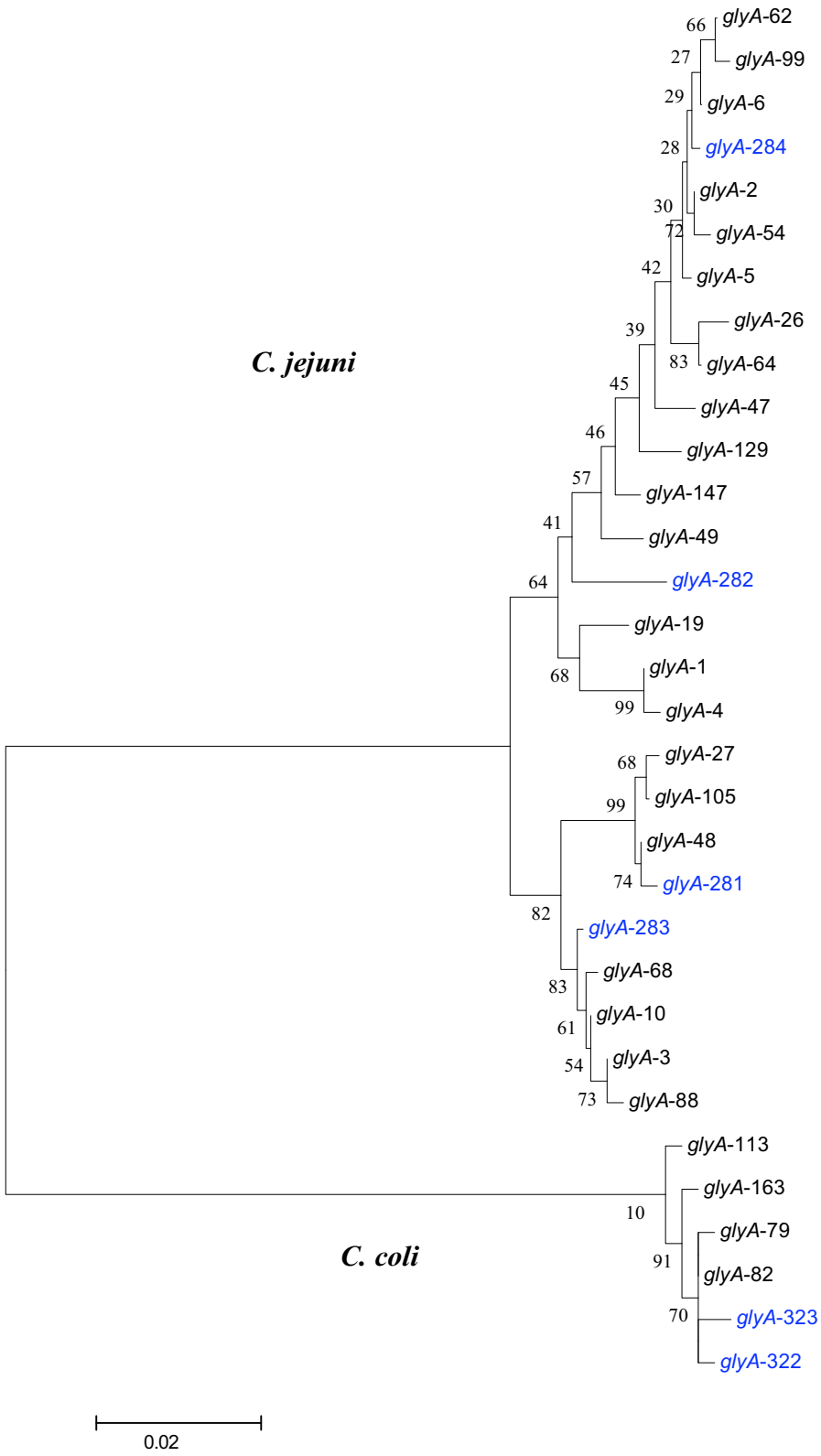
An ME tree (using the Kimura 2-parameter) was constructed for all *glnA* alleles in the New Zealand *Campylobacter* sample. Blue text indicates new alleles.

C. *gltA* locus



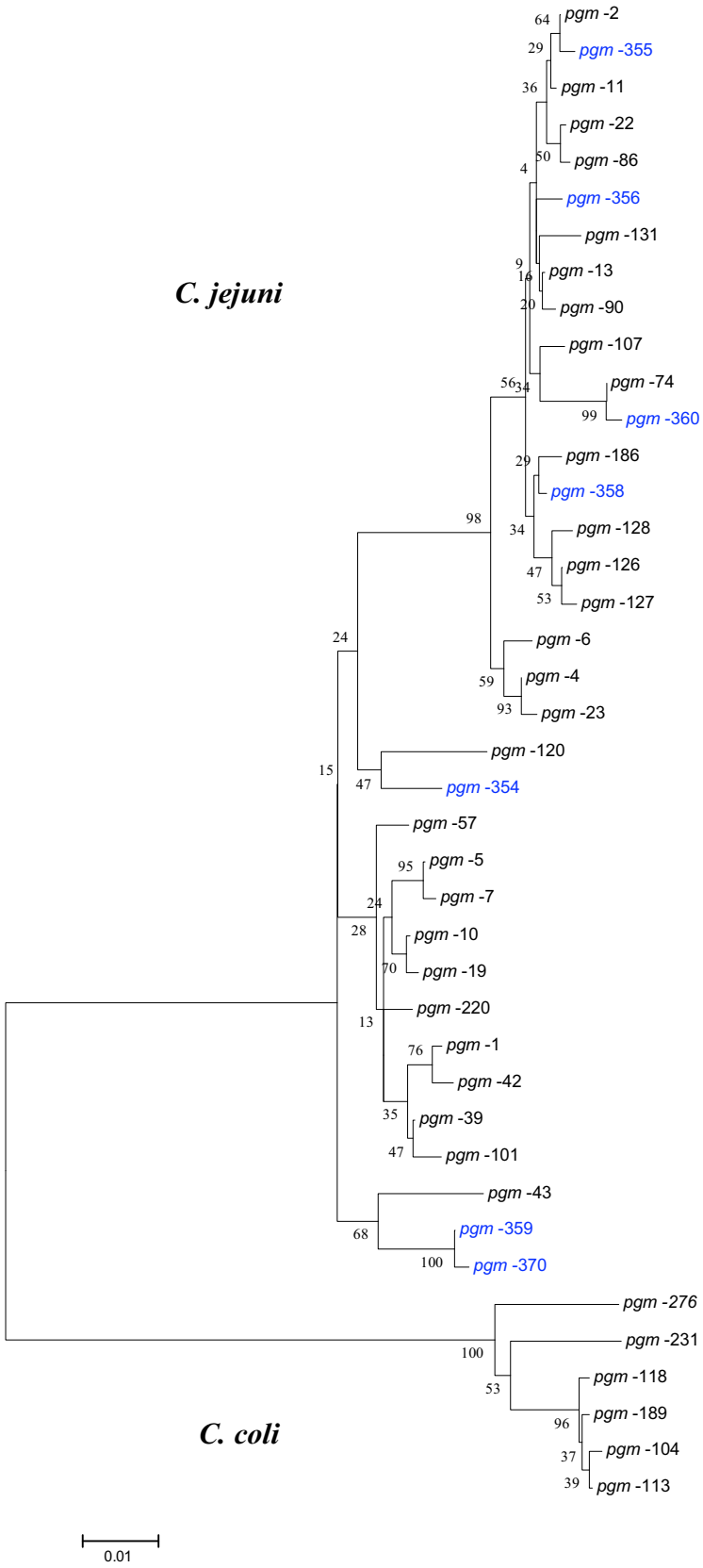
An ME tree (using the Kimura 2-parameter) was constructed for all *gltA* alleles in the New Zealand *Campylobacter* sample. Blue text indicates new alleles.

D. *glyA* locus



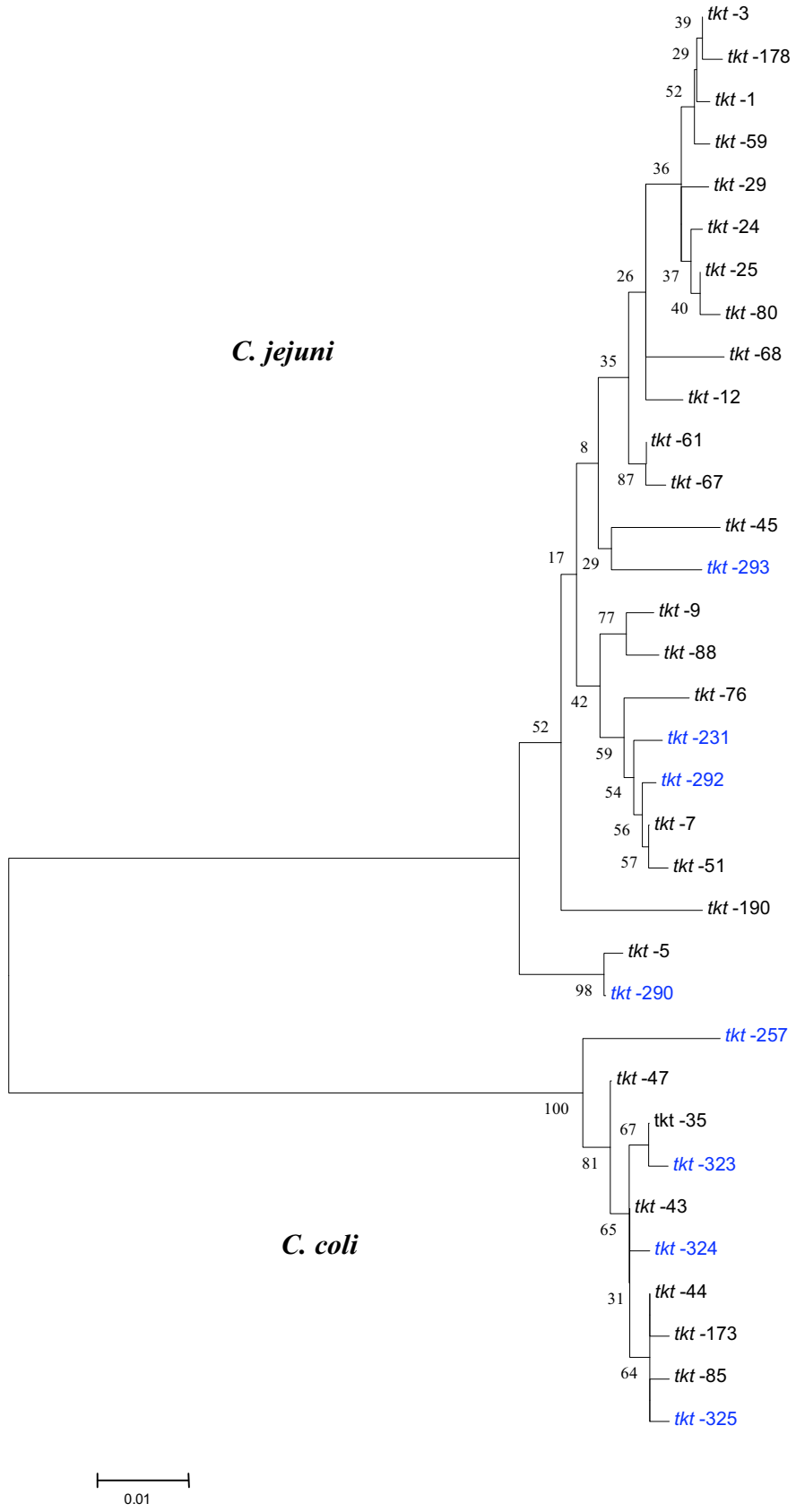
An ME tree (using the Kimura 2-parameter) was constructed for all *glyA* alleles in the New Zealand *Campylobacter* sample. Blue text indicates new alleles.

E. *pgm* locus



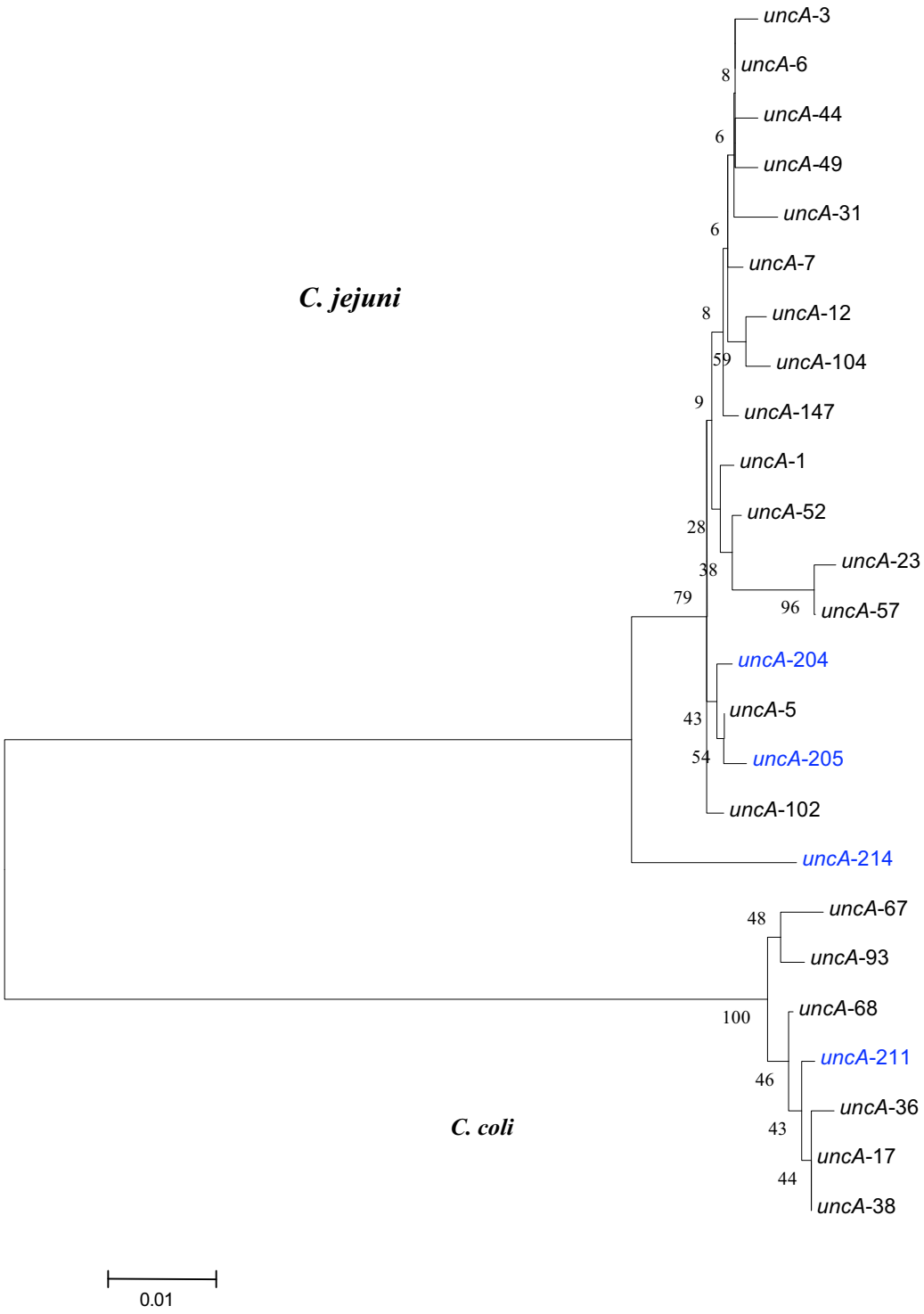
An ME tree (using the Kimura 2-parameter) was constructed for all *pgm* alleles in the New Zealand *Campylobacter* sample. Blue text indicates new alleles.

F. *tkt* locus



An ME tree (using the Kimura 2-parameter) was constructed for all *tkt* alleles in the New Zealand *Campylobacter* sample. Blue text indicates new alleles.

F. *uncA* locus



An ME tree (using the Kimura 2-parameter) was constructed for all *uncA* alleles in the New Zealand *Campylobacter* sample. Blue text indicates new alleles.

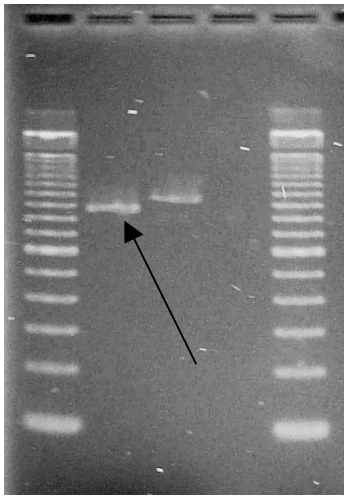
The ratio of non-synonymous to synonymous nucleotide substitutions (d_N/d_S) was calculated for each gene locus in both *C. jejuni* and *C. coli* samples (Table 4.1). d_N/d_S ratios were observed to be less than one for both species in the current study. The highest ratio observed in *C. jejuni* was at the *glnA* locus (d_N/d_S = 0.0773) and the lowest ratio was observed in the *uncA* locus (d_N/d_S = 0.0116). The d_N/d_S ratios observed in *C. coli* were between *glnA* (0.2741) and *uncA* (0).

Figure 4.3: Recombination event observed in allele *aspA*173

Scheme	*Campylobacter jejuni*
Locus	*aspA*
Analysis range	1 – 477
Sequences	*aspA*-2 and *aspA*-33 (derived: *aspA*-173)
Possible recombination	after nucleotide 195
Max Chi Squared	55
Significance, *P*	0.000

*aspA*2, asp173 and *aspA*33 were aligned, an HR event was occurred after nucleotide 195. *aspA*2 a *C. jejuni* (indicated by blue rectangles) associated allele and *aspA*33 a *C. coli* (indicated by green rectangles) associated allele produced a interspecies allele within a *C. jejuni* isolate.

Figure 4.4: *aspA* deletion in PH526



PH526 *aspA* PCR product arrow indicates a smaller MW band than expected (2% Agarose gel, 100bp ladder)

>*aspA*-PH526
ATGATAGGCGAAGATATACAAAGAGTATTAGAAGCTAGAAAATTAATTTTAGAGATCAAT
TTGGGTGGAACTGCTATTGGAACGGGAATTAATTCTCATCCTGATTATCCGAAGGTTGTA
GAAAGAAAAATAAGAGAAGTGACAGGTTTTGAATATACTGTGGCTGAGGATTTGATCGAG
GCGACTCAAGATACGGGAGCTTATGTACAAATTTCAGGTGTTTTAAAACGTGTTGCAACA
AAACTTTCTAAAGTATGTAATGACTTAAGACTTTTAAGCAGTGGTCCAAATTATTCCTGA
AGTAGTTAATCAAGTTTGTTATTTTGTTATTGGAGCAGATGTAACTGTAACTTTTGCTTG
TGAGGGTGGACAATTACAACTTAATGTTTTTGAACCAGTTGCA

>*aspA*-10
ATGATAGGCGAAGATATACAAAGAGTATTAGAAGCTAGAAAATTAATTTTAGAGATCAAT
TTGGGTGGAACTGCTATTGGAACGGGAATTAATTCTCATCCTGATTATCCGAAGGTTGTA
GAAAGAAAAATAAGAGAAGTGACAGGTTTTGAATATACTGTGGCTGAGGATTTGATCGAG
GCGACTCAAGATACGGGAGCTTATGTACAAATTTCAGGTGTTTTAAAACGTGTTGCAACA
AAACTTTCTAAAGTATGTAATGACTTAAGACTTTTAAGCAGTGGTCCAAAATGTGGTCTT
AATGAGATTAATCTTCCAAAAATGCAACCAGGTAGTTCTATCATGCCAGGTAAGGTAAAT
CCTGTTATTCCTGAAGTAGTTAATCAAGTTTGTTATTTTGTTATTGGAGCAGATGTAACT
GTAACTTTTGCTTGTGAGGGTGGACAATTACAACTTAATGTTTTTGAACCAGTTGCA

Whole *aspA* gene translated sequence from the Sanger website

MGTRKEHDFIGELEISDEVYYGVQTFRAVENFDISHDRLKDFPRFVRALARVKKAAAMAN
HELGLLDKNIQDAIIKACDKILEGGYYDQFVVDMIQGGAGTSTNMNANEVIANIGLELMG
HKKGEYQYLHPNDHVNLSQSTNDAYPTALHLALHDYLSDLAKAMEHLKKAYERKAEEFKD
VLKMGRTQLQDAVPMTLGREFKTFAVMIGEDIQRVLEARKLILEINLGGTAIGTGINSHP
DYPKVVERKIREVTGFEYTVAEDLIEATQDTGAYVQISGVLKRVATKLSKVCNDLRLLSS
GPKCGLNEINLPKMQPGSSIMPGKVNPVIPEVVNQVCYFVIGADVTVTFACEGGQLQLNV
FEPVVAYSLFNSVVMLEKAMYTLADKCIDGITANEKICSDFVYNSVGIVTALNPYIGYEN
SASIAKEAMNTGKRVADIALERGLLSKEQIDEILTPSNMLNPHMEAKK

* Yellow highlighting indicates where deletion has occurred, blue and red highlighting indicate the beginning and the end of the deletion.

Table 4.2: Diversity of STs with the *uncA*17 allele within NZ sample

CC	ST	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	Species
ST-61	61	1	4	2	2	6	3	17	*C. jejuni*
ST-61	432	1	4	2	2	6	1	17	*C. jejuni*
ST-61	578	1	4	2	2	2	3	17	*C. jejuni*
ST-61	2340	1	45	2	4	6	3	17	*C. jejuni*
ST-61	2341	1	1	2	2	6	3	17	*C. jejuni*
ST-61	2342	1	4	2	54	6	178	17	*C. jejuni*
ST-61	2357	1	14	2	2	6	3	17	*C. jejuni*
ST-61	2383	176	4	2	2	6	3	17	*C. jejuni*
ST-828	829	33	39	30	82	113	43	17	*C. coli*
ST-828	854	33	38	30	82	104	43	17	*C. coli*
ST-828	1016	33	38	30	82	118	43	17	*C. coli*
ST-828	1134	33	39	30	82	104	173	17	*C. coli*
ST-828	1191	33	39	30	82	189	47	17	*C. coli*
ST-828	1445	33	39	30	82	104	85	17	*C. coli*
ST-828	1595	33	38	30	79	104	43	17	*C. coli*
ST-828	2397	184	39	30	82	113	43	17	*C. coli*
ST-828	2534	33	38	65	79	113	47	17	*C. coli*
ST-828	3222	33	283	44	82	189	44	17	*C. coli*
ST-828	3224	33	285	30	322	104	85	17	*C. coli*
ST-828	3230	33	39	30	322	104	85	17	*C. coli*
ST-828	3232	32	39	30	82	104	324	17	*C. coli*
ST-828	3234	33	38	242	79	104	35	17	*C. coli*
ST-828	3297	33	66	30	82	104	173	17	*C. coli*
ST-828	3299	33	39	44	82	113	43	17	*C. coli*
U/A	1581	129	66	30	82	189	47	17	*C. coli*
U/A	2352	2	29	4	105	131	24	17	*C. jejuni*
U/A	3229	1	2	215	322	90	25	17	*C. jejuni*
U/A	3235	213	1	57	26	127	29	17	*C. jejuni*
U/A	3303	130	155	69	113	276	257	17	*C. coli*
U/A	3296	33	153	44	82	189	44	17	*C. coli*

4.3.2 Intra species HR in the New Zealand *Campylobacter* sample

Intra species HR was regularly observed in the New Zealand *Campylobacter* sample. To exclude possible vertical transfer as the method of inheritance, only the frequency of alleles between CCs, not within CCs were measured. Intra species HR between CCs was observed frequently in *C. jejuni*. HGT within *C. jejuni* was observed at each allele loci (examples shown in Table 4.3). The New Zealand *C. coli* population had few examples of intra species HGT due to the sample size and identified clonality of this species. Intra species HGT was seen in

C. coli at four allele loci *glnA*, *gltA*, *pgm* and *tkt* (*glnA*30, *glyA*82, *pgm*189, *tkt*35, *tkt*47 and *uncA*17).

Table 4.3: Intra species HGT within New Zealand *C. jejuni* isolates

aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	CC
37	4	4	48	13	25	23	2354	U/A
37	52	57	26	107	29	23	991	ST-692
37	253	75	48	126	25	3	2389	U/A
10	**2**	107	62	120	76	1	2535	U/A
1	**2**	3	4	5	9	3	42	ST-42
9	**2**	2	10	86	59	5	530	U/A
7	**2**	5	2	10	3	6	5	ST-353
9	**2**	4	62	4	5	6	257	ST257
1	**2**	215	322	90	25	17	3229	U/A
2	29	**4**	48	13	24	57	693	U/A
2	59	**4**	105	126	25	23	694	ST-1034
9	2	**4**	62	4	5	6	257	ST257
37	4	**4**	48	186	25	23	1607	U/A
175	251	**4**	105	10	25	102	2385	U/A
2	4	1	**2**	7	1	5	48	ST-48
1	4	2	**2**	6	3	17	61	ST-61
8	10	2	**2**	11	12	6	354	ST-354
9	2	4	**62**	4	5	6	257	ST-257
7	21	5	**62**	4	61	44	436	U/A
7	71	5	**62**	11	67	1	1972	U/A
10	2	107	**62**	120	76	1	2535	U/A
2	1	2	3	2	**3**	5	451	ST-21
1	4	2	2	6	**3**	17	61	ST-61
7	2	5	2	10	**3**	6	5	ST-353
9	25	2	10	22	**3**	6	52	ST-52
24	17	2	322	104	**3**	12	3227	ST-443
7	2	5	2	10	3	**6**	5	ST-353
9	25	2	10	22	3	**6**	52	ST-52
9	2	4	62	4	5	**6**	257	ST-257
8	10	2	2	11	12	**6**	354	ST-354
2	15	4	48	356	25	**6**	2382	U/A
173	4	1	68	11	290	**6**	2398	U/A

Bold indicates identical alleles in multiple STs; Green indicates *C. coli* genotypes, Blue indicates *C. jejuni* genotypes.

4.3.3 Comparison with the international *Campylobacter* database

Allelic diversity seen in isolates identified as *C. jejuni* and *C. coli* in the international MLST *Campylobacter* database were compared to allelic diversity present in the New Zealand *Campylobacter* isolates (Table 3.1). Large levels of genetic diversity were observed between the gene loci, for example when both *C. jejuni* and *C. coli aspA* alleles were combined 219 and 369 alleles were observed at the each loci, similarly the number of polymorphic sites within gene loci differed ranged from 247 (*glnA*) to 334 (*pgm*) (Table 3.1). The observations made in the New Zealand *Campylobacter* sample concerning the most frequently observed alleles at each gene locus, were similarly observed in the MLST *Campylobacter* database (Table 4.4). The most frequently identified alleles were *aspA*2 (n = 665) present in 19% of all *Campylobacter* isolates and *tkt*1 (n = 514) present in 15% of isolates within the international database (Figure 4.5). The majority of allele frequencies observed at each gene locus were similar between the New Zealand and international database samples.

Table 4.4: Most frequently identified alleles in the MLST *C. jejuni/C. coli* database

Gene loci	Allele	Number of alleles present in current samples	
		New Zealand (362 isolates)	International DB (3437 isolates)
aspA	*aspA*1	34 (9%)	221 (6%)
	*aspA*2	**119 (32%)**	665 (19.3%)
	*aspA*4	**43 (12%)**	202 (6%)
	*aspA*9	16 (4%)	190 (5.5%)
	*aspA*33	37 (10%)	**561 (16%)**
glnA	*glnA*1	84 (23%)	364 (10.5%)
	*glnA*2	29 (8%)	377 (11%)
	*glnA*7	42 (11.6%)	214 (6.2%)
	*glnA*38	13 (3.6%)	172 (5.0%)
	*glnA*39	44 (12.1%)	421 (12.2%)
gltA	*gltA*1	12 (3.3%)	240 (7.0%)
	*gltA*2	41 (11.3%)	427 (12.4%)
	*gltA*4	28 (7.7%)	225 (6.5%)
	*gltA*5	37 (10.2%)	441 (12.8%)
	*gltA*30	68 (18.7%)	551 (16.0%)

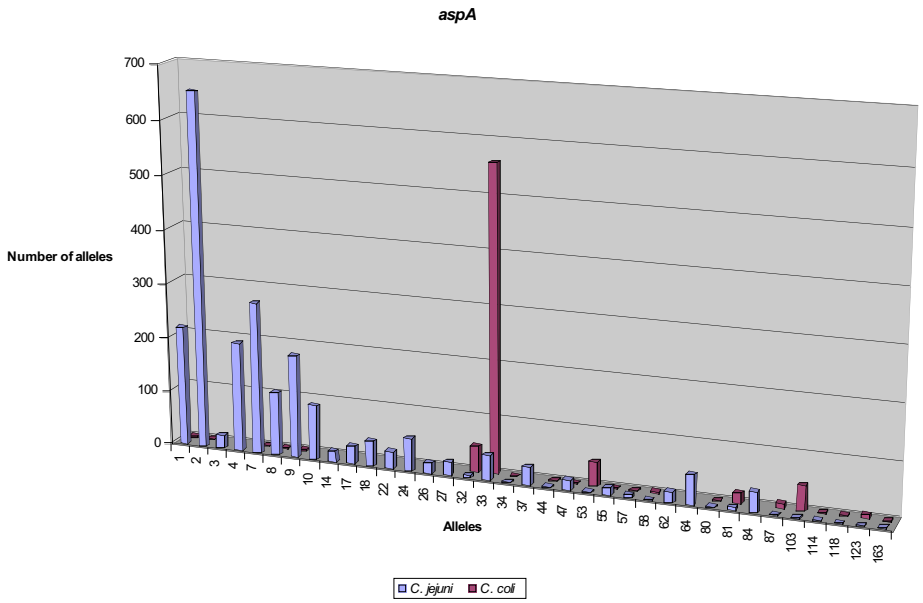
glyA	*glyA*2	47 (13%)	443 (12.8%)
	*glyA*3	76 (21%)	329 (9.6%)
	*glyA*4	53 (14.6%)	343 (9.9%)
	*glyA*79	5 (1.3%)	110 (3.2%)
	*glyA*82	**67 (8.1%)**	33 (0.96%)
pgm	*pgm*1	**36 (9.9%)**	140 (4.1%)
	*pgm*2	**89 (24.6%)**	411 (12.0%
	*pgm*6	24 (6.6%)	110 (3.2%)
	*pgm*104	32 (8.8%)	394 (11.4%)
	*pgm*113	24 (6.6%)	170 (4.9%)
tkt	*tkt*1	69 (19%)	514 (15%)
	*tkt*3	43 (12%)	514 (15%)
	*tkt*7	**44 (12.1%)**	179 (5.2%)
	*tkt*43	26 (7.1%)	231 (6.7%)
	*tkt*47	19 (5.2%)	134 (3.9%)
uncA	*uncA*1	49 (13.5%)	366 (10.6%)
	*uncA*3	14 (3.8%)	167 (4.8%)
	*uncA*5	**97 (27%)**	532 (15.5%)
	*uncA*6	29 (8%)	498 (14.5%)
	*uncA*17	**88 (24.3%)**	527 (15.3%)

Bold indicates frequencies of interest

Figure 4.5: Allelic diversity in international database

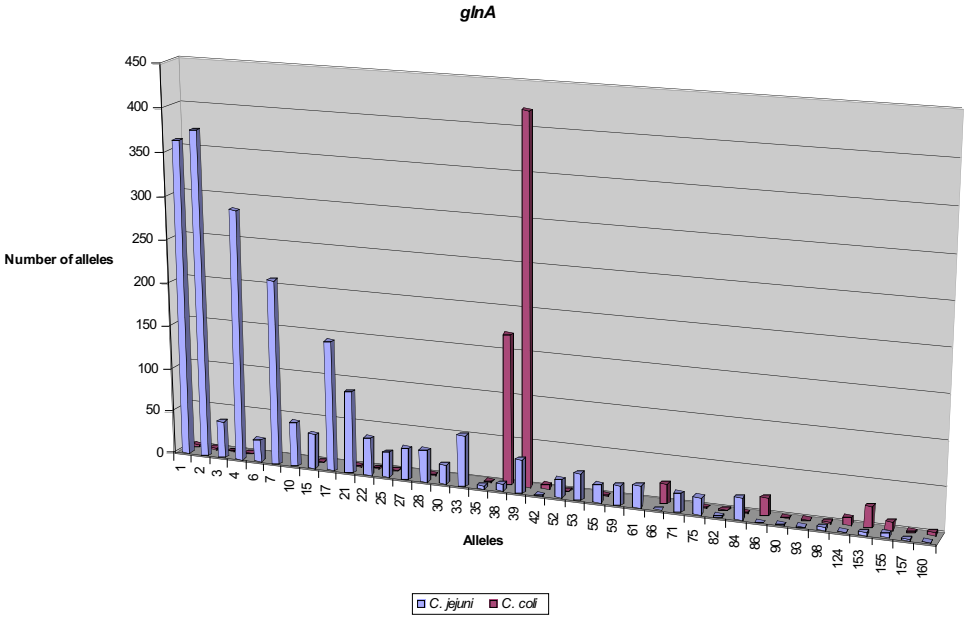
Allele frequencies for each of the seven housekeeping genes in the international database were calculated in START and graphed A-G. Intra species HGT was present at each loci characterised. Only alleles with frequencies higher than 20 alleles or the alleles appeared in both species are displayed

A. *aspA* locus



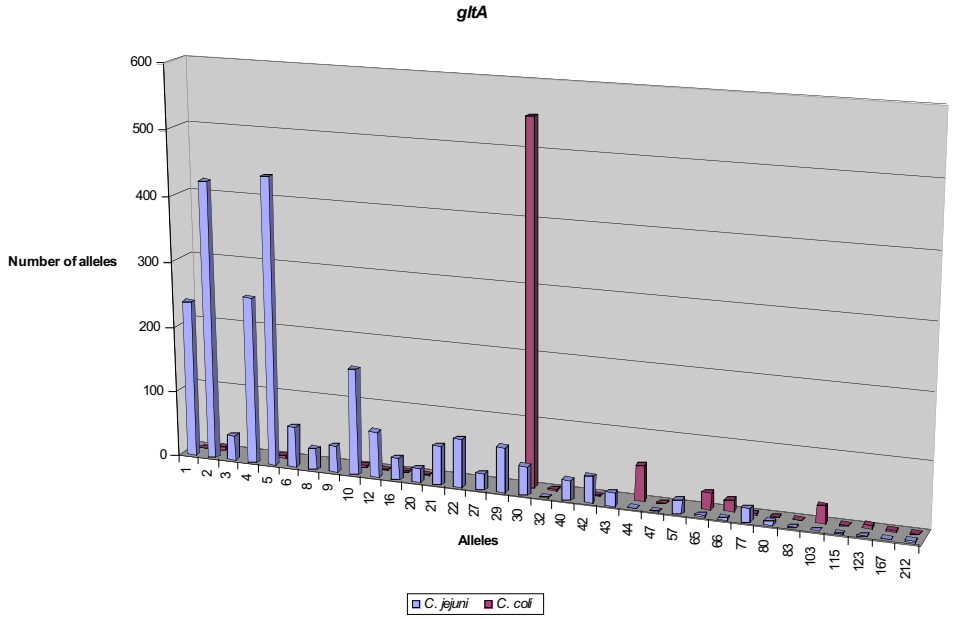
Note the HGT events at multiple alleles in this locus. Two alleles were dominant in the population *aspA*2 and *aspA*33 associated with *C. jejuni* and *C. coli* respectively.

B. *glnA* locus



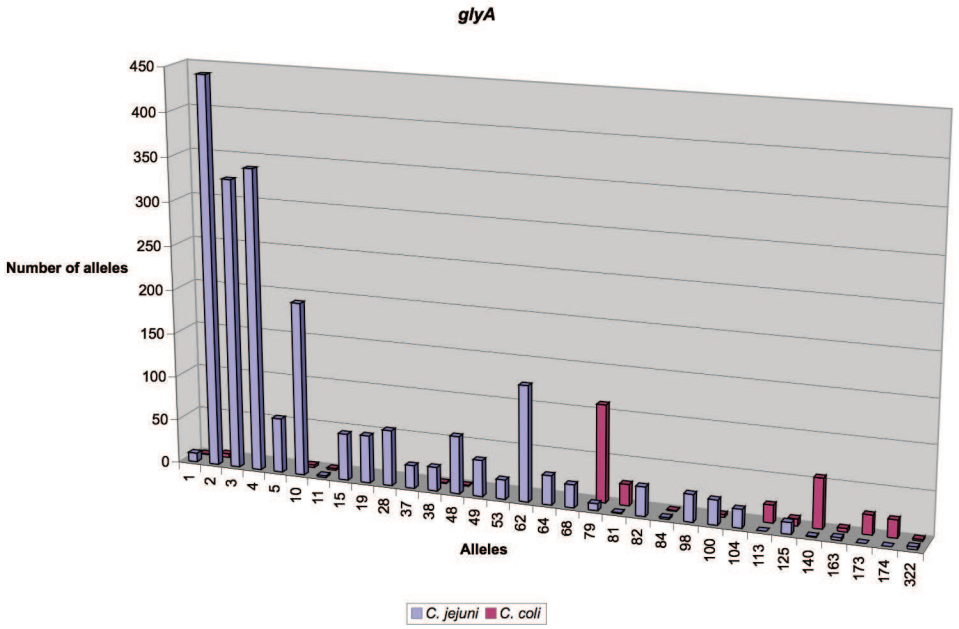
Note the HGT events at multiple alleles in this locus. Four alleles were considered dominant in the population *glnA*1, *glnA*2, *glnA*4 and *glnA*39.

C. *gltA* locus



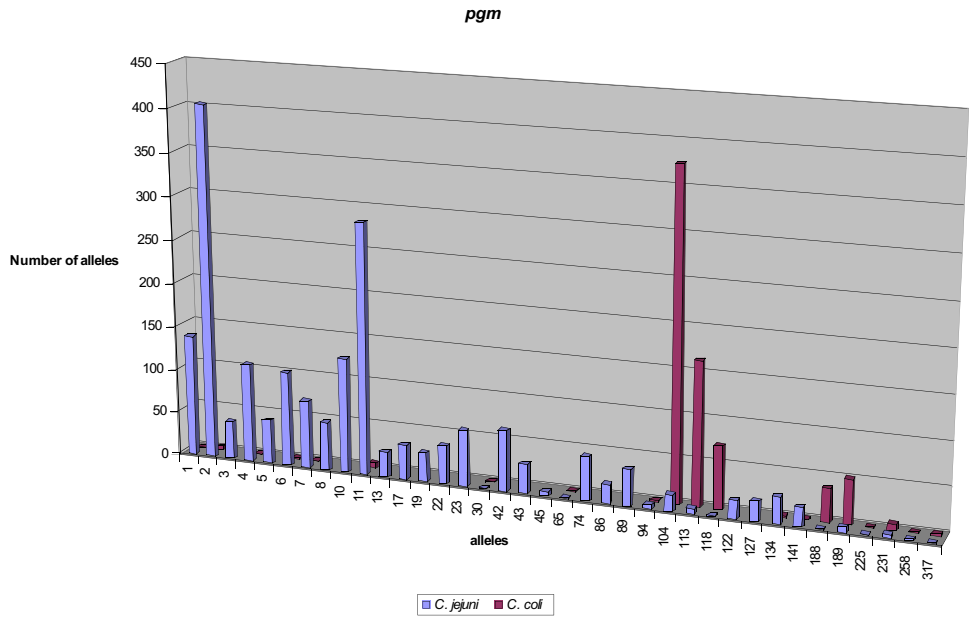
Note the HGT events at multiple alleles in this locus. Three alleles were considered dominant in the population *gltA*2, *gltA*5 and *gltA*30.

D. *glyA* locus



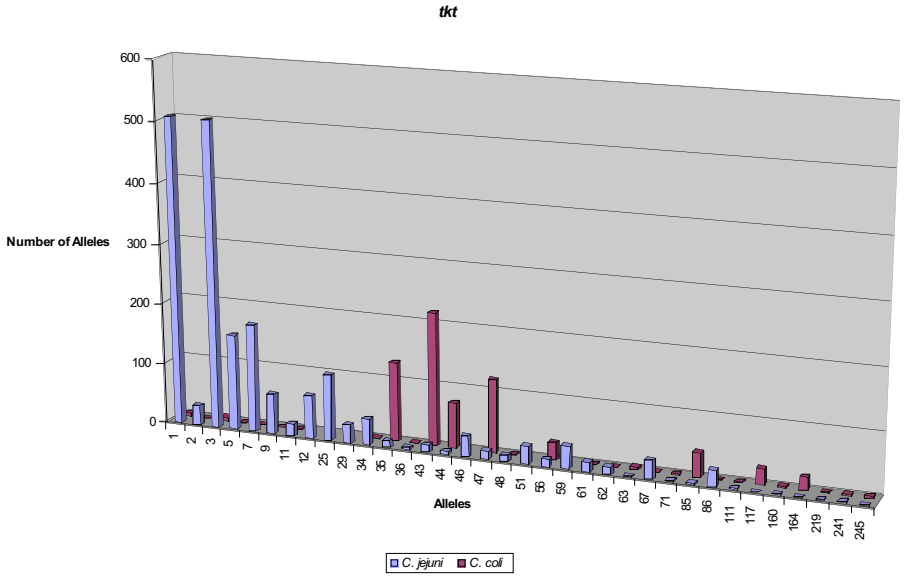
Note the HGT events at multiple alleles in this locus. Three alleles were considered dominant in the population *glyA*2, *glyA*3 and *glyA*4.

E. *pgm* locus



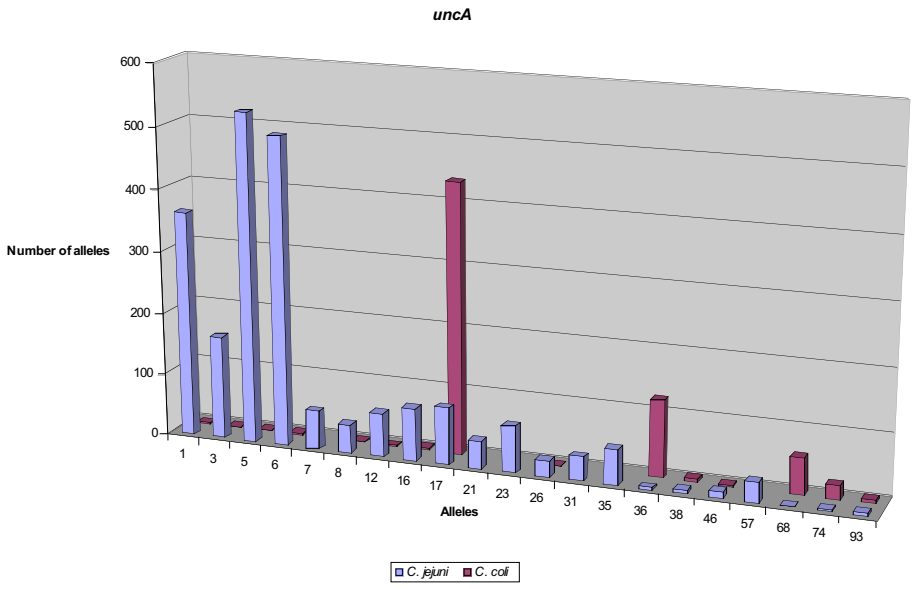
Note the HGT events at multiple alleles in this locus. Three alleles were considered dominant in the population *pgm*2, *pgm*11 and *pgm*104.

F. *tkt* locus



Note the HGT events at multiple alleles in this locus. Alleles *tkt*1 and *tkt*3 occurred at least twice a much as any other alleles present in the population.

G. *uncA* locus



Note the HGT events at multiple alleles in this locus. There were less HGT events occurring at this locus than other loci characterised, and less genotypes observed at frequencies greater than 20. Four alleles were dominant in the database *uncA*1, *uncA*5, *uncA*6 and *uncA*17.

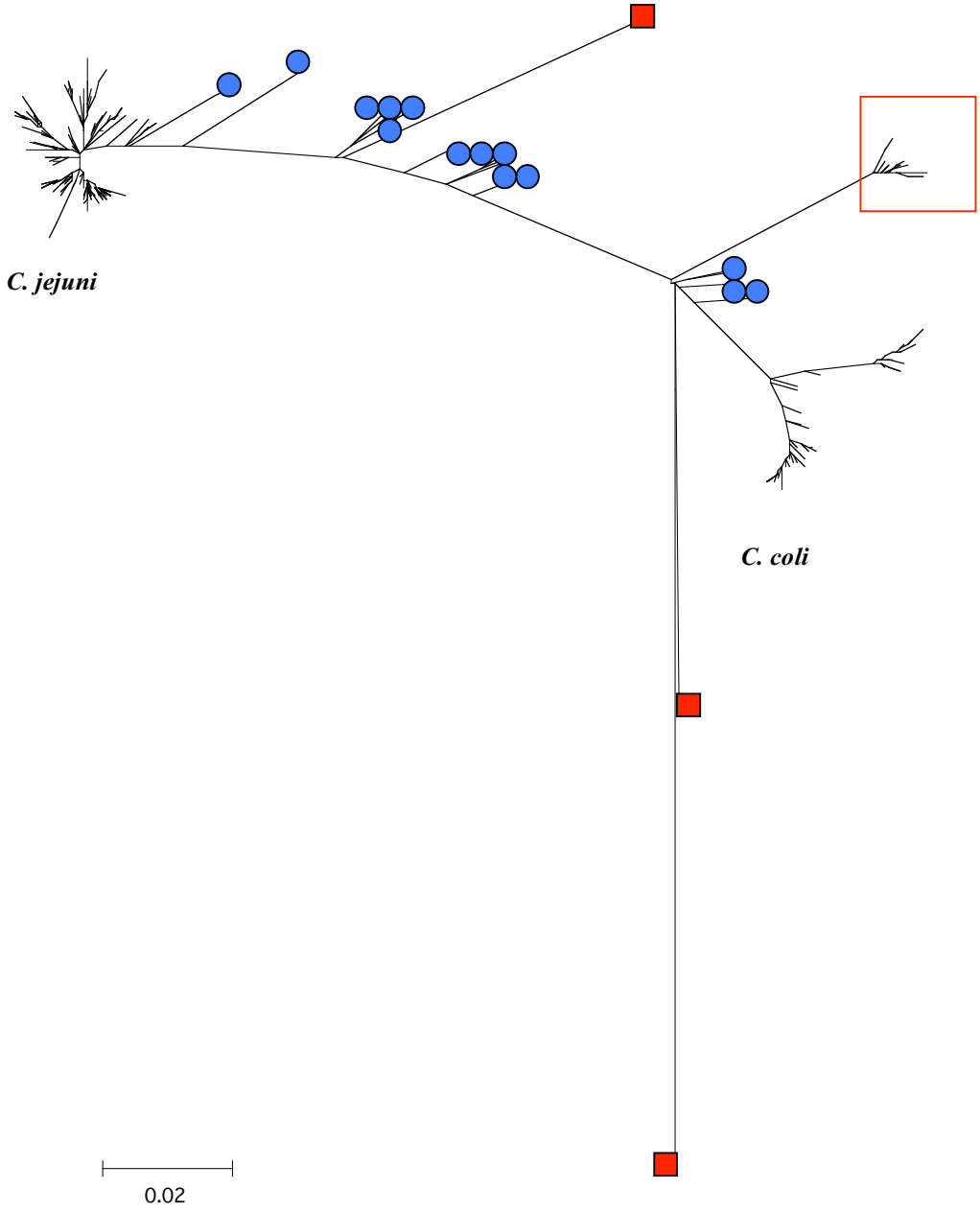
4.3.4 Recombination and allele diversity in international MLST database

Minimum evolution (ME) trees were constructed for each gene locus in the Mega v. 3.1 (126) program using the Kimura two-parameter model based on individual sequences downloaded from http://pubmlst.org and aligned using CLUSTALW found in MEGA v. 3.1 (126). The ME trees exhibited incongruence at each gene locus examined (Figure 4.6, A - G), alleles were observed at different intervals along the branch from *C. jejuni* to the *C. coli* clusters in all trees constructed. All allele loci sampled from the MLST database showed evidence of intra (Figure 4.6 blue circles) and inter species (Figure 4.6 red squares) recombination. The *aspA* locus contained the highest number of mosaic alleles, and the *glnA* locus the least. Groups of alleles at the *aspA* and *pgm* loci appeared to have arisen from HGT another *Campylobacter* species, (red rectangles Figure 4.6).

Figure 4.6: Allelic diversity in MLST database

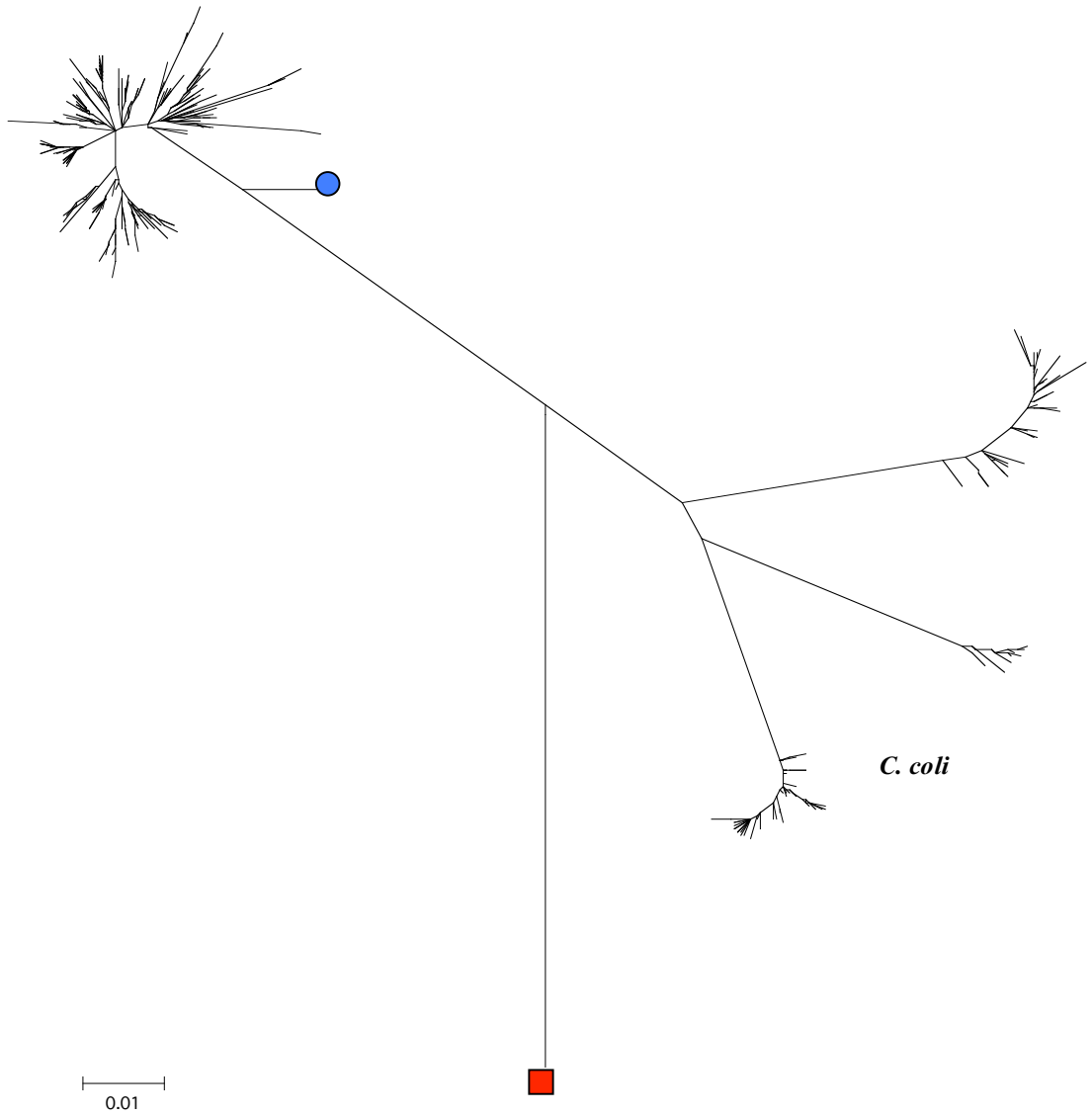
A ME tree, using the Kimura 2-parameter model and a bootstrap value of 1000, was constructed for each of the seven housekeeping gene loci. The ME trees indicated all alleles were closely related and the majority of allele loci produced congruent phylogenetic trees. Blue circles indicate recombinant alleles between *C. jejuni* and *C. coli*, red squares indicate possible HR events with other unidentified *Campylobacter* species, and red rectangles/squares with no fill indicate HGT events with species other than those characterised in the current study.

A. *aspA* locus



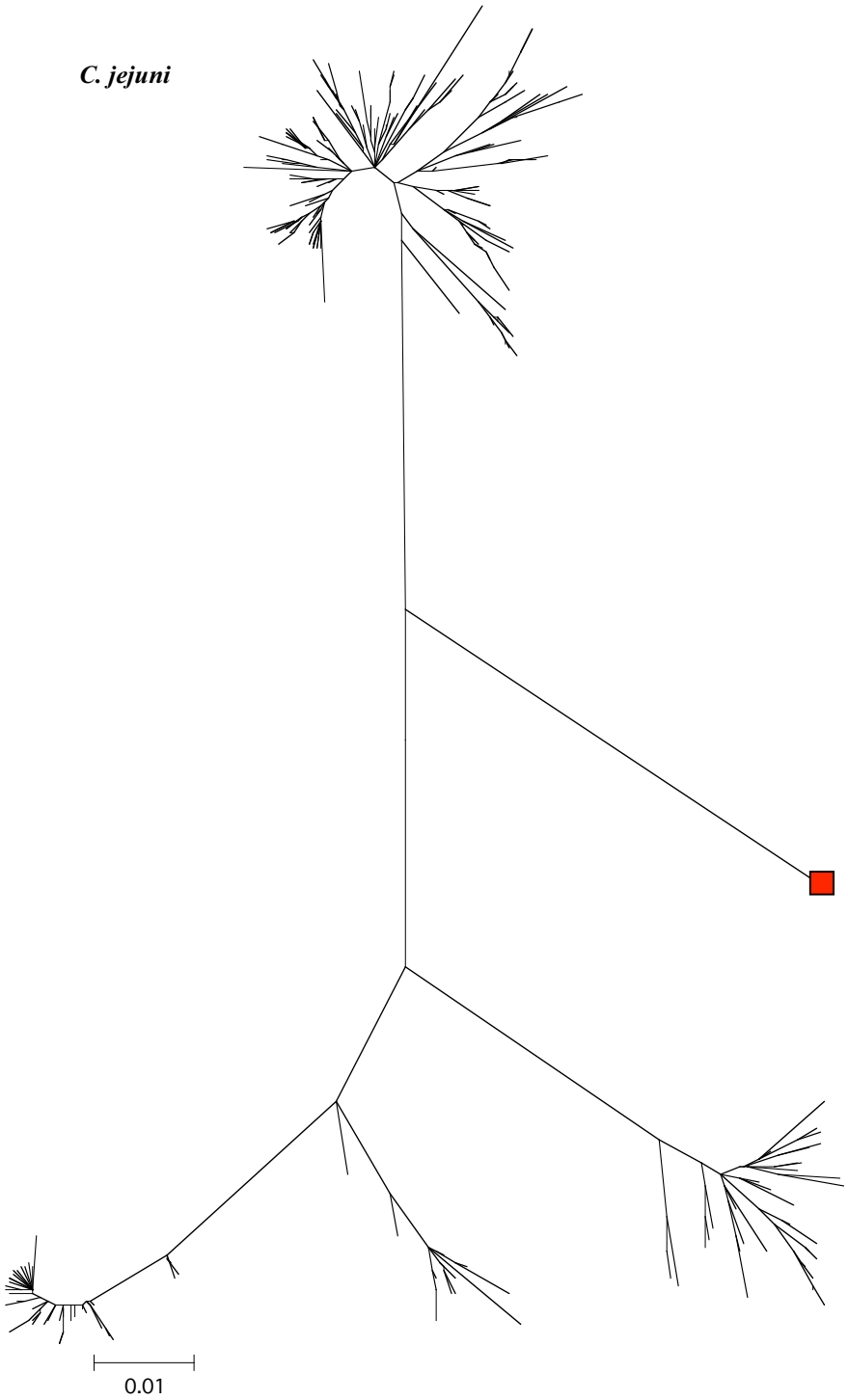
Note the HR events between *C. jejuni* and *C. coli*, but also the apparent either HGT and HR events between *C. jejuni* and *C. coli*, and other *Campylobacter* species.

B. *glnA* locus



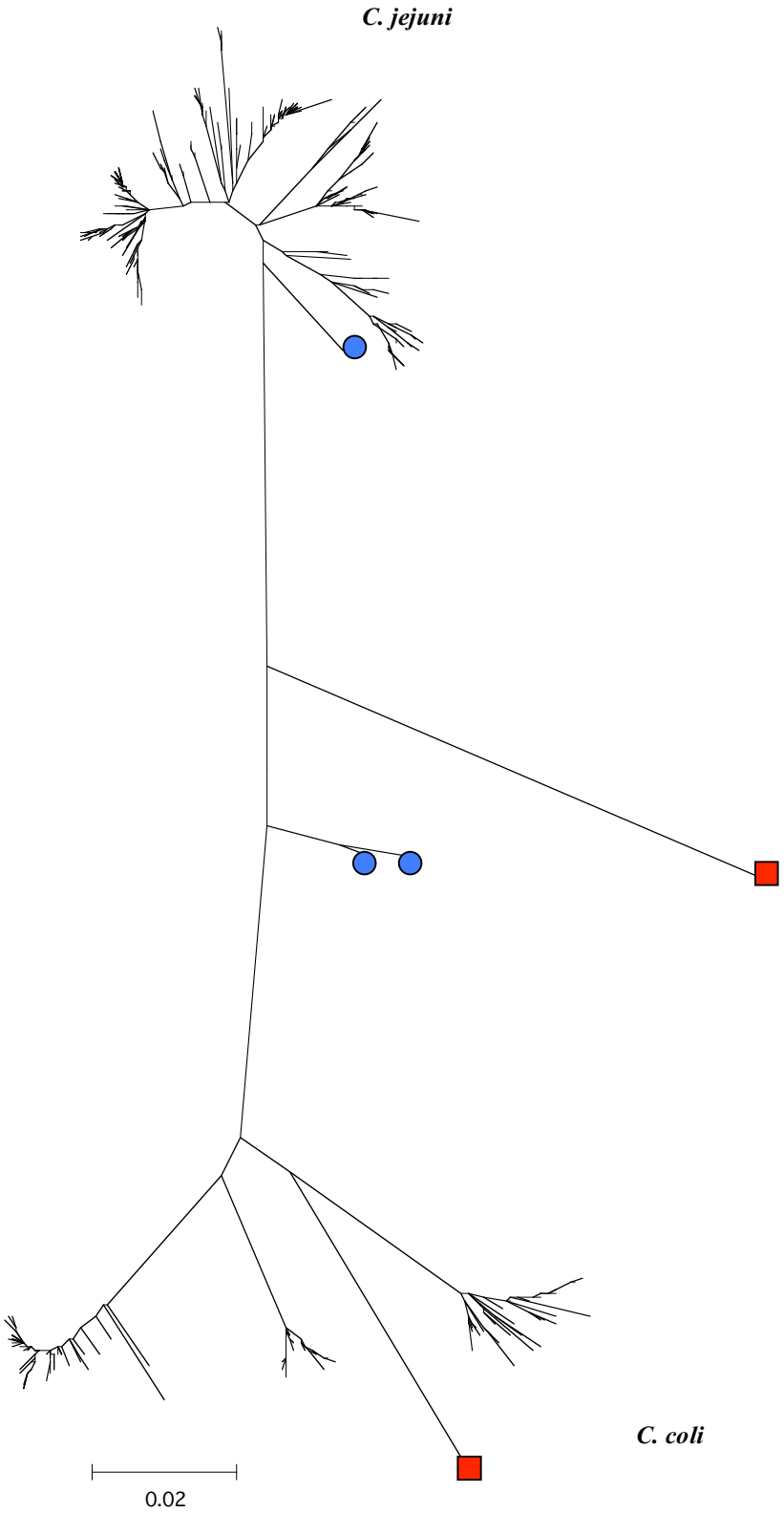
The *glnA* locus had few HR or HGT events compared to other loci in the MLST database. The *glnA* locus ME tree, with the two allele exceptions resolved into individual species.

C. *gltA* locus



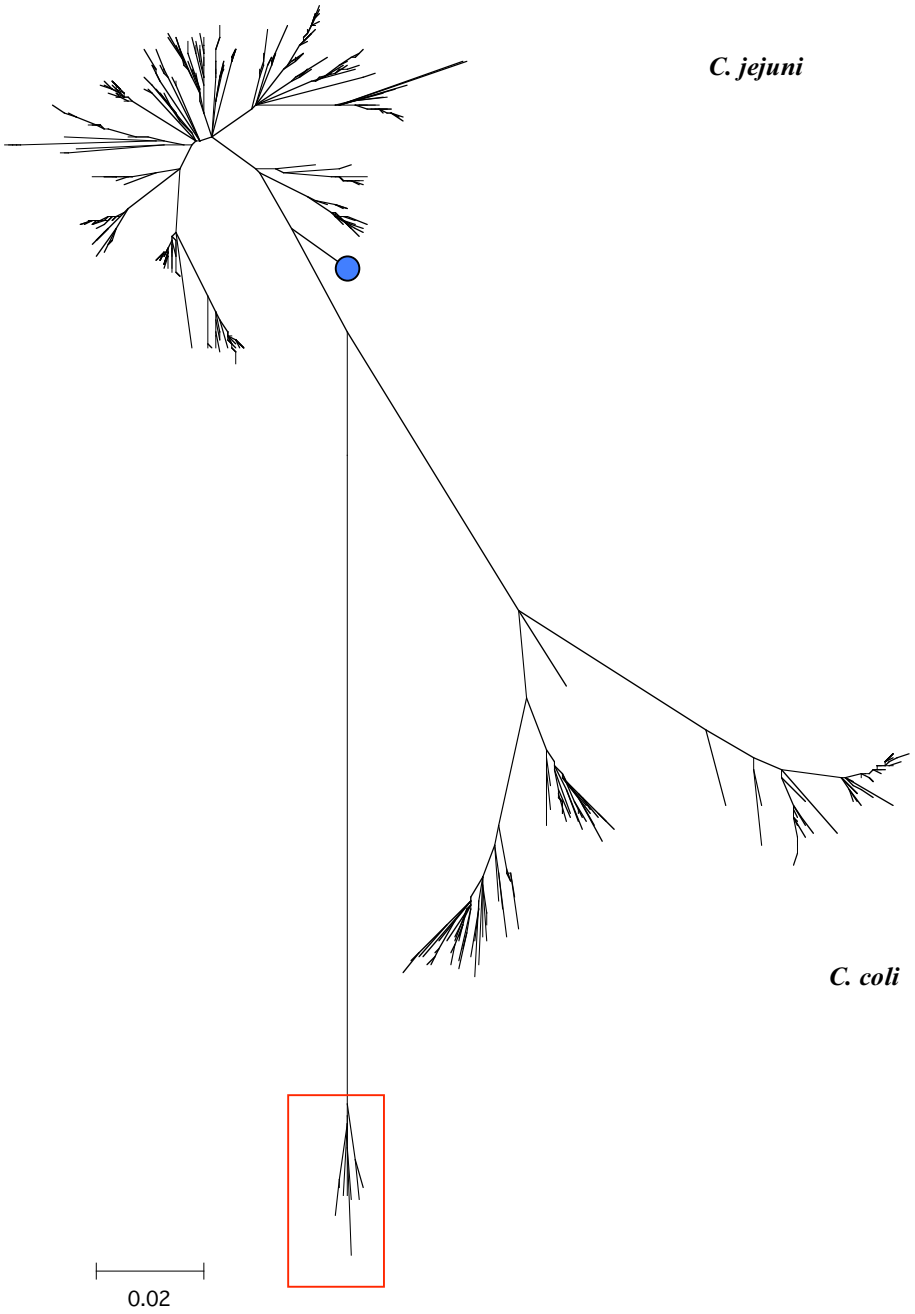
The *gltA* locus resolved into two species clusters with the exception of one HGT or HR event involving a *C. jejuni* isolate and another *Campylobacter* isolate.

D. *glyA* locus



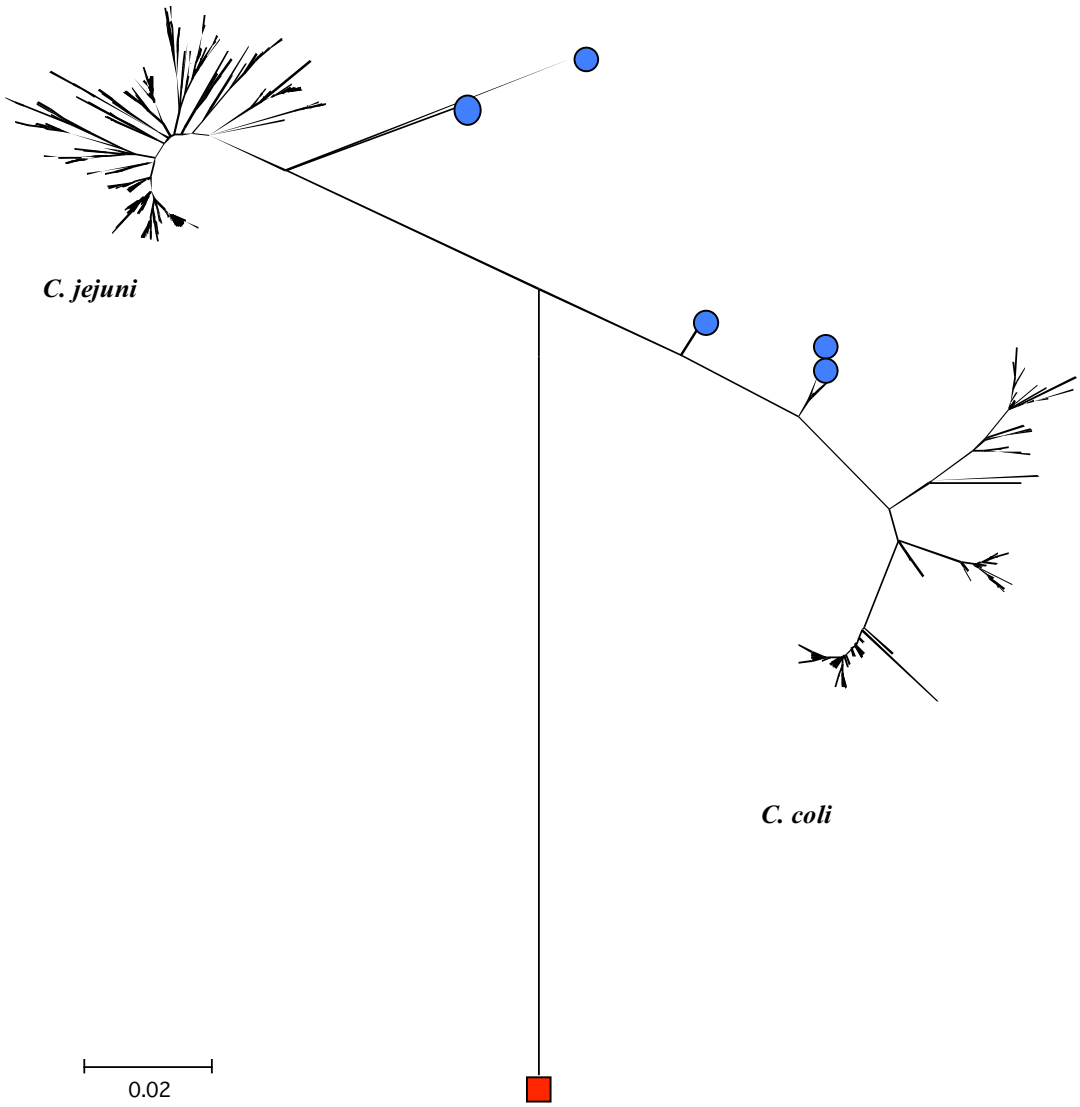
Multiple HR and HGT events were identified at the *glyA* locus, including at least two HGT events with other *Campylobacter* species.

E. *pgm* locus



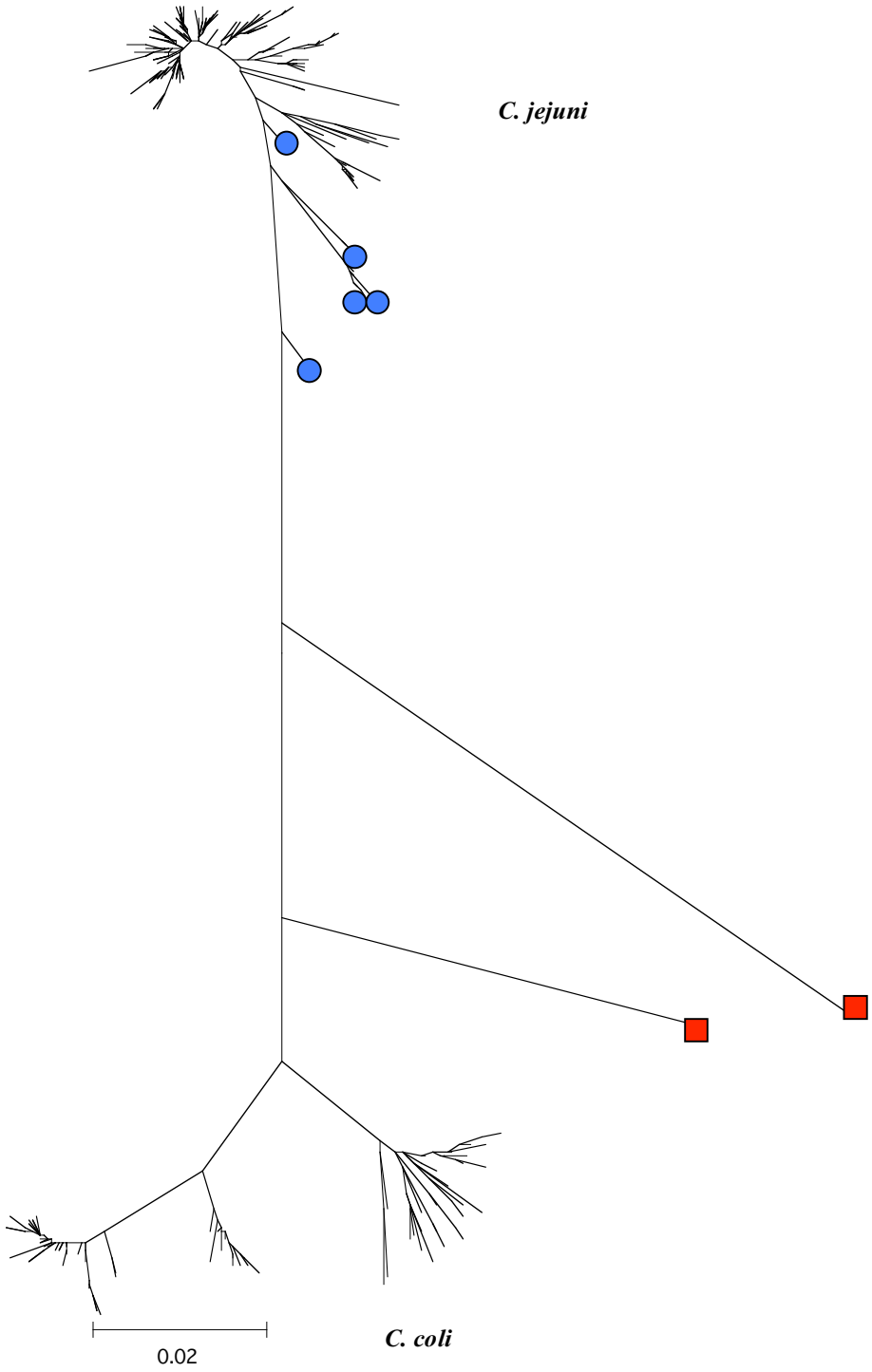
Note the red rectangle at this locus indicates a HGT event with *C. lari* identified as the contributing species identified by Miller (2005) *et al (* (149).

F. *tkt* locus



Multiple HR events were identified at the *tkt* locus, including at one HGT event with other *Campylobacter* species.

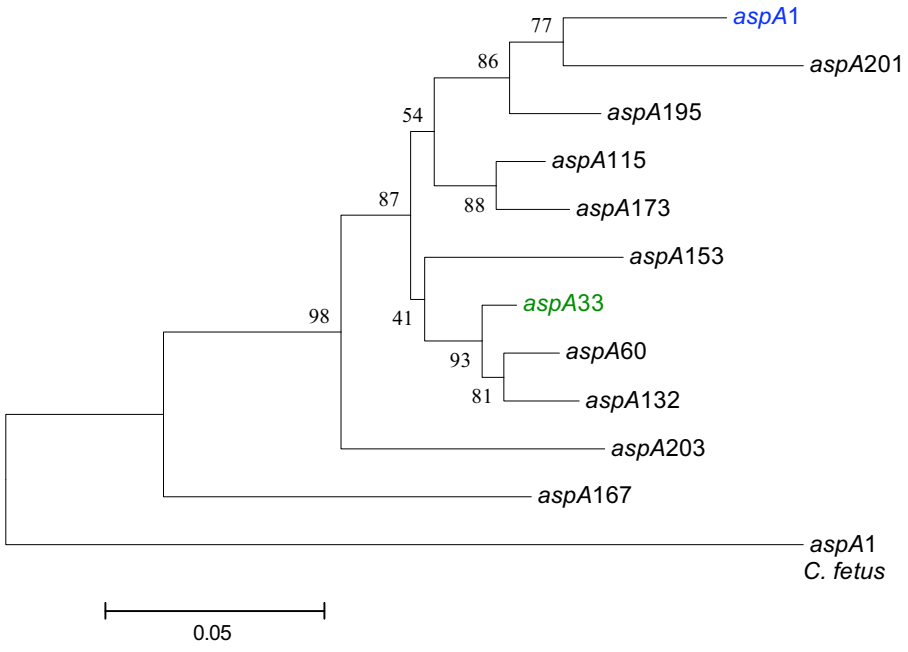
G. *uncA* locus



Multiple HR or HGT events were identified at the *uncA* locus, with two HR events with other unidentified *Campylobacter* species.

A selection of alleles from the *aspA* locus (Figure 4.6 A) found on the branch separating the two species clusters, were aligned with CLUSTALW in MEGA v 3.1 (126) and a ME tree constructed using *aspA*1 (*C. fetus*) as an outgroup (Figure 4.7). The distribution of allele sequences observed in Figure 4.7, suggests that *aspA*201, *aspA*195, *aspA*115, *aspA*173, *aspA*153, *aspA*60 and *aspA*132 were mosaic allele sequences derived from *C. jejuni* and *C. coli* parental strains, whereas *aspA*203 and *aspA*167 arose as a result of HGT with another undefined *Campylobacter* species. Maximum chi square analysis of selected *aspA* mosaic alleles (Table 5) indicated potential recombination sites between *C. jejuni* and *C. coli* in all alleles characterised.

Figure 4.7: *aspA* mosaic alleles



A ME tree (using the Kimura 2-parameter model) was constructed in MEGA v 3.1 (126) for a selection of *aspA* allele sequences which had been identified as either the result of HGT or HR events. *C. fetus* was used as an outgroup. Blue text indicates *C. jejuni* allele, green text indicates *C. coli* allele.

Table 4.4: Max Chi squared analysis of selected mosaic *aspA* alleles

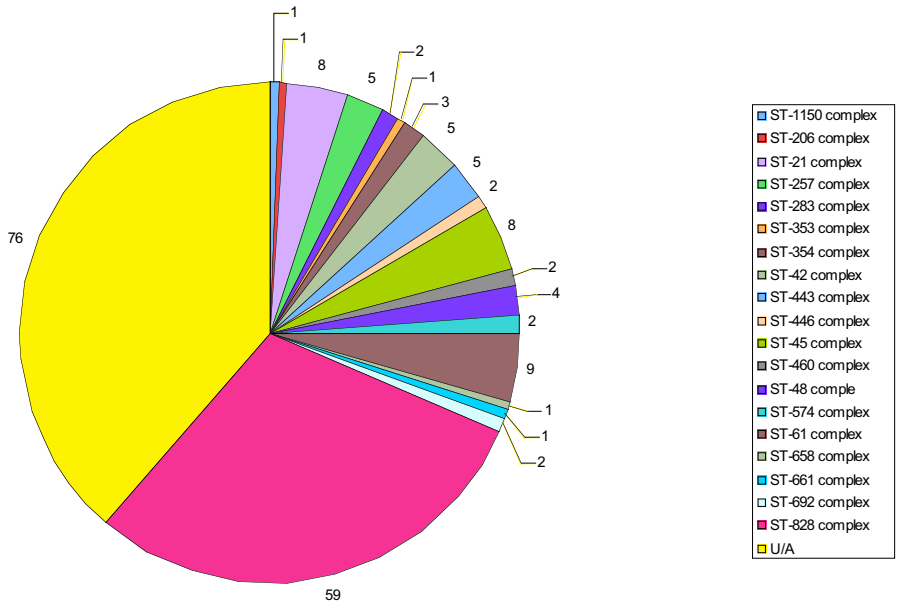
aspA allele	Max Chi^2	Parental sequences	Possible recombination site	Significance (P)
*aspA*60	49.59	*aspA*1/*aspA*33	After nucleotide 411	0.000
*aspA*115	52.16	*aspA*1/*aspA*33	After nucleotide 117	0.000
*aspA*132	32.86	*aspA*2/*aspA*33	After nucleotide 369	0.000
*aspA*195	13.68	*aspA*10/*aspA*33	After nucleotide 87	0.001

4.3.5 Recombination and ST diversity

In total 128 STs, 17 CCs and a large number of STs (n = 37) unassigned to CCs were identified in the New Zealand samples. Concatenated sequences of all STs identified in the New Zealand sample were aligned using CLUSTALW in MEGA v 3.1 (126), allowing a phylogenetic tree to be constructed using the minimum evolution method and the Kimura two-parameter model (Figure 4.9). A similar tree was constructed for concatenated sequences identified in the international database (Figure 4.10). Mosaic STs were observed in 6 *C. jejuni* (excluding ST-61, although ST-61 STs can be seen at the top of the box in Figure 4.9) and two *C. coli* isolates, these STs can be easily identified in the ME tree on the branch leading from the *C. jejuni* clusters to the *C. coli* clusters (Figure 4.9).

The international database has almost ten times more isolates than were present in the New Zealand *Campylobacter* sample, consequently a higher variety of both STs and CCs is present in the international database. The incongruence between species in Figure 4.10 suggests a large amount of HR is occurring at these gene loci. HR was observed both between species and within species at all gene loci sampled.

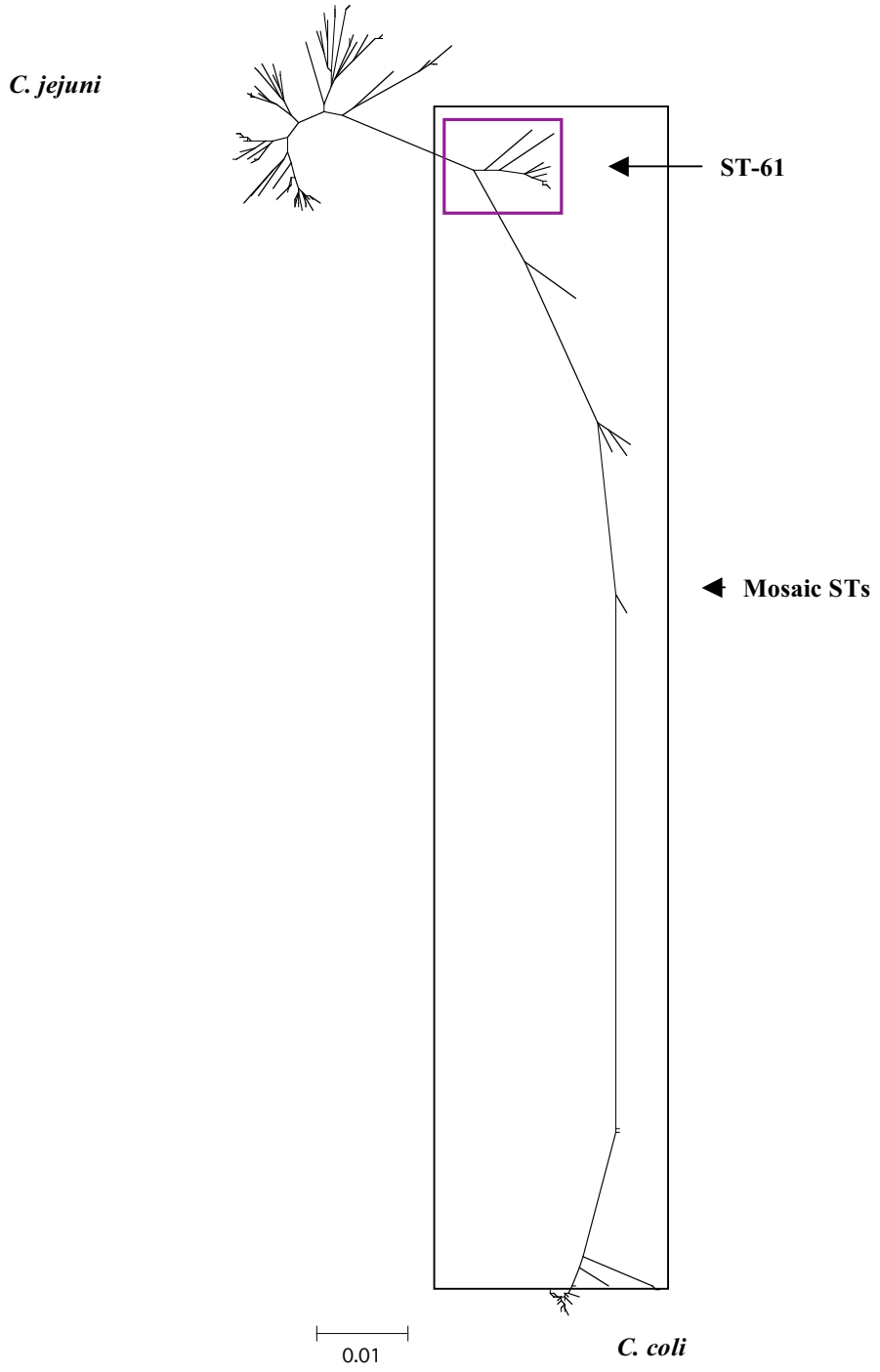
Figure 4.8: Distribution of mosaic STs in CCs



A pie chart was constructed to show the distribution of mosaic STs in different CCs represented in the international database. The majority of the mosaic STs arise out of the ST-828 complex and those STs not assigned to a clonal complex. The numbers represent the number of mosaic STs present in that CC.

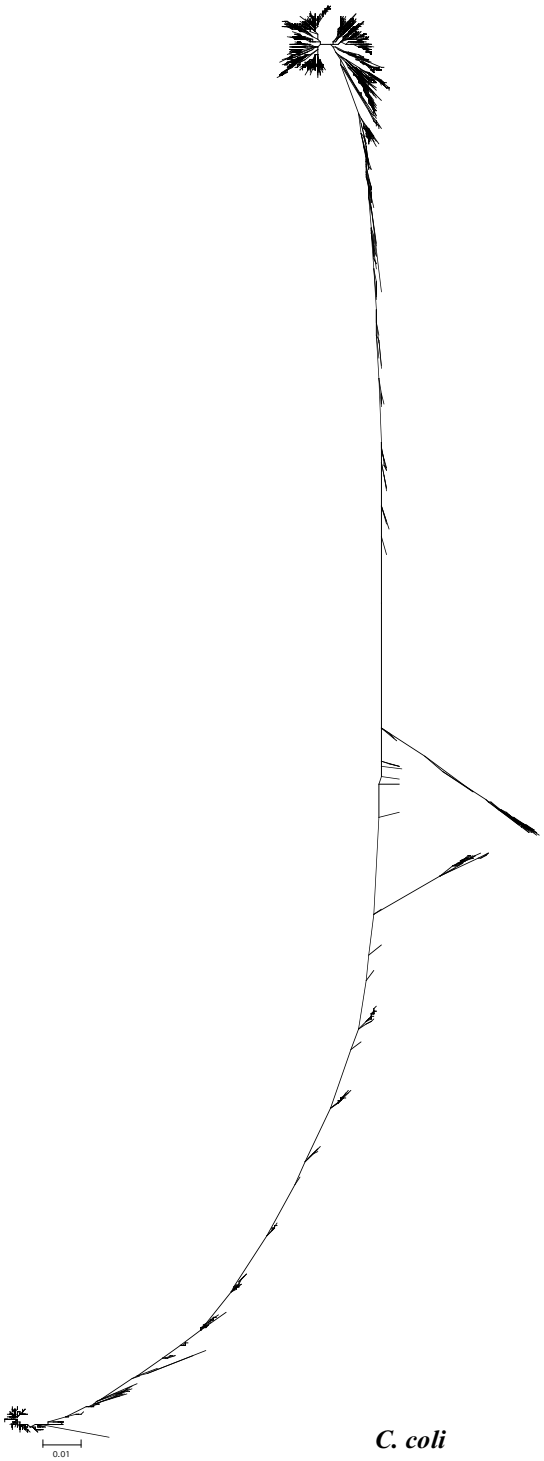
One hundred and ninety seven interspecies mosaic STs of *C. jejuni* and *C. coli*, were observed in the international database from a variety of CCs, excluding the majority of ST-61 isolates. Eighty *C. coli* isolates were observed to have between one and seven alleles associated with *C. jejuni* isolates (Table 4.5). Eight *C. jejuni* associated STs (ST-446, ST-61, ST-661, ST-574 and ST-257) were present in the *C. coli* mosaic sample speciated as *C. coli*. One hundred and twenty seven *C. jejuni* isolates were composed of between one and six *C. coli* alleles. Twenty one *C. jejuni* isolates were identified as having CC ST-828 genotypes, predominantly associated with *C. coli* isolates. CC ST-828 accounted for 40% of all mosaic STs identified in the MLST database.

Figure 4.9: ST diversity in New Zealand sample



A ME tree (Kimura-2 Parameter model) was constructed in Mega v 3.1 (126) using the concatenated sequences of the available STs identified as either *C. jejuni* or *C. coli* in New Zealand *Campylobacter* sample.

Figure 4.10: ST diversity observed in the international MLST database



A ME tree (Kimura-2 Parameter model) was constructed in Mega v 3.1 (126) using the concatenated sequences of the available STs identified as either *C. jejuni* or *C. coli* in the pubMLST database.

Table 4.5: Recombinant STs in MLST database

ST	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	Clonal Complex	Species
3104	184	39	1	82	7	43	5	U/A	*C. coli*
2316	1	4	2	2	6	286	17	ST-61 complex	*C. coli*
1244	1	1	2	2	225	3	17	ST-61 complex	*C. coli*
1993	7	53	2	10	11	3	17	ST-574 complex	*C. coli*
3032	9	2	2	38	113	5	6	ST-257 complex	*C. coli*
1129	103	110	2	140	104	164	79	ST-1150 complex	*C. coli*
2051	33	39	4	79	104	35	17	ST-828 complex	*C. coli*
2489	47	55	5	10	258	48	8	ST-446 complex	*C. coli*
2204	33	39	10	79	104	47	17	ST-828 complex	*C. coli*
2064	33	39	10	82	104	44	37	ST-828 complex	*C. coli*
3129	87	39	10	79	104	35	17	U/A	*C. coli*
1772	130	86	10	124	269	129	73	U/A	*C. coli*
2050	33	39	12	139	113	43	17	ST-828 complex	*C. coli*
1553	33	38	16	82	104	43	17	ST-828 complex	*C. coli*
2467	34	93	16	1	30	1	17	U/A	*C. coli*
2501	1	39	30	78	104	43	17	ST-828 complex	*C. coli*
2784	2	39	30	79	104	43	17	ST-828 complex	*C. coli*
3079	2	1	30	82	118	1	17	U/A	*C. coli*
3223	9	284	30	79	113	47	17	U/A	*C. coli*
2506	32	219	30	81	118	36	36	U/A	*C. coli*
3169	33	39	30	82	1	56	17	ST-828 complex	*C. coli*
1758	33	39	30	82	2	1	5	ST-828 complex	*C. coli*
1574	33	38	30	82	2	1	17	ST-828 complex	*C. coli*
2762	33	39	30	82	2	56	3	ST-828 complex	*C. coli*
1623	33	39	30	82	4	47	17	ST-828 complex	*C. coli*
2499	33	176	30	79	11	61	17	U/A	*C. coli*
2129	33	39	30	82	11	47	17	ST-828 complex	*C. coli*
2588	33	38	30	11	104	85	17	ST-828 complex	*C. coli*
1010	33	39	30	79	104	86	17	ST-828 complex	*C. coli*
2773	33	39	30	82	104	1	17	ST-828 complex	*C. coli*
2502	33	38	30	82	104	3	17	ST-828 complex	*C. coli*
3097	33	39	30	82	104	56	1	ST-828 complex	*C. coli*
1011	33	82	30	82	104	56	17	ST-828 complex	*C. coli*
2815	33	39	30	82	104	56	38	ST-828 complex	*C. coli*
3116	33	39	30	82	104	62	17	ST-828 complex	*C. coli*
935	33	39	30	115	104	35	42	ST-828 complex	*C. coli*
2587	33	39	30	13	113	43	17	ST-828 complex	*C. coli*
2241	33	39	30	79	113	1	17	ST-828 complex	*C. coli*
2621	33	39	30	81	113	7	17	ST-828 complex	*C. coli*
3096	33	3	30	82	113	3	17	ST-828 complex	*C. coli*
2503	33	39	30	82	113	3	17	ST-828 complex	*C. coli*
2816	33	39	30	82	113	43	38	ST-828 complex	*C. coli*
2623	33	39	30	82	113	56	12	ST-828 complex	*C. coli*
2470	33	28	30	82	113	56	17	ST-828 complex	*C. coli*
2003	33	39	30	82	113	214	38	ST-828 complex	*C. coli*
2617	33	39	30	82	189	43	42	ST-828 complex	*C. coli*
1655	33	39	30	82	189	219	17	ST-828 complex	*C. coli*
2608	33	124	30	139	189	47	38	U/A	*C. coli*
3177	33	39	30	272	189	56	38	U/A	*C. coli*

1681	53	38	30	81	118	71	36	U/A	*C. coli*
2194	57	39	30	79	104	35	17	ST-828 complex	*C. coli*
903	33	39	32	79	104	47	17	ST-828 complex	*C. coli*
1447	33	38	32	82	104	35	68	U/A	*C. coli*
2055	33	39	42	82	104	56	17	ST-828 complex	*C. coli*
3300	55	39	44	82	118	35	36	U/A	*C. coli*
1415	33	39	47	82	104	43	36	ST-828 complex	*C. coli*
1611	33	39	65	140	247	3	17	U/A	*C. coli*
1610	33	39	66	174	65	43	41	U/A	*C. coli*
2472	81	104	69	113	11	43	67	U/A	*C. coli*
2173	64	232	77	100	94	160	16	U/A	*C. coli*
958	2	75	80	48	142	34	1	ST-661 complex	*C. coli*
1420	32	39	115	115	104	85	17	U/A	*C. coli*
1757	103	110	122	140	104	168	46	U/A	*C. coli*
2474	81	39	128	82	113	47	38	U/A	*C. coli*
2020	138	166	144	262	230	191	67	U/A	*C. coli*
1576	128	175	152	197	245	208	133	U/A	*C. coli*
2485	123	21	175	82	273	234	185	U/A	*C. coli*
3123	163	215	186	220	286	208	155	U/A	*C. coli*
3109	123	90	187	223	317	245	198	U/A	*C. coli*
2002	121	213	190	218	299	241	175	U/A	*C. coli*
1982	148	206	195	224	282	239	190	U/A	*C. coli*
2491	64	22	212	100	134	233	16	U/A	*C. coli*
797	2	1	1	3	2	1	17	ST-21 complex	*C. jejuni*
2788	2	1	1	3	2	47	5	ST-21 complex	*C. jejuni*
2781	2	1	1	3	2	56	5	ST-21 complex	*C. jejuni*
2446	6	3	1	2	7	47	5	ST-48 complex	*C. jejuni*
2969	33	55	1	3	2	1	74	ST-21 complex	*C. jejuni*
3075	33	4	1	2	7	1	5	ST-48 comple	*C. jejuni*
3084	33	39	1	2	7	1	17	ST-48 complex	*C. jejuni*
1933	33	2	1	3	10	3	6	U/A	*C. jejuni*
2341	1	1	2	2	6	3	17	ST-61 complex	*C. jejuni*
425	2	2	2	2	2	3	17	ST-61 complex	*C. jejuni*
1940	2	217	2	5	2	268	5	U/A	*C. jejuni*
81	2	4	2	2	6	3	17	ST-61 complex	*C. jejuni*
500	3	4	2	2	6	3	17	ST-61 complex	*C. jejuni*
2596	4	7	2	10	11	3	17	ST-574 complex	*C. jejuni*
2029	7	1	2	2	4	3	17	ST-61 complex	*C. jejuni*
2693	7	4	2	2	6	3	17	ST-61 complex	*C. jejuni*
2592	7	17	2	15	23	3	17	ST-443 complex	*C. jejuni*
2609	7	17	2	15	23	43	12	ST-443 complex	*C. jejuni*
2459	7	17	2	15	23	56	12	ST-443 complex	*C. jejuni*
3211	10	23	2	19	6	18	17	U/A	*C. jejuni*
3212	19	4	2	2	6	3	17	ST-61 complex	*C. jejuni*
3227	24	17	2	322	104	3	12	ST-443 complex	*C. jejuni*
2979	33	10	2	10	1	12	6	ST-354 complex	*C. jejuni*
3140	33	2	2	2	10	3	1	U/A	*C. jejuni*
2124	33	17	2	15	23	3	12	ST-443 complex	*C. jejuni*
438	33	39	2	2	81	35	17	U/A	*C. jejuni*
2832	33	153	2	2	89	43	6	ST-460 complex	*C. jejuni*
2832	33	153	2	2	89	43	6	ST-460 complex	*C. jejuni*
155	34	4	2	43	13	3	6	ST-658 complex	*C. jejuni*

2243	57	17	2	2	11	47	6	ST-354 complex	*C. jejuni*
2268	103	84	2	10	119	178	26	U/A	*C. jejuni*
2888	114	2	2	2	11	5	6	ST-354 complex	*C. jejuni*
1013	1	2	3	4	5	9	17	ST-42 complex	*C. jejuni*
3197	1	4	3	4	5	9	17	ST-42 complex	*C. jejuni*
2664	1	2	3	4	5	56	3	ST-42 complex	*C. jejuni*
3008	33	2	3	4	5	9	3	ST-42 complex	*C. jejuni*
2352	2	29	4	105	131	24	17	U/A	*C. jejuni*
2456	9	2	4	62	4	3	17	ST-257 complex	*C. jejuni*
929	9	2	4	62	4	5	17	ST-257 complex	*C. jejuni*
2039	9	2	4	62	4	43	6	ST-257 complex	*C. jejuni*
2566	33	2	4	62	4	5	12	ST-257 complex	*C. jejuni*
3090	33	39	4	79	6	3	17	U/A	*C. jejuni*
3089	33	39	4	48	104	34	38	U/A	*C. jejuni*
2690	1	17	5	2	10	47	6	ST-353 complex	*C. jejuni*
2424	2	1	5	3	2	1	17	ST-21 complex	*C. jejuni*
3295	2	4	5	82	104	1	5	ST-206 complex	*C. jejuni*
2434	9	2	5	4	2	1	17	U/A	*C. jejuni*
2118	9	216	5	10	10	3	1	U/A	*C. jejuni*
158	10	27	5	44	52	41	17	U/A	*C. jejuni*
2907	47	55	5	10	23	120	17	ST-446 complex	*C. jejuni*
327	51	37	5	65	2	56	5	U/A	*C. jejuni*
2052	2	4	6	2	7	219	5	ST-48 complex	*C. jejuni*
1760	7	4	6	68	93	3	17	U/A	*C. jejuni*
2803	7	4	6	68	188	3	46	U/A	*C. jejuni*
1686	118	3	6	4	3	3	17	ST-22 complex	*C. jejuni*
726	2	2	10	4	7	71	1	U/A	*C. jejuni*
2590	4	7	10	4	5	47	1	ST-45 complex	*C. jejuni*
2600	4	7	10	4	42	43	1	ST-45 complex	*C. jejuni*
2691	4	7	10	4	42	47	1	ST-45 complex	*C. jejuni*
1685	4	7	10	4	121	7	17	ST-45 complex	*C. jejuni*
714	33	7	10	4	1	7	1	ST-45 complex	*C. jejuni*
3010	33	39	10	4	1	7	1	ST-45 complex	*C. jejuni*
3091	33	39	10	4	1	7	17	ST-45 complex	*C. jejuni*
2989	33	7	10	4	42	51	1	ST-283 complex	*C. jejuni*
3071	184	7	10	4	1	7	1	ST-45 complex	*C. jejuni*
1754	33	39	12	3	2	56	5	U/A	*C. jejuni*
3202	2	1	21	82	2	1	5	ST-21 complex	*C. jejuni*
348	33	1	21	3	2	1	5	ST-21 complex	*C. jejuni*
3086	33	39	21	79	2	123	17	U/A	*C. jejuni*
803	18	85	22	104	113	105	6	U/A	*C. jejuni*
802	18	100	22	104	113	105	6	U/A	*C. jejuni*
3200	1	157	30	4	5	9	17	ST-42 complex	*C. jejuni*
2746	1	39	30	82	104	3	3	ST-828 complex	*C. jejuni*
2777	1	39	30	82	104	9	3	ST-828 complex	*C. jejuni*
1869	2	1	30	5	2	1	5	ST-21 complex	*C. jejuni*
2774	2	39	30	82	104	56	17	ST-828 complex	*C. jejuni*
150	4	38	30	15	12	44	1	U/A	*C. jejuni*
151	4	42	30	4	51	43	1	U/A	*C. jejuni*
2565	4	1	30	82	113	44	17	U/A	*C. jejuni*
1682	10	186	30	62	260	223	6	U/A	*C. jejuni*
127	30	37	30	32	11	35	6	U/A	*C. jejuni*

139	32	38	30	41	49	35	28	U/A	*C. jejuni*
646	33	39	30	82	1	47	17	ST-828 complex	*C. jejuni*
3117	33	39	30	79	2	35	17	ST-828 complex	*C. jejuni*
3077	33	39	30	82	2	56	17	ST-828 complex	*C. jejuni*
57	33	39	30	2	6	47	17	ST-828 complex	*C. jejuni*
1529	33	39	30	82	7	47	17	ST-828 complex	*C. jejuni*
437	33	39	30	79	39	43	17	ST-828 complex	*C. jejuni*
138	33	39	30	2	48	35	17	ST-828 complex	*C. jejuni*
2502	33	38	30	82	104	3	17	ST-828 complex	*C. jejuni*
2660	33	39	30	82	104	56	65	ST-828 complex	*C. jejuni*
2669	33	160	30	272	104	56	17	ST-828 complex	*C. jejuni*
648	33	39	30	82	113	47	1	ST-828 complex	*C. jejuni*
647	33	39	30	82	113	47	3	ST-828 complex	*C. jejuni*
2689	33	39	30	82	189	56	17	ST-828 complex	*C. jejuni*
2665	33	39	30	82	189	56	38	ST-828 complex	*C. jejuni*
1362	33	124	30	84	189	47	74	U/A	*C. jejuni*
445	53	38	30	81	65	71	36	U/A	*C. jejuni*
2527	81	155	30	82	231	3	93	U/A	*C. jejuni*
2528	81	155	30	163	231	1	93	U/A	*C. jejuni*
2526	81	155	30	163	231	3	93	U/A	*C. jejuni*
2533	81	155	30	163	231	190	93	U/A	*C. jejuni*
1084	87	39	30	82	104	44	74	ST-828 complex	*C. jejuni*
2684	87	39	30	82	189	43	17	ST-828 complex	*C. jejuni*
2697	165	39	30	82	118	35	17	ST-828 complex	*C. jejuni*
357	3	39	32	79	79	43	17	U/A	*C. jejuni*
625	58	7	40	4	42	3	38	ST-283 complex	*C. jejuni*
2799	7	78	42	82	106	12	8	U/A	*C. jejuni*
1366	33	2	42	4	90	25	8	U/A	*C. jejuni*
284	54	39	48	3	2	44	5	U/A	*C. jejuni*
3284	7	17	52	10	89	164	6	U/A	*C. jejuni*
2622	33	115	57	26	127	29	17	ST-692 complex	*C. jejuni*
3235	213	1	57	26	127	29	17	ST-692 complex	*C. jejuni*
555	33	39	65	79	111	7	17	U/A	*C. jejuni*
2363	33	39	66	82	2	1	174	U/A	*C. jejuni*
3092	33	38	66	82	5	43	17	ST-828 complex	*C. jejuni*
1457	2	165	73	147	220	190	104	U/A	*C. jejuni*
2402	27	255	77	18	25	160	16	U/A	*C. jejuni*
886	53	38	83	82	104	43	17	ST-828 complex	*C. jejuni*
1254	64	93	100	3	175	143	16	U/A	*C. jejuni*
1349	84	140	115	144	199	136	87	U/A	*C. jejuni*
2657	81	155	167	277	338	43	17	U/A	*C. jejuni*
3109	123	90	187	223	317	245	198	U/A	*C. jejuni*
3168	163	215	210	218	4	241	198	U/A	*C. jejuni*
2681	163	215	210	218	324	241	198	U/A	*C. jejuni*
3229	1	2	215	322	90	25	17	U/A	*C. jejuni*

C. coli associated CC

C. coli associated allele

Allele found in equal numbers in both *C. jejuni* and *C. coli*

C. jejuni associated CC

C. jejuni associated allele

*Full details of the Mosaic STs can be found in Appendix 3

4.3.6 Linkage Disequilibrium

Linkage disequilibrium was calculated for the New Zealand sample and the MLST database sample using the START programme. Both sample groups were examined for linkage disequilibrium, in all groups the observed variance (V_O) was greater than the maximum variance obtained in 1000 trials, implying significant linkage disequilibrium was present.

Table 4.6: Linkage Disequilibrium

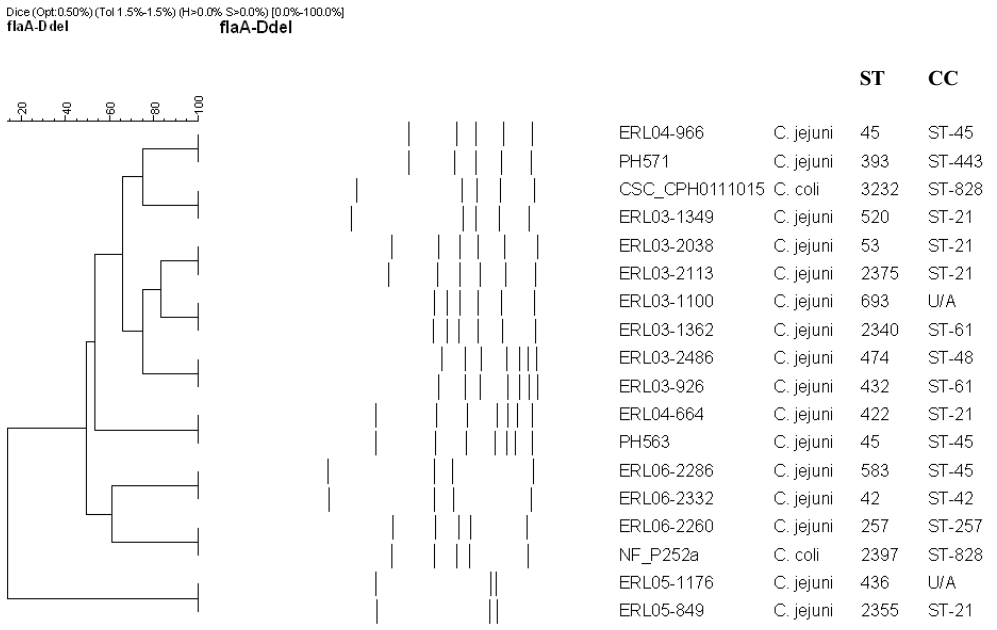
Species	No of isolates	Ve	Vo	IA
MLST Database				
C. jejuni	2559	0.5406	1.0379	0.9201
C. coli	878	1.3453	2.5289	0.8798
New Zealand				
C. jejuni	276	1.4438	3.3943	1.351
C. coli	83	1.3453	3.7949	3.185

Vo = Observed variance, Ve = Expected variance, I_A = Index of association

4.3.7 *flaA* genotyping

Three hundred and thirty nine *Campylobacter* isolates in the current New Zealand sample were characterised by *flaA* PCR RFLP and 88 *flaA* genotypes were identified. The most commonly identified *flaA* types in the New Zealand sample were *flaA*20 (n = 32), *flaA*36 (n = 32) and *flaA*60 (n = 33).

Figure 4.11: Inter and Intra species recombination at the *flaA* locus

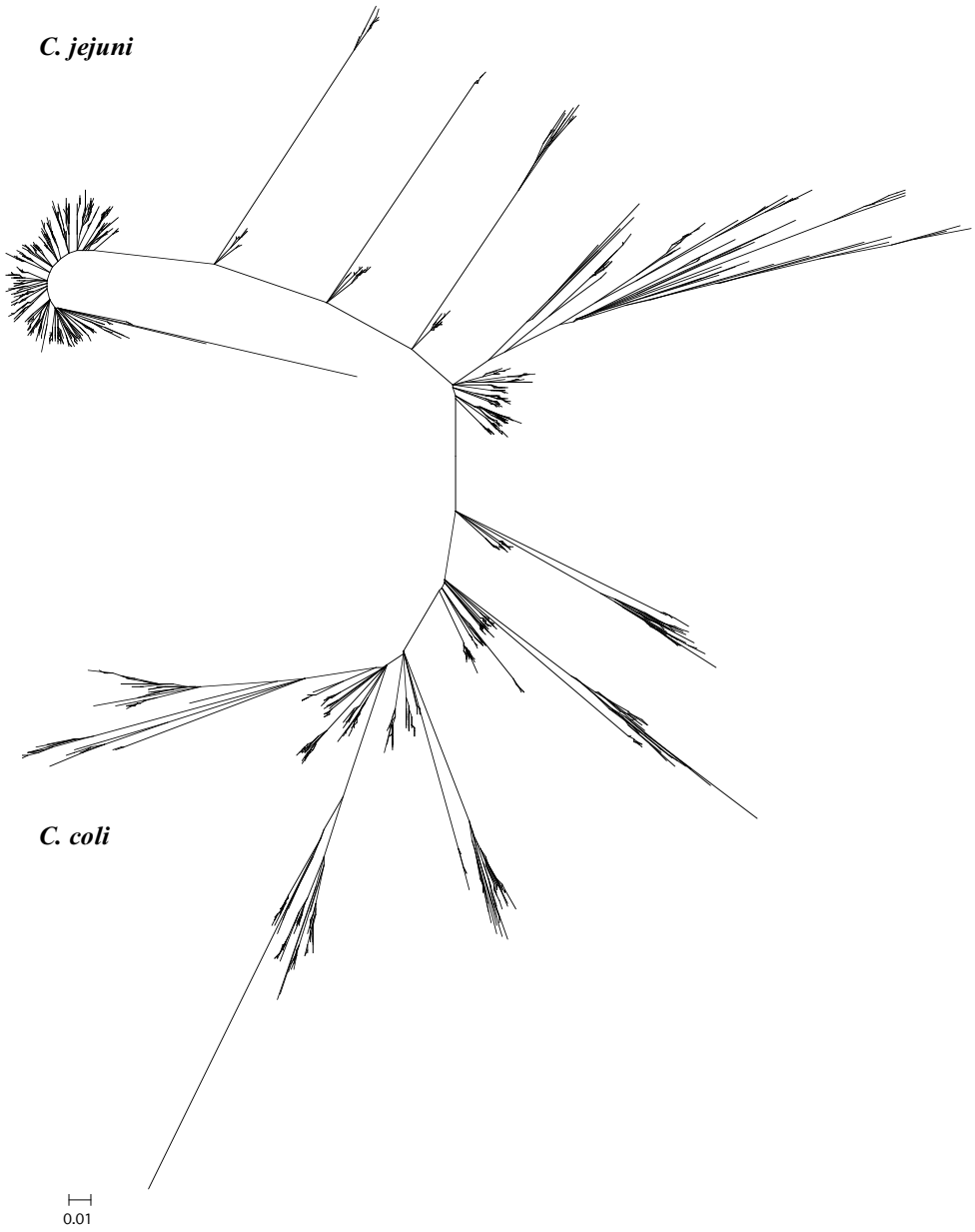


An UPMGA tree was constructed using the Dice coefficient of *flaA* PCR RFLP profiles showing inter and intra species HGT at the *flaA* locus.

HGT was observed at the *flaA* locus between *C. coli* and *C. jejuni.* The *flaA*60 genotype was associated with *C. jejuni* ST-257 and *C. coli* ST-2397, and the *flaA*28 genotype was associated with *C. jejuni* ST-520 and *C. coli* ST-3232 (Figure 4.11). Intra and inter species HGT was observed in the New Zealand *Campylobacter* sample between multiple isolates and STs (Figure 4.11). *C. jejuni* isolates accounted for the majority of HGT events at this locus. No international database of RFLP fingerprints for the *flaA* locus was available for comparison with the current *flaA* genotypes, however examination of the *flaA* short variable region (SVR) database was possible. A ME tree of all available *flaA* SVR sequences was constructed and compared to the MLST housekeeping genes (Figure 4.12). Although *flaA* SVR database isolates (n = 1014) clustered into *C. jejuni* and *C. coli* groups, evidence of HR at this locus was frequently observed. The Tajima's D statistic was calculated for the *flaA* locus based on the

international *flaA* SVR database data. An unexpected negative value of (-0.78307) for Tajima's D statistic was calculated in dnaSP (182) for this locus.

Figure 4.12: *flaA* SVR genotypes in the international *flaA* SVR database



A ME tree (using the Kimura 2-parameter model) of all *flaA* SVR sequences in the *flaA* SVR database (n = 1014) was constructed in MEGA v3.1. Note the much increased spread of alleles between the two species clusters.

4.4 Discussion

The genetic plasticity of *Campylobacter*, *C. jejuni* in particular, is well documented, with many studies describing the underlying mechanisms involved in the generation of genetic variability (8, 16, 53, 70, 96, 119, 151, 225). Given the instability of the *C. jejuni* genome, proven natural transformability (222), and occupation of identical ecological niches, it is unsurprising that HGT and HR have been identified between *C. jejuni*, *C. coli* and *C. lari* (149). Both interspecies and intraspecies HR and HGT, have been identified in *C. jejuni* and *C. coli* at multiple loci including both housekeeping (*aspA*, *pgm* and *uncA*) and non-housekeeping genes (*tetO* and *flaA*). HR and HGT has been identified in the flagellin genes *flaA* and *flaB* (96, 224), and HGT has also been identified at the *tetO* (16), *aspA* (148), *pgm* (149) and *uncA* (47) loci. The development of *C. jejuni* and *C. coli* MLST schemes to genotype *Campylobacter* isolates allows the accurate characterisation of geographically and temporally diverse *Campylobacter* populations (136). MLST assumes that if HR or HGT events were to occur the use of multiple gene loci should decrease the impact of those events and the relationships observed between bacterial isolates would still be valid (135).

Eight interspecies mosaic STs, one mosaic gene (*aspA*173) (Chapter 5) and one large deletion in an *aspA* allele (PH526) (Chapter 3) were observed in the New Zealand *Campylobacter* sample in the current study. Interspecies HR and HGT had been identified in *Campylobacter* MLST housekeeping genes, however the interspecies mosaic STs were unexpected as the number and identity of loci involved were different to previously reported (47, 70, 148, 149, 210). The identification of a large deletion in *C. jejuni* isolate PH526 was also unexpected;

the allele was the first identification of a large deletion in an *aspA* allele. These findings caused an examination of the entire *C. jejuni* and *C. coli* MLST database for similar HGT or HR events, potentially influencing how *Campylobacter* isolates were characterised.

4.4.1 Evidence of homologous recombination in *Campylobacter* housekeeping genes

Feil *et al* (2001) defined three signs that HR is occurring within a bacterial population namely: incongruent phylogenetic trees, mosaic genes and linkage equilibrium (71) . HGT and HR events were investigated in the MLST database and the New Zealand *Campylobacter* samples using phylogenetic minimum evolution trees (ME), index of association (I_A), maximum chi square analysis and d_N/d_S ratios. All isolates present in the MLST database were presumed to be correctly speciated.

4.4.2 Incongruent *C. jejuni* and *C. coli* phylogenetic trees

Incongruent phylogenetic trees, that is the sequences identified within the dataset which do not cluster into expected groups, suggests a high level of recombination resulting in trees where no evolutionary signal is seen (71). Clonal bacteria such as *S. enterica*, frequently resolve into fully congruent trees (69). *C. jejuni* with a weakly clonal population (50), and *C. coli* with a highly clonal population structure would then be expected to have relatively congruent allele locus trees, using the MLST scheme.

The current study characterised the same gene fragments in both *C. jejuni* and *C. coli* allowing in one ME tree to be constructed, with the expectation this would

demonstrate congruence between the species clusters. Incongruent individual gene locus trees from the New Zealand *Campylobacter* sample and the international MLST database were identified, predominantly at the *aspA*, *glnA* and *uncA* loci. These results were confirmed when the entire MLST database was examined at the *aspA*, *glnA*, *glyA*, *pgm* and *uncA* examples of mosaic genes and therefore incongruent trees were identified.

4.4.3 Mosaic genes

Mosaic genes are the result of HR between two closely related species (71), such as *C. jejuni* and *C. coli.* One mosaic gene allele, *aspA*173, was identified in the New Zealand sample and most likely arose as a result of a HR event between *aspA*2, a *C. jejuni* allele and *aspA*33 a *C. coli* allele. The deletion identified in the *aspA* allele of a *C. jejuni* isolate, PH526, either resulting from an incomplete HR event, or could from an incomplete non-HR event with a less closely related *Campylobacter*. Mosaic genes were identified at all gene loci in the MLST database, the interesting result was the identification of possible HGT events with *Campylobacter* species other than *C. jejuni* or *C. coli* (Figure 4.6).

STs seen in the New Zealand *Campylobacter* sample and the international MLST database were considered mosaic if one or more alleles identified within the ST was from an alternate species. The number of interspecies mosaic STs was surprising with HGT events identified not only between *C. jejuni* and *C. coli* isolates, but also between other *Campylobacter* species. Interspecies HGT has been identified, with *C. lari* associated *pgm* alleles observed within *C. jejuni* (149), and *C. coli* associated alleles in *C. jejuni* isolates (47, 148). In the current study without other *Campylobacter* reference alleles for all loci, accurate

identification of the parental alleles or from which species the HGT event occurred was not possible.

The high levels of HGT and HR occurring within selectively neutral *Campylobacter* housekeeping genes suggest that the level of HGT and HR occurring in non housekeeping genes such as the *flaA* locus may be higher. The results of the current study examining the *flaA* locus indicate that intra and interspecies HR and HGT events occur frequently. The incongruent ME tree constructed from sequences in the *flaA* SVR database indicates that although clusters of *C. jejuni* and *C. coli* associated SVR genotypes can be identified, there are large numbers of mosaic genes present at this locus. This could be interpreted as inter species HR events occurring frequently at the *flaA* locus. HGT events with the *flaA* SVR sequences could not be identified in the *flaA* SVR database, due to limited information concerning the sequence and the isolate from which it was characterised.

4.4.4 Linkage disequilibrium

Linkage equilibrium, where alleles show no evidence of association, has traditionally been associated with freely recombining bacteria (202). Conversely, linkage disequilibrium was associated with bacteria that were thought to undergo HR infrequently (71). Linkage disequilibrium has previously been identified in *C. jejuni* housekeeping genes (189), indicating that HR was not occurring at a high enough frequency to cause the disruption of gene linkage. The current study confirms this observation, with a low I_A value observed in the MLST database. The higher I_A value observed in the New Zealand sample for both *C. jejuni* and *C. coli* samples could be a reflection of the sample size as compared to the

international MLST database sample size. The use of linkage disequilibrium as a measure of recombination should be treated with caution, as high levels of recombination are needed to ensure linkage equilibrium (202).

4.4.5 The neutrality of housekeeping genes within the *C. jejuni* and *C. coli* MLST schemes

Dingle *et al* (2001) identified very low d_N/d_S values for the housekeeping genes chosen in the *C. jejuni/C. coli* scheme, indicating negative or purifying selection was occurring (50). Examination of the entire *C. jejuni/C.coli* database confirmed the observations of Dingle *et al* (2001) (50). The current study challenges the concept of near neutrality within the housekeeping genes as proposed by Maiden *et al* (1998) (136). The d_N/d_S ratios calculated for each gene loci in the current study indicate these genes cannot be characterised as selectively neutral.

A d_N/d_S ratio of more than one indicates positive selection, d_N/d_S equal to one indicates neutral selection and d_N/d_S less than one indicates negative or purifying selection (103, 237). Within the international MLST database and the New Zealand sample, all loci d_N/d_S ratios were observed to be less than one. This strongly suggests these genes are not selectively nearly neutral, and are under negative selection, that is any mutation that is strongly deleterious to the enzyme will be removed (167). The frame-shift deletion seen in the *aspA* allele in PH526 appears deleterious to the growth of the isolate on nutrient rich agar (PH526 repeatedly low growth compared to other *C. jejuni* isolates in identical conditions), suggesting that under normal conditions this allele would be removed from the population by natural selection.

The negative selection observed at these loci ranged from very strong negative selection at the *C. coli uncA* locus (0.0), to weak negative selection at the *C. coli tkt* locus (0.0915). The initial finding of a zero value for the d_N/d_S ratio at the *C. coli uncA* locus (Table 4.1) was unusual. The d_N/d_S ratio for the *uncA* loci, using the entire MLST database was calculated (0.007). The findings of low d_N/d_S values for all these loci were supported by the literature, although little comment has been made of these findings and no reports examining the entire database.

4.4.6 *flaA* SVR genotyping

The expectation prior to the examination of the *flaA* SVR database was that the *flaA* locus would be under positive selection (24, 227). The FlaA subunit, encoded by the *flaA* gene, is an integral part of the *Campylobacter* flagella (87), and any mutation or HR event in the *flaA* gene that increased the growth or survival of the bacteria would be positively selected for. When Tajima's D statistic was calculated for this locus, using the *flaA* SVR database, a negative value was observed. A negative Tajima's D statistic can be interpreted either as an indicator of negative or purifying selection occurring at the locus (78, 103), or that there has been a recent transmission bottleneck (28). Given the flagellum is a target for the immune system, particularly the human immune system, it is unlikely that one flagellum phenotype is going to be highly conserved. It is more likely that a transmission bottleneck has occurred at this particular region of the *flaA* locus.

4.4.7 Bacterial Species Identification

The accuracy of bacterial species identification is extensively debated in the literature (36, 82, 90-92, 130, 234). Unlike multicellular animals there can be few phenotypic or ecological differences between closely related bacteria, and high levels of HR and HGT within and between 'species', can obfuscate the genetic signal separating clusters of isolates (82, 91, 205, 234). Multiplex PCR using a single gene for the identification of *Campylobacter* species, as well as phenotypic methods such as hippurate hydrolysis, are used as the definitive methods of speciating *Campylobacter* isolates in the research laboratories (personal communication, C.E. Pope and C. Nicol). Although no published evidence exists, the results of examining the MLST data base suggests that HGT or HR may occur at the locus eg the *hipO* locus, used for speciation of *C. jejuni* and *C. coli* (Table 4.5), causing misidentification of *Campylobacter* isolates. Unless other confirmatory subtyping techniques are used to speciate the isolate in question, it may be incorrectly identified.

The mosaic genes and genotypes, and the incongruent phylogenetic ME trees identified in New Zealand and the international MLST database would suggest that *C. jejuni* and *C. coli* are 'fuzzy' species (91). Currently *C. jejuni* and *C. coli* are characterised as two separate species by DDH (144). Although no DDH has been performed speciating the mosaic isolates identified in the MLST database, the existence of a large number of mosaic genes and genotypes has implications for defining *Campylobacter* species. Potentially these two species represent a spectrum of genotypes from the same bacterial lineage or conversely the strains that appear in the middle of the *C. jejuni* and *C. coli* clusters could be considered

sub species variants (personal communication, P. Carter and C. Pope). Further work to characterise these isolates is required.

4.4.8 Effect of homologous recombination and horizontal transfer on subtyping schemes

Subtyping schemes assume that different populations of bacteria exhibit both unique phenotypic and genotypic characteristics (personal communication, C.E. Pope); an assumption that allows the characterisation of bacteria for public health tracing of outbreaks and population structure studies. This assumption is violated when characterising closely related bacteria such as *C. jejuni* and *C. coli*. The two species are so closely related that only one phenotypic characteristic, hippurate hydrolysis, is commonly used to differentiate the species, and even then approximately 5% of all *C. jejuni* isolates are hippurate negative (124, 154). Incorrect speciation by hippurate hydrolysis was identified in the current study. Six *C. jejuni* isolates and one *C. coli* isolate were misidentified in the ESR database, it became readily apparent after MLST genotyping they were either the incorrect species or a mosaic genotype. The isolates were further speciated with the multiplex PCR method of Wang *et al* (221), and were identified as either *C. jejuni* or *C. coli* based on the methods available. Further confirmation of these observations were obtained by examining the MLST database for mosaic STs, where *C. jejuni* or *C. coli* associated STs appeared to be incorrectly speciated, indicating that an HGT event had possibly occurred at the gene locus used for speciation. The results of the current study reinforce the need for multiple genotyping methods to be used to characterise bacterial isolates to ensure correct speciation and correct identification of bacterial relationships.

Although the assumption of selective neutrality of the *Campylobacter* housekeeping genes has been proven to be incorrect, MLST still remains the only subtyping scheme that directly examines sequence data at multiple loci and can be used to characterise bacterial population structures. The direct analysis of sequence data is ultimately the most accurate method for the identification of bacterial relationships. The value of direct DNA analysis has been demonstrated in the current study by the identification of HR and HGT events that might affect subtyping schemes if only one subtyping method is used to characterise or speciate bacteria.

CHAPTER 5: COMPARATIVE ANALYSIS OF MOLECULAR SUBTYPING TOOLS TO CHARACTERISE OUTBREAKS OF *CAMPYLOBACTER*

5.1 Introduction

Campylobacter species are the foremost aetiological agents of bacterial gastroenteritis in both New Zealand and the developed world (77). There are currently many subtyping methods, both phenotypic and genotypic to characterise these relatively biochemically inert, Gram-negative bacteria (226). The majority of the genotyping methods and the equipment necessary to characterise *Campylobacter* are not readily accessible to non-reference medical laboratories, therefore the majority of *Campylobacter* isolates remain uncharacterised other than at the genus level (personal communication, C.E. Pope). Genotyping techniques such as PFGE and *flaA* PCR-RFLP, allow characterisation of *Campylobacter* outbreaks, but have limited applications for population studies. With the development of MLST for *C. jejuni* and *C. coli*, the characterisation of geographically diverse *Campylobacter* populations is now possible (37, 56, 121, 138, 146, 148, 213).

Previous attempts to characterise *Campylobacter* outbreaks and population studies using both phenotypic and genotypic techniques have indicated possible animal associations, and illustrated the widespread nature of this bacteria in the environment (38, 44, 46, 121, 183, 201). Until recently the number of published studies that characterised New Zealand *Campylobacter* populations was limited by the availability of molecular techniques. With the development of MLST more

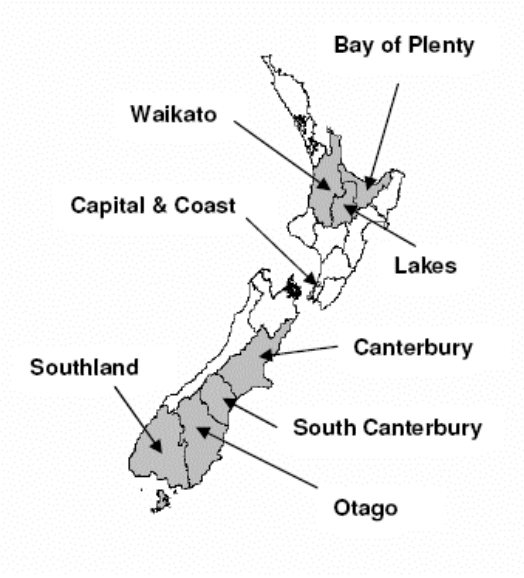
information is available to describe *Campylobacter* in New Zealand and elucidate possible animal and environmental transmission routes.

During the winter of 2006, the rate of reported campylobacteriosis cases increased well over the expected rate (11), in the majority of New Zealand's district health boards (DHBs), and was not restricted to any ethnic or age group. In the current study MLST, PFGE (*KpnI*) and *flaA* PCR RFLP were used to characterise 112 *Campylobacter* isolates (collected in July/August 2006) that had been previously described by PFGE (*SmaI*) and Penner serotyping.

5.2 Methods

All *Campylobacter* isolates used in this study originated from eight DHBs across New Zealand (Table 5.1). Each medical laboratory sent 25 consecutive, non-linked *Campylobacter* isolates for further phenotypic and genotypic characterisation.

Figure 5.1: Representative DHB's



New Zealand DHBs that participated in the current study are shown on the map of New Zealand indicating the geographical spread of isolates obtained.

Of the 125 isolates initially received 13 were excluded on the basis of either, no viable *Campylobacter* species present or repetition of a previous sample sent. *Campylobacter* isolates not conforming to known *C. jejuni* characteristics underwent *Campylobacter* identification multiplex PCR to speciate the isolate. All methods are fully described in Chapter 2.

Table 5.1: Origins of "Outbreak" isolates

Origin	Associated DHB	Species	
		C. jejuni	***C. coli***
TGML	Bay of Plenty	11	0
WNML	Capital & Coast	23	2
CHML	Canterbury, South Canterbury	22	2
RODI	Lakes	9	0
DNSC	Otago, Southland	20	0
HMNL	Waikato	22	1
Total		107	5

5.3 Results

MLST analysis identified a total of 11 CCs and one group of isolates that were unassigned to a CC, 25 STs and 82 alleles (Table 2). Four novel STs and two novel *aspA* alleles (*aspA*184, *aspA*173) were identified. CC ST-21 (n = 32) and CC ST-48 (n = 34) represented 59% of all isolates. Two dominant STs were found in the sample, ST-474 (CC ST-48) and ST-190 (CC ST-21) that were represented 44% (n = 50) of all isolates. All 112 *Campylobacter* isolates in the current study were typeable using the MLST subtyping method. Concatenated ST allele sequences were downloaded from http://pubmlst.org/campylobacter/, aligned in CLUSTALW and a neighbour joining tree constructed using MEGA v 3.1 (126) (Figure 5.2).

Cluster analysis of the MRFPs using BioNumerics (90% similarity, 0.5% optimization, 1.5% band tolerance) identified 17 groups of two or more isolates (n = 81) (Figure 5.3). Fifteen of the 17 MRFP clusters contained isolates arising from multiple DHBs (n = 74). MRFP analysis identified three clusters, J (n = 7), K (n = 9) and L (n = 7) within the ST-474 genotype (n = 23). Isolates within the ST-474 clusters arose from multiple DHBs. ST-190 also contained three clusters of isolates as identified by MRFP analysis (n = 16). All ST-190 isolates were digested with a second enzyme, *KpnI*, again the patterns within the clusters were indistinguishable. Six of the *SmaI* MRFP clusters labelled B, K, L, M, P and S were undescribed the Pulsenet Aotearoa database. Two isolates were unable to be typed by PFGE (Table 5.4).

Table 5.2: MLST Profiles of outbreak survey isolates

ERL #	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST	CC
ERL06-2307	7	2	5	2	10	3	6	5	ST-353
ERL06-2420	2	1	1	3	2	1	5	21	ST-21
ERL06-2261	1	2	3	4	5	9	3	42	ST-42
ERL06-2304	1	2	3	4	5	9	3	42	ST-42
ERL06-2332	1	2	3	4	5	9	3	42	ST-42
ERL06-2340	1	2	3	4	5	9	3	42	ST-42
ERL06-2268	4	7	10	4	1	7	1	45	ST-45
ERL06-2357	4	7	10	4	1	7	1	45	ST-45
ERL06-2423	4	7	10	4	1	7	1	45	ST-45
ERL06-2377	4	7	10	4	1	7	1	45	ST-45
ERL06-2295	2	4	1	2	7	1	5	48	ST-48
ERL06-2329	2	1	12	3	2	1	5	50	ST-21
ERL06-2351	2	1	12	3	2	1	5	50	ST-21
ERL06-2339	2	1	12	3	2	1	5	50	ST-21
ERL06-2330	2	1	12	3	2	1	5	50	ST-21
ERL06-2372	2	1	12	3	2	1	5	50	ST-21
ERL06-2378	2	1	12	3	2	1	5	50	ST-21
ERL06-2305	2	1	12	3	2	1	5	50	ST-21
ERL06-2359	2	1	12	3	2	1	5	50	ST-21
ERL06-2257	9	25	2	10	22	3	6	52	ST-52
ERL06-2272	9	25	2	10	22	3	6	52	ST-52
ERL06-2298	9	25	2	10	22	3	6	52	ST-52
ERL06-2341	9	25	2	10	22	3	6	52	ST-52
ERL06-2343	2	1	21	3	2	1	5	53	ST-21
ERL06-2345	2	1	21	3	2	1	5	53	ST-21

ERL06-2358	2	1	21	3	2	1	5	53	ST-21
ERL06-2355	2	1	21	3	2	1	5	53	ST-21
ERL06-2267	1	4	2	2	6	3	17	61	ST-61
ERL06-2249	2	1	5	3	2	3	5	190	ST-21
ERL06-2258	2	1	5	3	2	3	5	190	ST-21
ERL06-2275	2	1	5	3	2	3	5	190	ST-21
ERL06-2291	2	1	5	3	2	3	5	190	ST-21
ERL06-2309	2	1	5	3	2	3	5	190	ST-21
ERL06-2327	2	1	5	3	2	3	5	190	ST-21
ERL06-2367	2	1	5	3	2	3	5	190	ST-21
ERL06-2374	2	1	5	3	2	3	5	190	ST-21
ERL06-2331	2	1	5	3	2	3	5	190	ST-21
ERL06-2336	2	1	5	3	2	3	5	190	ST-21
ERL06-2337	2	1	5	3	2	3	5	190	ST-21
ERL06-2364	2	1	5	3	2	3	5	190	ST-21
ERL06-2356	2	1	5	3	2	3	5	190	ST-21
ERL06-2274	2	1	5	3	2	3	5	190	ST-21
ERL06-2334	2	1	5	3	2	3	5	190	ST-21
ERL06-2369	2	1	5	3	2	3	5	190	ST-21
ERL06-2342	2	1	5	3	2	3	5	190	ST-21
ERL06-2376	2	1	5	3	2	3	5	190	ST-21
ERL06-2289	9	2	4	62	4	5	6	257	ST-257
ERL06-2365	9	2	4	62	4	5	6	257	ST-257
ERL06-2571	9	2	4	62	4	5	6	257	ST-257
ERL06-2260	9	2	4	62	4	5	6	257	ST-257
ERL06-2354	9	2	4	62	4	5	6	257	ST-257
ERL06-2255	8	10	2	2	11	12	6	354	ST-354
ERL06-2270	8	10	2	2	11	12	6	354	ST-354
ERL06-2290	8	10	2	2	11	12	6	354	ST-354
ERL06-2297	8	10	2	2	11	12	6	354	ST-354
ERL06-2417	8	10	2	2	11	12	6	354	ST-354
ERL06-2328	8	10	2	2	11	12	6	354	ST-354
ERL06-2363	8	10	2	2	11	12	6	354	ST-354
ERL06-2418	8	10	2	2	11	12	6	354	ST-354
ERL06-2421	8	10	2	2	11	12	6	354	ST-354
ERL06-2366	7	21	5	62	4	61	44	436	U/A
ERL06-2375	7	21	5	62	4	61	44	436	U/A
ERL06-2422	7	21	5	62	4	61	44	436	U/A
ERL06-2250	2	4	1	2	2	1	5	474	ST-48
ERL06-2253	2	4	1	2	2	1	5	474	ST-48
ERL06-2256	2	4	1	2	2	1	5	474	ST-48
ERL06-2262	2	4	1	2	2	1	5	474	ST-48
ERL06-2265	2	4	1	2	2	1	5	474	ST-48
ERL06-2266	2	4	1	2	2	1	5	474	ST-48
ERL06-2287	2	4	1	2	2	1	5	474	ST-48
ERL06-2288	2	4	1	2	2	1	5	474	ST-48
ERL06-2292	2	4	1	2	2	1	5	474	ST-48
ERL06-2293	2	4	1	2	2	1	5	474	ST-48
ERL06-2296	2	4	1	2	2	1	5	474	ST-48
ERL06-2306	2	4	1	2	2	1	5	474	ST-48
ERL06-2308	2	4	1	2	2	1	5	474	ST-48

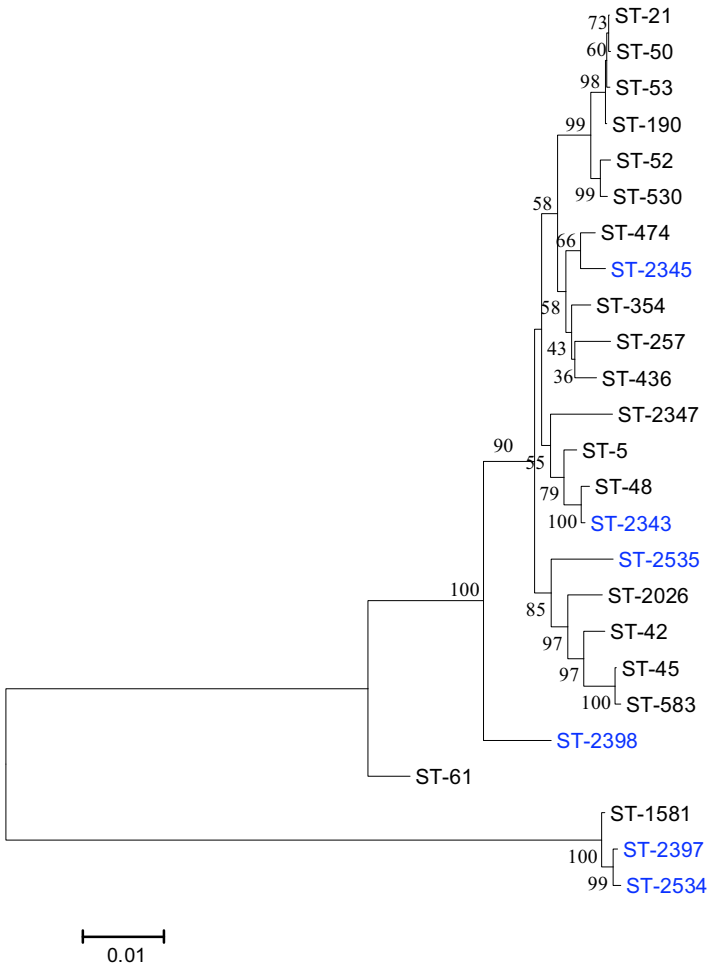
ERL06-2310	2	4	1	2	2	1	5	474	ST-48
ERL06-2312	2	4	1	2	2	1	5	474	ST-48
ERL06-2313	2	4	1	2	2	1	5	474	ST-48
ERL06-2325	2	4	1	2	2	1	5	474	ST-48
ERL06-2338	2	4	1	2	2	1	5	474	ST-48
ERL06-2352	2	4	1	2	2	1	5	474	ST-48
ERL06-2361	2	4	1	2	2	1	5	474	ST-48
ERL06-2368	2	4	1	2	2	1	5	474	ST-48
ERL06-2370	2	4	1	2	2	1	5	474	ST-48
ERL06-2373	2	4	1	2	2	1	5	474	ST-48
ERL06-2549	2	4	1	2	2	1	5	474	ST-48
ERL06-2271	2	4	1	2	2	1	5	474	ST-48
ERL06-2326	2	4	1	2	2	1	5	474	ST-48
ERL06-2415	2	4	1	2	2	1	5	474	ST-48
ERL06-2424	2	4	1	2	2	1	5	474	ST-48
ERL06-2551	2	4	1	2	2	1	5	474	ST-48
ERL06-2412	2	4	1	2	2	1	5	474	ST-48
ERL06-2416	2	4	1	2	2	1	5	474	ST-48
ERL06-2419	2	4	1	2	2	1	5	474	ST-48
ERL06-2259	9	2	2	10	86	59	5	530	U/A
ERL06-2286	4	7	10	4	42	51	1	583	ST-45
ERL06-2353	129	66	30	82	189	47	17	1581	U/A
ERL06-2344	10	1	16	19	10	5	7	2026	ST-403
ERL06-2371	2	4	5	2	10	1	5	2343	ST-48
ERL06-2413	2	4	5	2	2	5	5	2345	ST-206
ERL06-2335	2	4	5	2	2	5	5	2345	ST-206
ERL06-2362	2	4	4	105	10	25	57	2347	U/A
ERL06-2252	**184**	39	30	82	113	43	17	2397	ST-828
ERL06-2254	**184**	39	30	82	113	43	17	2397	ST-828
ERL06-2269	**184**	39	30	82	113	43	17	2397	ST-828
ERL06-2311	**173**	4	1	68	11	290	6	2398	U/A
ERL06-2360	33	38	65	79	113	47	17	2534	ST-828
ERL06-2263	10	2	107	62	120	76	1	2535	U/A
ERL06-2273	10	2	107	62	120	76	1	2535	U/A
ERL06-2294	10	2	107	62	120	76	1	2535	U/A

Bold type indicates novel alleles, Red STs indicate STs with novel alleles, Blue STs indicate novel STs composed of previously described alleles.

Analysis of the *flaA* locus of the 112 isolates identified 25 RFLP patterns (Table 5.3) with between three and seven bands identified in each isolate. The most commonly identified *flaA* RFLP type were *flaA*36 (n = 33) and *flaA*14 (n = 17). Digestion with *DdeI* indicated that the *flaA* genotypes within each ST-474 cluster were identical, although the *flaA* genotypes within all ST-474 isolates were not the same. Similarly isolates within the ST-190 genotype indistinguishable *flaA* RFLP

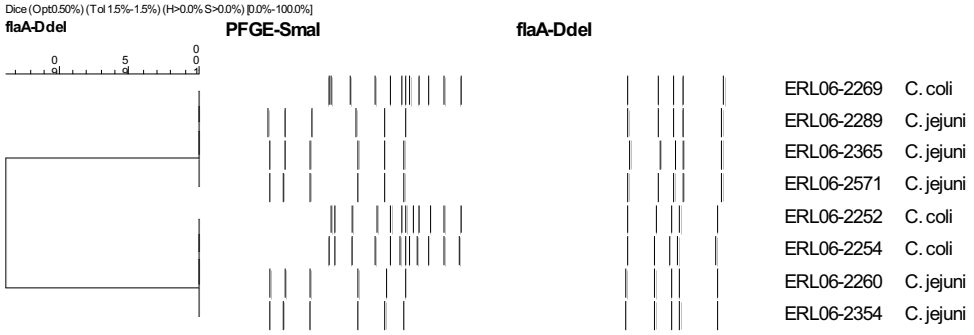
patterns were identified. Six HGT events were observed at this locus; both intra species and one interspecies were observed in the current study (Figure 5.4). The only single example of inter species HGT was that of *flaA*60 which was identified in multiple isolates of ST-257 and ST-2397.

Figure 5.2: NJ tree of MLST STs found in sample



A ME tree (Kimura 2-parameter model) constructed in MEGA v 3.1 using concatenated sequences of each MLST ST present in the current study. The STs in blue text indicate novel STs.

Figure 5.3: Distribution of *flaA*60



An UPMGA tree (using the Dice coefficient, (90% similarity, 0.5% optimization, 1.5% band tolerance)) was constructed in BioNumerics of the *flaA* genotype *flaA*60 and previously characterized PFGE MRPs (*SmaI*). The UPMGA tree was drawn used the *flaA-DdeI* RPs as the basis for the tree.

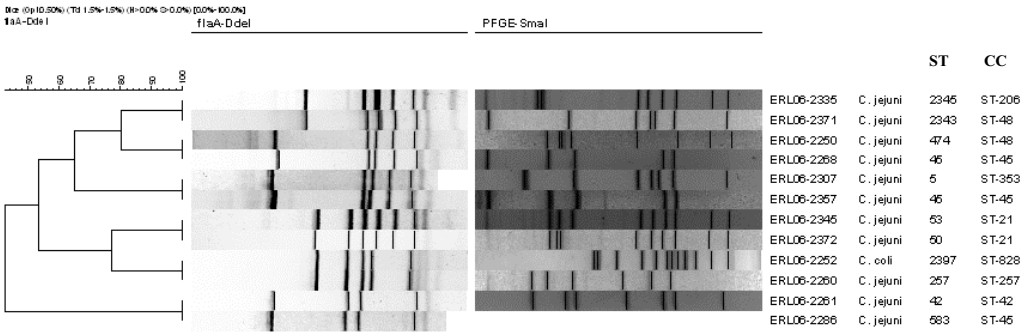
Table 5.3: *flaA* genotype profiles of outbreak survey isolates

***flaA* type**	**MLST STs**
*flaA*8	2535
*flaA*9	2398
*flaA*14	190
*flaA*15	2026
*flaA*19	474, 21
*flaA*20	50
*flaA*21	2347
flaA 22	1581
flaA 26	45
flaA 36	45, 474, 5
flaA 38	354
flaA 39	530
flaA 41	354
flaA 42	354
flaA 45	2534
flaA 57	2345, 2343
flaA 60	2397, 257
flaA 62	53, 50
flaA 91	53
flaA 67	52
flaA 76	45
flaA 82	42, 583
flaA 89	436
flaA 81	48
flaA 52	474

Fifteen different serogroups were identified using the Penner serotyping method, although 17 out of 112 isolates were untypable. The most common serogroups identified were the O: 4-complex (n = 36) and the O: 2 (n = 23) serogroups. The

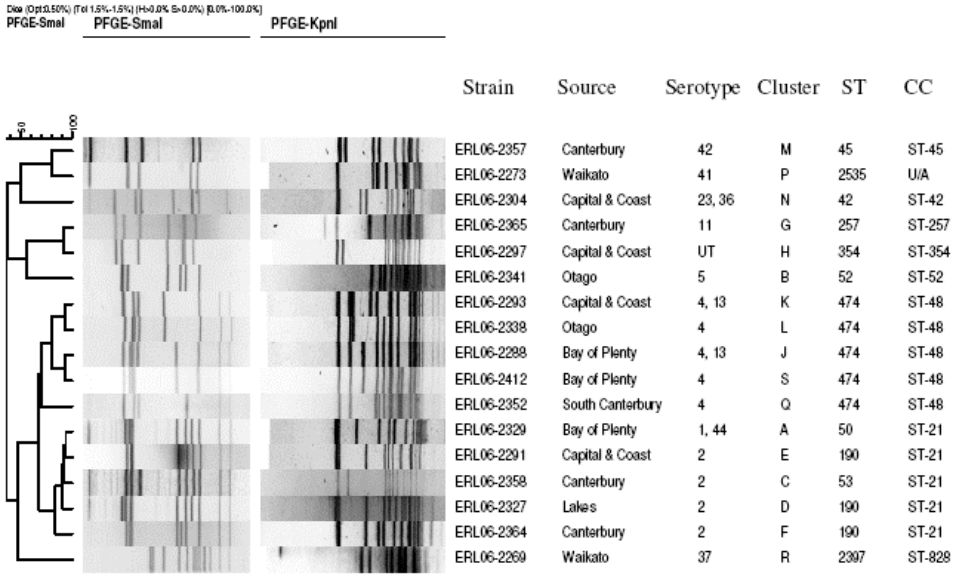
majority of isolates within all clusters as defined by MRFP and MLST analysis had identical serogroups.

Figure 5.4: Intra and inter species HGT events at the *flaA* locus



An UPMGA tree (using the Dice coefficient, (90% similarity, 0.5% optimization, 1.5% band tolerance)) was constructed in BioNumerics of the HGT events occurring within *flaA* genotypes and previously characterized PFGE MRPs (*SmaI*). The UPMGA tree was drawn used the *flaA-DdeI* RPs as the basis for the tree. Several attempts were made to characterize ERL06-2286, however it was unable to be characterized by *KpnI* or *SmaI* due to DNAse's present within the isolate.

Figure 5.5: UPGMA tree of MRFP gel clusters



An UPMGA tree (using the Dice coefficient, (90% similarity, 0.5% optimization, 1.5% band tolerance)) was constructed in BioNumerics based on PFGE MRPs (*SmaI* and *KpnI*). The UPMGA tree was based on the *SmaI* MRPs, each pattern shown is a representative of a cluster of isolates with the same MRPs, CCs, STs and Penner serotypes, the majority of these clusters had the same *flaA* genotype (not shown) within the cluster also.

Table 5.4: Typeability of outbreak survey isolates

Method		Typeable		Untypeable	
		C. jejuni	*C. coli*	*C. jejuni*	*C. coli*
flaA PCR RFLP (*DdeI*)		107	5	0	0
MLST		107	5	0	0
Penner Serotyping		98	3	15	2
PFGE	*KpnI*	105	5	2	0
	SmaI	105	5	2	0

Table 5.5: Simpson's Index of diversity

Subtyping Method	Number of types	Discrimination Index
flaA PCR RFLP	33	0.898
PFGE *SmaI*	47	0.975
PFGE *KpnI*	66	0.992
Penner serotyping	17	0.904
MLST	25	0.878

The ability of subtyping methods to type *Campylobacter* isolates used in the current study was identified by the Simpson's Index of Diversity (Table 5.5) (104). The most discriminatory method identified was that of PFGE using *KpnI* (0.992), followed by PFGE using *SmaI* (0.975), the least discriminatory subtyping methods were *flaA* PCR RFLP (0.898) and MLST (0.878).

5.5 Discussion

Campylobacter infections are rarely attributed to outbreaks, in New Zealand, as with other developed countries (77). In 2006, 38 *Campylobacter* outbreaks were reported comprising 260 cases, the remaining 13,587 reported cases were considered sporadic (12). The ratio of outbreaks to sporadic cases implies either a significant number of sporadic cases or under reporting of outbreaks occurring in New Zealand. Due to the sheer volume of campylobacteriosis cases, samples received are not routinely speciated further than the genus level (personal

communication, C. Pope), consequently recognition of potential outbreaks is often missed.

The relative biochemical inertness and fastidious of growth and transport conditions for *Campylobacter* isolates prevent quick identification using biochemical tests (159). Recent New Zealand studies used both phenotypic and genotypic methods to identify outbreaks, and to investigate possible transmission routes for *Campylobacter* infections (4, 21, 38, 67, 83, 102, 186). PFGE, using the restriction enzymes *SmaI* and *KpnI* for greater discrimination between *Campylobacter* isolates, identified outbreak clusters in Christchurch, New Zealand (102). The recognition of indistinguishable clusters within a defined geographical location over a limited time period, suggests that campylobacteriosis outbreaks in New Zealand may occur more frequently than previously thought (102).

Studies in other developed countries characterising isolates using sequence based methods such as MLST, in addition to band based methods such as PFGE and *flaA* PCR RFLP, identified both temporal and geographical clusters of *Campylobacter* isolates (56, 75, 146). While MLST has been used to characterise *Campylobacter* populations in both developing and developed countries, the lower discriminatory power of this technique has limited applications for outbreaks. In the current study, *Campylobacter* isolates collected within a discrete time period from geographically diverse locations were characterised, using both phenotypic and genotypic subtyping methods. Indistinguishable isolate clusters were identified across both the North and South Islands of New Zealand.

Previous studies have suggested that one restriction enzyme alone, such as *SmaI* may not be discriminatory enough to discern accurate relationships between isolates and it may be necessary to use a second restriction enzyme to provide further verification (102, 145). *SmaI* and *KpnI* were used to construct PFGE MRPs of the *Campylobacter* isolates in the current study, with the two-enzyme analysis of each isolate supporting the clusters identified by the UPMGA/Dice coefficient analysis. Although the majority of isolates were correctly identified by *SmaI* MRP analysis, the use of two enzymes in the current study is supported by the identification of a *SmaI* pattern, Sm1 that was composed of two different STs.

Geographically diverse isolates were obtained from eight DHBs spread across the North and South Islands. Clusters (n =17) of at least two isolates of *C. jejuni* or *C. coli* that were indistinguishable by MLST, PFGE and *flaA* PCR-RFLP were identified. Several clusters were found to be composed of isolates derived from multiple DHBs, an unusual finding that could be the result of exposure to multiple sources associated with common risk factors, or common source outbreaks arising from single point sources widely available throughout New Zealand. The increase observed in New Zealand reported campylobacteriosis cases was absent in Australia and any other country in the Asia-Pacific region. Due to the number of isolates associated with each cluster, possible sources of *Campylobacter* and transmission routes could not be gathered from epidemiological information gathered. The widespread distribution of these clusters gives credence to the theory that these were not simple local point source outbreaks, but rather they reflect a common point source or sources that were widely spread through out

New Zealand via a food e.g. poultry, distribution network (personal communication, Phil Carter). Similar incidences of food distribution networks spreading enteropathgenic bacteria include to *S. enterica* serotype Enteritidis outbreak associated with raw almonds (107), and E. *coli* O157:H7 which was associated with prewashed spinach (10).

Two dominant genotypes were observed in the current study, ST-474 and ST-190, these strains were most likely associated with common point sources. ST-474 was identified in seven of the eight DHBs and ST-190 identified in all eight DHBs. Analysis of the two dominant genotypes demonstrated two MRFPs not previously identified in New Zealand, K and L, all other ST-474 and ST-190 clusters had been previously characterised in the Pulsenet Aotearoa *Campylobacter* database, D (number of isolates = 1), F (n = 23), E (n = 44). Genotyping data (Chapter 3 and 4) indicate that *Campylobacter* strains in New Zealand are stable over long time periods, which may lead to incorrect assumptions about the nature of relationships between isolates in outbreak situations. Previous studies using molecular subtyping methods (PFGE, AFLP) have also suggested that particular genotypes are stable over time (96, 138, 139).

A number of CCs have previously been associated with human infection, CC ST-21, CC ST-48, CC ST-257 and CC ST-354 (35, 50, 120, 126), and in the current study similar results were observed. Isolates with genotypes belonging to CC ST-21, ubiquitous amongst avian, mammalian and environmental sources, have been identified in up to a third of reported human campylobacteriosis cases (138, 146, 201). In a recently published study CC ST-48 was identified in

approximately one third of human derived *Campylobacter* isolates, comparable to findings in the current study (146), other studies have observed a much lower rate of incidence (5-10%) for CC ST-48 reasons are not clear for the disparity of findings (48, 118, 189, 201). ST-474, the dominant CC ST-48 sequence type, and dominant MLST ST in the current study is not commonly identified in the literature. ST-474 has been rarely identified outside of New Zealand, this genotype is present in approximately 10% of isolates characterised in New Zealand human and poultry isolates, this genotype has also been observed in sheep and cattle derived isolates (unpublished work, McTavish and Carter). Potentially endemic strains have been observed in Australia, Curacao and Senegal (56, 121, 146), ST-474 may represent a ST endemic to New Zealand, further work may provide valuable information on virulence and survival of isolates with this genotype.

All *Campylobacter* isolates characterised in this study were typeable by MLST and *flaA* PCR RFLP, three isolates (2.6%) were unable to be characterised by PFGE and 17 isolates (15.1%) were untypeable by Penner serotyping. These results agree with previous studies suggesting that Penner serotyping is unable to fully subtype large groups of *Campylobacter* isolates (74, 162) and inadequate to draw conclusions about bacterial relatedness if used as the sole subtyping technique. HGT events had occurred both inter and intra species between *C. jejuni* and *C. coli*; if one locus is the target of a subtyping method such as *flaA* incorrect assumptions about the relatedness may be made (226).

CHAPTER 6: GENERAL DISCUSSION AND FURTHER WORK

6.1 Significance of the current study

Campylobacter jejuni and *Campylobacter coli* are two of the most prevalent bacterial enteropathogens in New Zealand, accounting for large proportion of all reported bacterial gastroenteritis cases each year (15). The economic cost of these enteropathogens is staggering, estimated in 2000 to be $55.1 million per year (192) and this will have risen with the increase in cases observed in New Zealand each year. Although these enteropathogens account for the majority of bacterial gastroenteritis in New Zealand, little is known about the transmission routes or the population structure of *Campylobacter* in New Zealand.

The current work represents the first attempt at characterising *C. jejuni* and *C. coli* in New Zealand using multiple direct and indirect methods of genotyping. The use of multiple genotyping techniques namely MLST, *Campylobacter* identification multiplex PCR, PFGE and *flaA* PCR RFLP and multiple gene loci identified groups of *C. jejuni* and *C. coli* present internationally and endemic to New Zealand (Chapters three, four and five). MLST was used for the first time to describe genotypes present in the New Zealand *C. jejuni* and *C. coli* culture collections held at ESR and Massey University, and in an outbreak in the winter of 2006. Indirect methods of genotyping were also used to characterise the *Campylobacter* isolates (*C. coli* (PFGE and *flaA* PCR RFLP), *C. jejuni* (*flaA* PCR RFLP). The resultant work represents a very well characterised group of isolates that provides a base for further work to be carried out in both identifying

transmission routes and in characterising particularly prevalent and apparently virulent *C. jejuni* isolates such as ST-474 (Chapter 5).

A representative sample of New Zealand *Campylobacter* isolates characterised with MLST was compared to the international MLST database. Identification of internationally very rare and unique genotypes to New Zealand isolated from human clinical isolates and environmental water samples, was a significant finding of the current study. The isolation of one particularly dominant, internationally rare, New Zealand genotype, ST-474, in 28% of all human clinical isolates was important (142). Correspondence with other research groups and reviews of current literature, suggest that ST-474 is internationally rare and relatively unique to New Zealand. The isolation of a single genotype responsible for a large proportion of campylobacteriosis cases in New Zealand may allow further work to limit the prevalence of this genotype (Chapter 5). The journal article resulting from work conducted in Chapter 5 can be found in Appendix 2.

Identification of two New Zealand unique clusters of *Campylobacter* isolates (one *C. jejuni* and one *C. coli*) isolated from environmental water sources, unique in New Zealand and internationally, may indicate *Campylobacter* isolates that have adapted to an unusual environment, or that the differences observed were sampling artefacts due to effective population size of *C. jejuni* and *C. coli* (Chapter 3). These findings were supported with the isolation of the founder genotype ST -2381 from the Manawatu river in 2007 by French *et al* (unpublished findings), over six years and a large geographical distance from the original isolation river, the Ashburton river in the South Island of New Zealand.

The characterisation of *C. jejuni* isolates that had been previously mistyped as *C. coli* lead to the identification of mosaic STs both in the New Zealand *Campylobacter* population (also confirmed by Wong & Carter, unpublished results) and in the international database. These results suggested that what was observed in terms of HGT and HR in the relatively small sample of New Zealand isolates was also representative of the international *Campylobacter* populations. Two *aspA* alleles identified and described in the New Zealand *Campylobacter* sample were direct evidence of HR occurring in the New Zealand sample between *C. jejuni* and *C. coli* housekeeping genes (Chapter 4). The findings of HR and HGT in both the New Zealand sample and the international database in both housekeeping and surface gene loci have implications for species boundaries and subtyping of *C. jejuni* and *C. coli*. These isolates may either represent a spectrum of a single species rather than two separate species, or that the isolates with mosaic genotypes may need to be considered as a new subspecies variant.

6.2 The use of MLST as a subtyping tool

The current work began as part of a study to ascertain whether MLST was a valid subtyping tool for day to day surveillance of *Campylobacter* isolates in New Zealand. Previous work suggested that MLST has a lower discriminatory levels between bacterial isolates than PFGE (69), the results of the current study confirm this. The inherent value in the use of MLST as an active surveillance method lies in the direct analysis and sampling of multiple loci using sequence data, and in being able to share that data electronically via the internet with international researchers (136). The more gene loci sampled the less impact HR or HGT will

have on distorting the hypothesised bacterial relationships. The revelation that HGT and HR is more common in *Campylobacter* than previously thought will, and should be, taken into account when typing these isolates, multiple subtyping methods need to be used as a part of an active public health monitoring system.

The results presented here suggest that using MLST has value as a confirmatory scheme for identifying bacterial relationships. MLST and *flaA* SVR have been identified as having approximately the same level of discriminatory ability as PFGE (183). This observation suggests that although the current cost of MLST is prohibitive in terms of the large amount of reported cases of campylobacteriosis in New Zealand, if *flaA* SVR, the cost of which is less prohibitive, was used as a front line subtyping method for active surveillance MLST could then be used as a confirmatory method of outbreak occurrence. Combined use to characterise previously speciated (using a multiplex PCR for identification) *Campylobacter* isolates would result in eight gene loci being sampled which should be enough to correctly speciated and subtype most *Campylobacter* isolates.

6.3 Characterisation of *Campylobacter* outbreaks in New Zealand

Prevailing theory in *Campylobacter* literature suggests that outbreaks of campylobacteriosis are rare and relatively easily traceable to a food or water source (111). Reported outbreaks in New Zealand literature (excluding the public health surveillance website) would suggest that the characterisation and defining the source of the outbreak is rare (Chapter 1). Results of the current work challenge this theory. Identification of clusters of indistinguishable isolates from diverse geographical locations suggests that outbreaks are occurring, but due to

the absence of an active surveillance system in place they are not being tracked, nor the origin traced (Chapter 4). Perceived outbreaks are reported regularly in the monthly surveillance reports (found at http://www.surv.esr.cri.nz/), these outbreaks may be true outbreaks, however, without molecular evidence of relationships between the identified bacteria the identification of outbreak clusters are not as accurate as they could be. The current work indicates that an active surveillance system for both campylobacteriosis cases and retail poultry products in New Zealand would identify and describe outbreaks far more accurately than occur presently.

6.4 Further work

The current study has identified a number of significant findings that need further investigation. Identification of a New Zealand specific cluster of *C. jejuni* isolated from environmental water sources in both the North and the South islands separated by a period of six years, suggests that either the animal or protozoal reservoir, potentially native to New Zealand, has not yet been identified or that these particular genotypes have adapted to an aquatic environment. No evidence has been published to suggest that *C. jejuni* or *C. coli* can live in an aquatic environment, other than within protozoa (200). *Campylobacter* is known to survive for up to 160 days in fresh water (181), but growth has not been demonstrated. These results warrant further investigation.

The observation that one ST (ST-474) is responsible for a large amount of human disease was unexpected. Previous work (Chapter 3) had not shown a significant amount of ST-474 in the ERL collection, further investigation revealed the international rarity of this genotype. Further work needs to be carried out on this

important genotype, including research into the virulence, survival (including biofilm formation) and possible reservoirs of this genotype.

The HGT and HR observed in the current body of work suggest that multiple forms of subtyping are need to correctly speciate and cluster *Campylobacter* isolates. With the improvement and decreased cost of high throughput sequence technology, the use of *flaA* SVR as a first instance active surveillance method, and MLST as a confirmatory method needs to be investigated for statistical validity.

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APPENDIX ONE

TE Buffer

1M Tris (pH 8.0)	5 mL
0.5M EDTA (pH 5.0)	1 mL
SDW	494 mL

Cell Lysis Buffer

1M Tris (pH 8.0)	25 mL
0.5M EDTA (pH 5.0)	50 mL
10% Sarcosyl	50 mL
SDW	375 mL

PEG

PEG	10 gm
NaCl	7.3 gm
SDW	Up to 50 mL

Sequence Reaction Cleanup Mastermix

Ethanol 96%	1 mL
3M Sodium Acetate (pH 5.2)	40 µL
DNase/RNase free water	200 µL

All other enzymes and buffers used were obtained from suppliers and not made in house.

APPENDIX TWO:

Journal article arising out of work that contributed to Chapter 5.

McTavish, SM., Pope, CE., Nicol, C., Sexton, K., French, N., Carter, PE. Wide geographical distribution of internationally rare Campylobacter clones within New Zealand. ***Epidmiol. Infect***. 2007 Nov 21;:1-9 [Epub ahead of print].

APPENDIX THREE:

All information regarding these isolates was obtained from the pubMLST *Campylobacter jejuni* and *Campylobacter coli* website

Id	Isolate	ST	Country	Year	Disease	Source	Epidemiology	Penner	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	CC
3802	2173	2316	Scotland	2005	G	human stool	sporadic case	.	1	4	2	2	6	286	17	ST-61
2583	89-18	1244	USA	.	.	cattle	.	.	1	1	2	2	225	3	17	ST-61
3986	Jun-50	2501	Canada	2006	.	cattle	.	.	1	39	30	78	104	43	17	ST-828
2422	BB1221	958	UK	2004	C	chicken	sporadic case	.	2	75	80	48	142	34	1	ST-661
4321	4701	2784	Scotland	.	G	human stool	.	2	2	39	30	79	104	43	17	ST-828
4644	11624	3079	UK	2000	.	.	.	.	2	1	30	82	118	1	17	U/A
3466	FSA05.280127	1993	England	2005	G	human stool	sporadic case	.	7	53	2	10	11	3	17	ST-574
4572	LMQS-928	3032	Italy	2005	.	chicken	.	.	9	2	2	38	113	5	6	ST-257
4804	ERL97-00454	3223	New Zealand	1997	.	human stool	.	Untypable	9	284	30	79	113	47	17	U/A
2772	648	1420	USA	2003	C	pig	.	.	32	39	115	115	104	85	17	U/A
3991	06-1498	2506	Canada	2005	.	manure	.	.	32	219	30	81	118	36	36	U/A
4662	12826	3097	UK	2006	.	.	.	.	33	39	30	82	104	56	1	ST-828
4299	6044B	2762	UK	2005	C	cattle	.	.	33	39	30	82	2	56	3	ST-828
3151	972/03	1758	Spain	2003	.	chicken	.	.	33	39	30	82	2	1	5	ST-828
4075	117	2623	UK	.	.	.	.	No value	33	39	30	82	113	56	12	ST-828
2942	dfvf1834	1574	Denmark	2002	C	chicken	.	.	33	38	30	82	2	1	17	ST-828
4310	8369	2773	UK	2006	C	cattle	.	.	33	39	30	82	104	1	17	ST-828
3721	FG1147	2241	UK	2001	G	.	.	.	33	39	30	79	113	1	17	ST-828
3987	Jun-96	2502	Canada	2006	.	chicken offal or meat	.	.	33	38	30	82	104	3	17	ST-828
3988	Jun-90	2503	Canada	2005	.	chicken offal or meat	.	.	33	39	30	82	113	3	17	ST-828
4661	12762	3096	UK	2006	.	.	.	.	33	3	30	82	113	3	17	ST-828
4073	969	2621	UK	.	.	.	.	No value	33	39	30	81	113	7	17	ST-828
3526	120	2051	Scotland	2005	G	human stool	sporadic case	.	33	39	4	79	104	35	17	ST-828
2921	dfvf13225	1553	Denmark	2001	C	pig	.	.	33	38	16	82	104	43	17	ST-828
4082	C125	2587	UK	.	.	chicken offal or meat	.	.	33	39	30	13	113	43	17	ST-828
3525	117	2050	Scotland	2005	G	human stool	sporadic case	.	33	39	12	139	113	43	17	ST-828

2993	RM4794	1623	Canada	2001	G	human stool	sporadic case	.	33	39	30	82	4	47	17	ST-828
3604	1878	2129	Scotland	2005	G	human stool	sporadic case	.	33	39	30	82	11	47	17	ST-828
3679	4391	2204	Scotland	2006	G	human stool	sporadic case	.	33	39	10	79	104	47	17	ST-828 complex
2303	Penner 54	903	Unknown	.	.	.	.	.	33	39	32	79	104	47	17	ST-828
4742	4665	3169	Scotland	2006	G	human stool	sporadic case	.	33	39	30	82	1	56	17	ST-828
2348	84	1011	UK	2000	C	human stool	sporadic case	.	33	82	30	82	104	56	17	ST-828
3530	145	2055	Scotland	2005	G	human stool	sporadic case	.	33	39	42	82	104	56	17	ST-828
3950	436	2470	UK	.	.	.	.	.	33	28	30	82	113	56	17	ST-828
4681	12705	3116	UK	2006	.	.	.	.	33	39	30	82	104	62	17	ST-828
4083	C138	2588	UK	.	.	chicken offal or meat	.	.	33	38	30	11	104	85	17	ST-828
2347	268	1010	UK	2000	G	human stool	sporadic case	.	33	39	30	79	104	86	17	ST-828
3028	RM4985	1655	USA	2003	G	human stool	sporadic case	.	33	39	30	82	189	219	17	ST-828
2767	794	1415	USA	2003	C	pig	.	.	33	39	47	82	104	43	36	ST-828
3539	184	2064	Scotland	2005	G	human stool	sporadic case	.	33	39	10	82	104	44	37	ST-828
4353	4760	2816	Scotland	.	G	human stool	.	2	33	39	30	82	113	43	38	ST-828
4352	4525	2815	Scotland	.	G	human stool	.	2	33	39	30	82	104	56	38	ST-828
3476	FSA05.280810	2003	England	2005	G	human stool	sporadic case	.	33	39	30	82	113	214	38	ST-828
2250	00-2810	935	Canada	2000	C	cattle	carrier	34	33	39	30	115	104	35	42	ST-828
4116	359	2617	UK	.	.	chicken offal or meat	.	.	33	39	30	82	189	43	42	ST-828
2980	101B	1611	Senegal	2002	.	chicken offal or meat	.	.	33	39	65	140	247	3	17	U/A
3984	409289	2499	England	2006	G	human stool	general outbreak	.	33	176	30	79	11	61	17	U/A
4107	50246	2608	England	.	.	chicken offal or meat	.	.	33	124	30	139	189	47	38	U/A
4750	6044	3177	Scotland	2005	.	cattle	environmental	.	33	39	30	272	189	56	38	U/A
2979	60C	1610	Senegal	2001	.	chicken offal or meat	.	.	33	39	66	174	65	43	41	U/A
2811	1038	1447	USA	2003	C	pig	.	.	33	38	32	82	104	35	68	U/A
3947	135	2467	UK	.	.	.	.	.	34	93	16	1	30	1	17	U/A
3969	683	2489	UK	.	.	.	.	.	47	55	5	10	258	48	8	ST-446
3052	1074E	1681	England	2000	C	chicken	carrier	.	53	38	30	81	118	71	36	U/A
4881	CPH013243c	3300	New Zealand	2001	.	sheep	.	.	55	39	44	82	118	35	36	U/A
3669	4165	2194	Scotland	2006	G	human stool	sporadic case	.	57	39	30	79	104	35	17	ST-828
3648	3759	2173	Scotland	2006	G	human stool	sporadic case	.	64	232	77	100	94	160	16	U/A

3971	719	2491	UK	.	.	.	.	.	64	22	212	100	134	233	16	U/A
3954	488	2474	UK	.	.	.	.	.	81	39	128	82	113	47	38	U/A
3952	255	2472	UK	.	.	.	.	.	81	104	69	113	11	43	67	U/A
4702	182	3129	Scotland	2005	G	human stool	sporadic case	.	87	39	10	79	104	35	17	U/A
2467	37747	1129	USA	1999	.	chicken	.	.	103	110	2	140	104	164	79	ST-1150
3150	83/04	1757	Spain	2004	.	chicken	.	.	103	110	122	140	104	168	46	U/A
3475	FSA05.280539	2002	England	2005	.	environmental waters	environmental	.	121	213	190	218	299	241	175	U/A
3965	123	2485	UK	.	.	.	.	.	123	21	175	82	273	234	185	U/A
4674	JD23-40	3109	Scotland	.	.	.	.	.	123	90	187	223	317	245	198	U/A
2944	dfvf1912	1576	Denmark	2002	C	chicken	.	.	128	175	152	197	245	208	133	U/A
3179	FSA04.281215	1772	England	2004	.	environmental waters	environmental	.	130	86	10	124	269	129	73	U/A
3493	FSA04.280601	2020	England	2004	.	environmental waters	environmental	.	138	166	144	262	230	191	67	U/A
3455	FSA04.281491	1982	England	2004	.	environmental waters	environmental	.	148	206	195	224	282	239	190	U/A
688	6897b	3123	Scotland	2005	.	wild bird	environmental	.	163	215	186	220	286	208	155	U/A
4669	CPH0712418A	3104	New Zealand	2007	.	chicken offal or meat	.	.	184	39	1	82	7	43	5	U/A
4227	710	2690	UK	2006	.	chicken	.	.	1	17	5	2	10	47	6	ST-353
4201	521	2664	UK	2006	.	cattle	.	.	1	2	3	4	5	56	3	ST-42
2350	81	1013	UK	2004	C	cattle	carrier	.	1	2	3	4	5	9	17	ST-42
4770	VC86	3197	England	2003	C	cattle	.	.	1	4	3	4	5	9	17	ST-42
4773	VC54	3200	Scotland	2003	C	cattle	.	.	1	157	30	4	5	9	17	ST-42
3822	ERL03-2487	2341	New Zealand	2003	.	potable/drinking water	environmental	4,13,16,50	1	1	2	2	6	3	17	ST-61
2583	89-18	1244	USA	.	.	cattle	.	.	1	1	2	2	225	3	17	ST-61
4283	R15-S115	2746	UK	2003	C	sheep	carrier	.	1	39	30	82	104	3	3	ST-828
4314	8712	2777	UK	2006	C	cattle	.	.	1	39	30	82	104	9	3	ST-828
4810	ERL96-01429	3229	New Zealand	1996	.	human stool	.	41	1	2	215	322	90	25	17	U/A
4876	ERL96-02902	3295	New Zealand	1996	.	human stool	.	37	2	4	5	82	104	1	5	ST-206
3339	12133	1869	UK	2001	.	human unspecified	.	.	2	1	30	5	2	1	5	ST-21
4775	VC156	3202	England	2003	C	cattle	.	.	2	1	21	82	2	1	5	ST-21
4325	5134	2788	Scotland	.	G	human stool	.	2	2	1	1	3	2	47	5	ST-21
4318	4615	2781	Scotland	.	G	human stool	.	2	2	1	1	3	2	56	5	ST-21

1985	leaI2462	797	UK	2001	C	cattle	carrier	.	2	1	1	3	2	1	17	ST-21
3904	299	2424	UK	.	.	.	.	.	2	1	5	3	2	1	17	ST-21
3527	129	2052	Scotland	2005	G	human stool	sporadic case	.	2	4	6	2	7	219	5	ST-48
776	1443	425	UK	1993	G	human stool	sporadic case	4,13,50	2	2	2	2	2	3	17	ST-61
1117	ShpSm5	81	UK	.	C	sheep	carrier	.	2	4	2	2	6	3	17	ST-61
4311	8377	2774	UK	2006	C	cattle	.	.	2	39	30	82	104	56	17	ST-828
1914	48293	726	UK	2001	.	chicken offal or meat	.	.	2	2	10	4	7	71	1	U/A
3409	55	1940	Netherlands	1998	C	chicken	carrier	.	2	217	2	5	2	268	5	U/A
3833	PH424	2352	New Zealand	2001	.	environmental waters	environmental	1,44	2	29	4	105	131	24	17	U/A
2825	001A-160	1457	Canada	2001	G	human stool	.	.	2	165	73	147	220	190	104	U/A
959	89 116	500	UK	1988	C	cattle	carrier	.	3	4	2	2	6	3	17	ST-61
571	P54	357	Unknown	.	.	.	.	54	3	39	32	79	79	43	17	U/A
4098	C344	2600	UK	.	.	chicken offal or meat	.	.	4	7	10	4	42	43	1	ST-45
4088	C192	2590	UK	.	.	chicken offal or meat	.	.	4	7	10	4	5	47	1	ST-45
4228	714	2691	UK	2006	.	chicken	.	.	4	7	10	4	42	47	1	ST-45
3059	001A-124	1685	Canada	2001	G	human stool	.	.	4	7	10	4	121	7	17	ST-45
4094	C295	2596	UK	.	.	chicken offal or meat	.	.	4	7	2	10	11	3	17	ST-574
187	p49	151	USA	.	G	human stool	sporadic case	49	4	42	30	4	51	43	1	U/A
186	p46	150	Canada	.	G	human stool	sporadic case	46	4	38	30	15	12	44	1	U/A
4053	218	2565	UK	.	G	human stool	sporadic case	.	4	1	30	82	113	44	17	U/A
3926	107	2446	UK	.	.	.	.	.	6	3	1	2	7	47	5	ST-48
4108	52665	2609	England	.	.	chicken offal or meat	.	.	7	17	2	15	23	43	12	ST-443
3939	316	2459	UK	.	.	.	.	.	7	17	2	15	23	56	12	ST-443
4090	C212	2592	UK	.	.	chicken offal or meat	.	.	7	17	2	15	23	3	17	ST-443
3504	M,05,280745	2029	England	2005	G	human stool	sporadic case	.	7	1	2	2	4	3	17	ST-61
4230	721	2693	UK	2006	.	sheep	.	.	7	4	2	2	6	3	17	ST-61
4865	CJ258	3284	Italy	.	.	chicken	.	.	7	17	52	10	89	164	6	U/A
4336	4664	2799	Scotland	.	G	human stool	.	2	7	78	42	82	106	12	8	U/A
3153	88/04	1760	Spain	2004	.	chicken	.	.	7	4	6	68	93	3	17	U/A
4340	4955	2803	Scotland	.	G	human stool	.	2	7	4	6	68	188	3	46	U/A
3514	80	2039	Scotland	2005	G	human stool	sporadic case	.	9	2	4	62	4	43	6	ST-257
3936	290	2456	UK	.	.	.	.	.	9	2	4	62	4	3	17	ST-257

2244	00-6389	929	Canada	2000	G	human stool	sporadic case	11	9	2	4	62	4	5	17	ST-257
3593	1756	2118	Scotland	2005	G	human stool	sporadic case	.	9	216	5	10	10	3	1	U/A
3914	300	2434	UK	.	.	.	.	.	9	2	5	4	2	1	17	U/A
3056	610/03	1682	Spain	2003	.	environmental waters	.	.	10	186	30	62	260	223	6	U/A
4784	VC85	3211	Scotland	2003	C	cattle	.	.	10	23	2	19	6	18	17	U/A
194	p35	158	USA	.	C	marmoset	carrier	35	10	27	5	44	52	41	17	U/A
1990	leaI864	802	UK	2001	C	other animal	carrier	.	18	100	22	104	113	105	6	U/A
1991	leaI879	803	UK	2001	.	environmental waters	environmental	.	18	85	22	104	113	105	6	U/A
4785	VC301	3212	England	2003	C	cattle	.	.	19	4	2	2	6	3	17	ST-61
4808	ERL96-00871	3227	New Zealand	1996	.	human stool	.	37	24	17	2	322	104	3	12	ST-443
3883	M,05.280394	2402	UK	2005	.	environmental waters	environmental	.	27	255	77	18	25	160	16	U/A
167	p34	127	Unknown	.	C	marmoset	carrier	34	30	37	30	32	11	35	6	U/A
182	p59	139	Unknown	.	C	pig	carrier	59	32	38	30	41	49	35	28	U/A
503	185H	348	Netherlands	1998	C	chicken	carrier	.	33	1	21	3	2	1	5	ST-21
4509	11904	2969	UK	2001	.	.	.	.	33	55	1	3	2	1	74	ST-21
4054	220	2566	UK	.	G	human stool	sporadic case	.	33	2	4	62	4	5	12	ST-257
4529	12688	2989	UK	2006	.	.	.	.	33	7	10	4	42	51	1	ST-283
4519	12611	2979	UK	2006	.	.	.	.	33	10	2	10	1	12	6	ST-354
4548	13125	3008	UK	2006	.	.	.	.	33	2	3	4	5	9	3	ST-42
3599	1866	2124	Scotland	2005	G	human stool	sporadic case	.	33	17	2	15	23	3	12	ST-443
1902	48300	714	UK	2001	.	chicken offal or meat	.	.	33	7	10	4	1	7	1	ST-45
4550	13158	3010	UK	2006	.	.	.	.	33	39	10	4	1	7	1	ST-45
4656	13176	3091	UK	2006	.	.	.	.	33	39	10	4	1	7	17	ST-45
4369	G11a	2832	USA	.	.	.	.	.	33	153	2	2	89	43	6	ST-460
4640	11206	3075	UK	2000	.	.	.	.	33	4	1	2	7	1	5	ST-48
4649	12669	3084	UK	2006	.	.	.	.	33	39	1	2	7	1	17	ST-48
4369	G11a	2832	USA	.	.	.	.	.	33	153	2	2	89	43	6	ST-460
4074	412	2622	UK	.	.	.	.	No value	33	115	57	26	127	29	17	ST-692 complex
1505	SH1	648	UK	.	C	sheep	carrier	.	33	39	30	82	113	47	1	ST-828
1501	Cattle9	647	UK	.	C	cattle	carrier	.	33	39	30	82	113	47	3	ST-828
3987	Jun-96	2502	Canada	2006	.	chicken offal or meat	.	.	33	38	30	82	104	3	17	ST-828
4682	12215	3117	UK	2002	.	.	.	.	33	39	30	79	2	35	17	ST-828

181	p56	138	Israel	.	G	human stool	sporadic case	56	33	39	30	2	48	35	17	ST-828
4657	13225	3092	UK	2006	.	.	.	.	33	38	66	82	5	43	17	ST-828
807	80171	437	Netherlands	1996	G	human stool	sporadic case	.	33	39	30	79	39	43	17	ST-828
1500	Cattle1	646	UK	.	C	cattle	carrier	.	33	39	30	82	1	47	17	ST-828
236	p51	57	Unknown	.	.	.	.	51	33	39	30	2	6	47	17	ST-828
2897	11760	1529	England	.	G	human stool	.	.	33	39	30	82	7	47	17	ST-828
4642	12087	3077	UK	2001	.	.	.	.	33	39	30	82	2	56	17	ST-828
4206	540	2669	UK	2006	.	cattle	.	.	33	160	30	272	104	56	17	ST-828
4226	704	2689	UK	2006	.	chicken	.	.	33	39	30	82	189	56	17	ST-828
4202	522	2665	UK	2006	.	cattle	.	.	33	39	30	82	189	56	38	ST-828
4197	436	2660	UK	2006	.	cattle	.	.	33	39	30	82	104	56	65	ST-828
4713	1068	3140	Scotland	2005	G	human stool	sporadic case	.	33	2	2	2	10	3	1	U/A
3146	974/03	1754	Spain	2003	.	chicken	.	.	33	39	12	3	2	56	5	U/A
3403	11902	1933	UK	2001	.	human unspecified	.	.	33	2	1	3	10	3	6	U/A
2750	K8	1366	Pakistan	.	G	human stool	sporadic case	.	33	2	42	4	90	25	8	U/A
4655	13171	3090	UK	2006	.	.	.	.	33	39	4	79	6	3	17	U/A
1176	F090	555	Australia	1999	G	human stool	sporadic case	.	33	39	65	79	111	7	17	U/A
808	3181	438	UK	1991	G	human stool	sporadic case	NT	33	39	2	2	81	35	17	U/A
4651	12822	3086	UK	2006	.	.	.	.	33	39	21	79	2	123	17	U/A
4654	13170	3089	UK	2006	.	.	.	.	33	39	4	48	104	34	38	U/A
2746	44464	1362	UK	.	G	human stool	sporadic case	.	33	124	30	84	189	47	74	U/A
3848	M04, 280865	2363	England	2004	G	human stool	sporadic case	.	33	39	66	82	2	1	174	U/A
191	ps15	155	Unknown	.	.	.	.	15	34	4	2	43	13	3	6	ST-658
4446	LMQS-704	2907	Germany	2006	.	chicken offal or meat	.	.	47	55	5	10	23	120	17	ST-446
519	451814	327	UK	1998	.	lamb offal or meat	.	NT	51	37	5	65	2	56	5	U/A
2215	H69901	886	UK	2003	G	human stool	sporadic case	.	53	38	83	82	104	43	17	ST-828
817	450767	445	UK	1998	.	beef offal or meat	.	NT	53	38	30	81	65	71	36	U/A
465	451192	284	UK	1998	.	lamb offal or meat	.	56	54	39	48	3	2	44	5	U/A
3724	FG1141	2243	UK	2001	G	.	.	.	57	17	2	2	11	47	6	ST-354
1691	Ch146	625	UK	2000	C	chicken	carrier	.	58	7	40	4	42	3	38	ST-283
2593	01-4:116	1254	Sweden	2001	.	wild bird	environmental	.	64	93	100	3	175	143	16	U/A
4194	385	2657	UK	2005	.	.	.	.	81	155	167	277	338	43	17	U/A
4016	Jun-85	2528	Canada	2005	.	environmental	.	.	81	155	30	163	231	1	93	U/A

						waters										
4015	Jun-84	2527	Canada	2005	.	environmental waters	.	.	81	155	30	82	231	3	93	U/A
4014	Jun-83	2526	Canada	2005	.	environmental waters	.	.	81	155	30	163	231	3	93	U/A
4021	Jun-82	2533	Canada	2006	.	environmental waters	.	.	81	155	30	163	231	190	93	U/A
2688	00-S2:10	1349	Sweden	2000	.	wild bird	environmental	.	84	140	115	144	199	136	87	U/A
4221	677	2684	UK	2006	.	chicken	.	.	87	39	30	82	189	43	17	ST-828
2415	AnnaH2	1084	UK	2004	C	cattle	carrier	.	87	39	30	82	104	44	74	ST-828
3746	11710	2268	UK	2000	.	human unspecified	.	.	103	84	2	10	119	178	26	U/A
4427	E60941	2888	Luxembourg	2006	.	chicken offal or meat	.	.	114	2	2	2	11	5	6	ST-354
3061	001A-285	1686	Canada	2004	G	human stool	.	.	118	3	6	4	3	3	17	ST-22
4674	JD23-40	3109	Scotland	.	.	.	.	.	123	90	187	223	317	245	198	U/A
4741	4663	3168	Scotland	2006	G	human stool	sporadic case	.	163	215	210	218	4	241	198	U/A
4218	651	2681	UK	2006	.	chicken	.	.	163	215	210	218	324	241	198	U/A
4234	782	2697	UK	2006	.	pig	.	.	165	39	30	82	118	35	17	ST-828
4636	CPH0711853	3071	New Zealand	2007	.	chicken offal or meat	.	.	184	7	10	4	1	7	1	ST-45
4816	CPH014735	3235	New Zealand	2001	.	environmental waters	.	.	213	1	57	26	127	29	17	ST-692

G - Gastroenteritis
C - Carrier