THE ISOLATION AND GENOTYPIC CHARACTERISATION OF *CAMPYLOBACTER JEJUNI* FROM ENVIRONMENTAL MATRICES

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List of Abbreviations

AFLP	amplified fragment length polymorphism
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
Bp	base pair(s)
CCDA	(blood-free) charcoal-cefoperazone-deoxycholate agar
CBA	Columbia blood agar
CCUG	Culture Collection, University of Göteborg
CDT	Cytolethal distending toxin
cfu g^{-1}	colony forming units per gram
CHO	tissue culture cell lines derived from Chinese hamster ovary cells
ddH_2O	double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERL	Enteric Reference Laboratory (ESR, New Zealand)
ESR	Environmental Science and Research Ltd.
(x) g	(times) gravity
GBS	Guillian Barré Syndrome
HeLa	tissue culture cell lines derived from human cervical cancer cells
Kb	kilobase
LPS	lipopolysaccharide
MLST	multi-locus sequence typing
MPN	Most probable number
MPN gfw^{-1}	Most Probable Number per gram of fresh weight (e.g. of intestinal contents)
MW	molecular weight
NCTC	National Collection of Type Cultures
NZRM	New Zealand Reference Culture Collection, Medical Section
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PFGE	pulsed field gel electrophoresis
RAPD	random amplified polymorphic DNA
RE	restriction enzyme
RFLP	restriction fragment length polymorphism

RTQ-PCR	real-time quantitative PCR
TBE	Tris Borate EDTA Buffer
UPGMA	unweighted pairs geometrics matched analysis
Vero cells	tissue culture cell lines derived from a green monkey kidney carcinoma
VBNC	viable but non culturable

Abstract

Infection by *Campylobacter* is the most notified gastrointestinal disease in New Zealand. Reliable recovery and identification of campylobacters is challenging. Improved and validated methods are needed to increase the power of subtyping and epidemiological studies to trace the sources and transmission routes of *Campylobacter*. An enrichment-PCR method for the isolation and detection of *C. jejuni* and *C. coli* was developed and sensitivity levels determined in 13 environmental matrices, including animal faeces, food and water. Less than ten cells per sample of either *C. jejuni* or *C. coli* could be detected, except for rabbit faeces where the minimum number of cells detected per sample was greater than ten cells for *C. coli* (range 3-32 cells). The sensitivity of the method was comparable to that determined for the conventional methods in the same matrices. Application of the method to retail chicken carcasses (n =204) determined a prevalence of 27.5% *C. jejuni* and 1% *C. coli*. River water assays (n = 293) found 55.3% of samples to contain *C. jejuni* and 4.1% *C. coli*. Furthermore, the enrichment-PCR assay was shown to identify up to three subtypes in individual water samples.

It was proposed that the identification of non-dominant subtypes carried by a chicken carcass may aid the identification of subtypes implicated in human cases of campylobacteriosis. An average of twenty-three *C. jejuni* isolates from each of ten retail chicken carcass were subtyped by PFGE using the two restriction enzymes *Sma*I and *Kpn*I. Fifteen subtypes, in total, were identified from the ten carcasses. One subtype was identified on three carcasses. Five carcasses carried a single subtype, three carcasses carried two subtypes each and two carcasses carried three subtypes each. Some of the subtypes carried by an individual carcass were shown to be clonally related raising the question of *in vivo* recombination events during host passage. Comparison of *C. jejuni* subtypes from chickens with those isolated from human clinical cases revealed three of the fifteen subtypes correlated with those from human cases. None of the minority subtypes were identified in human case isolate data, suggesting that the lack of identification of non-dominant subtypes from chicken carcasses may not hinder the investigation of campylobacteriosis outbreaks.

1 Introduction

1.1 The Genus *Campylobacter*

In 1886, Escherich described an unidentified spiral bacterium in the faeces of children suffering from diarrhoea. This bacterium could not be isolated on solid media. Further observations of a similar organism were made by German authors, in particular, over the following decade. Interest in this unculturable organism waned until the second half of the twentieth century when initial investigations placed the bacterium in the genus *Vibrio* due to its spiral shape and physiology (see Park 2002 for historical detail). Subsequent research led to the formation of the new genus *Campylobacter* and following on the work of others (Butzler 2004), Skirrow showed that *Campylobacter jejuni* was an aetiologic agent of diarrhoeal illness in humans (Skirrow 1977).

Over the last thirty years *Campylobacter* has become recognised as the major cause of gastroenteritis in developed countries, as the number of cases far exceeds those attributed to the better known pathogens in the genus *Salmonella* (Table 2). *Campylobacter* belongs to the family Campylobacteraeae, which also includes *Helicobacter*, *Arcobacter* and *Wolinella* (Nachamkin 1995, Corry *et al.* 1995). This family belongs to the epsilon division of the class *Proteobacteria*, also known as ribosomal RNA superfamily VI (On 2001). Currently, 16 species and six subspecies of *Campylobacter* are recognised (On 2001) including the more recent additions of *C. hominis* and *C. lanienae* which were both identified in human faeces (Logan *et al.* 2000, Lawson *et al.* 1998), and *C. helveticus* (Stanley *et al.* 1992) which was originally isolated from cats and dogs. The thermotolerant subgroup of the campylobacters, which includes *C. jejuni* and *C. coli, C. lari and C. upsaliensis* grow optimally at 42°C and comprise the group of campylobacters most important as pathogens of humans (Corry *et al.* 1995). *C. jejuni* and *C. coli* are the two pathogenic species that cause the majority of human cases of campylobacteriosis (80-90% and 5-10% respectively) in developed countries (Lawson *et al.* 1999).

1.1.1 Basic morphology and physiology of *Campylobacter*

The Greek language provides the source of the word *Campylobacter*, meaning a curved rod, and refers to the S-shaped or spiral morphology of cells less than 48 hours old (Griffiths and Park 1990). Older cells form a coccoid shape, which is associated with reduced viability and the controversial issue of viable but non-culturable cells (refer Section 1.1.5) (Beumer *et al.* 1992, Moran and Upton 1986, Ng *et al.* 1985). Campylobacters are gram negative and the size of the spiral shaped cells ranges from 0.5 to 8.0 μm long and 0.2 to 0.5 μm wide. Most organisms have a single polar flagellum, although some are identified with a flagellum at each pole (Griffiths and Park 1990). Microscopical identification is aided by observation of its 'corkscrew-like' motility with movements including rapid jerking and instantaneous reversing. These features are thought to enhance the mobility of *Campylobacter* as it moves through the viscous mucous to colonise the membrane surfaces of the alimentary tract (Skirrow 2000).

Campylobacters are oxidase positive and most of the themotolerant group are catalase positive except for *C. upsaliensis* which is either negative or weakly positive. They are assacharolytic organisms, unable to oxidize or ferment carbohydrates and therefore require alternative carbon sources for growth (Barros-Velázquez *et al.* 1999). Amino acids have been suggested as likely carbon sources and *C. jejuni* has been shown to utilise serine, aspartate, glutamate and proline (Leach *et al.* 1997). Organic acids including lactic, succinic and malic acids have been shown to enhance the growth of *Campylobacter* isolates when added as either individual or mixed supplements to basal broth media (Hinton 2006). The assacharolytic feature of campylobacters limits the biochemical tests available for their differentiation.

1.1.2 Characteristics of the *Campylobacter* genome

The genome sizes of *C. jejuni* and *C. coli* are approximately 1.6 to 1.7 megabases and the genome is present as a single circular DNA molecule with a low GC ratio of approximately 30% (Taylor *et al.* 1992, Owen 1983). It was the low GC ratio and assacharolytic nature of *Campylobacter* that first led to its differentiation from the genus *Vibrio* (Moore *et al.* 2005, On 2001). The first *C. jejuni* genome sequence (strain NCTC11168) was published in 2000 by Parkhill *et al*. and revealed a relative lack of organisation of genes into operons or clusters apart from those involved in ribosomal protein production and cell surface modifications. Consistent

with the assacharolytic phenotype of *C. jejuni*, there were few genes identified for the degradation of carbohydrates. Three copies of the ribosomal RNA operon were identified and no functional insertion sequence elements, retrons or prophages were evident. There was also a surprising lack of repetitive DNA sequences with only four repeated sequences identified. A standout feature of the genome was the presence of hypervariable sequences, where tracts of DNA revealed variation at single points between otherwise identical clones. Four-fold sequencing of several variants showed no differences to the original sequence, confirming that the differences were not due to artefacts in the sequencing method. This finding was further supported by direct PCR analysis and denatured polyacylamide gel electrophoresis that identified a conserved number of polymorphic forms within a single gene of a strain and this polymorphism was observed within single colonies (Wassenaar *et al.* 2002). These differences have not been associated with detectable phenotypes but are proposed to be variations conferring a survival advantage during infection of a host.

Understanding of the genetic polymorphisms leading to the high divergence of *C. jejuni* subtypes observed in the environment (Siemer *et al.* 2004, Hopkins *et al.* 2004) has increased rapidly with studies showing regions of high recombination potential and regions of gene conservation (Poly *et al.* 2005, 2004; Miller *et al.* 2005, Karlyshev *et al.* 2005, Taboada *et al.* 2004, Prendergast *et al.* 2004, Schouls *et al.* 2003, Sails *et al.* 2003b, de Boer *et al.* 2002, Pearson *et al.* 2000). By comparison, the genome of *C. coli* is recognised as relatively homogeneous (On and Harrington 2000, Duim *et al.* 1999).

Plasmids have been identified in *Campylobacter* strains (Taylor 1992a). For example, mutational studies have shown that a plasmid pVir (37-kb) carries genes important in the invasion of host intestinal cells. Another plasmid, pTet (45-kb), confers resistance to the antibiotic tetracycline (Poly *et al.* 2005, Bacon *et al.* 2002, Bacon *et al.* 2000).

1.1.3 Incidence of species of *Campylobacter* implicated in campylobacteriosis

A United Kingdom survey of isolates from 3,378 human faecal samples used PCR detection to identify 493 (14.5%) samples as positive for *Campylobacter* (Lawson *et al.*, 1999). When identified to the species level by PCR, 89% of isolates were *C. jejuni* and 18% were *C. coli*. These data included 19 samples that were positive for a mixed infection of *C. jejuni* and *C. coli*. The other *Campylobacter* species present were *C. upsaliensis* (2%), *C. hyointestinalis* (0.6%)

and *C. lari* (0.2%). The generally accepted figure for prevalence of *Campylobacter* species in human clinical cases of campylobacteriosis in the developed world is 80-90% *C. jejuni* and approximately 10% *C. coli* (Tauxe 1992, Skirrow 1990). Figures for New Zealand clinical cases of campylobacteriosis are difficult to obtain, as most clinical laboratories do not identify *Campylobacter* to the species level. A study conducted in the Ashburton District of the South Island, however, did report isolation rates of 90% for *C. jejuni* and 10% for *C. coli* from 61 human clinical specimens (Devane *et al.* 2005).

Over a ten year period at the Red Cross Children's Hospital in Cape Town, South Africa, stools from 19,535 paediatric patients suffering from diarrhoea were examined for causative agents of diarrhoea (Lastovica *et al*. 2000). The percentage of infections caused by thermotolerant and other campylobacters from the South African study is presented in Table 1. The differences in prevalence found compared to studies in European countries may be due to the restricted age range, the large number of samples tested, geographical differences and/or the different isolation methods used between studies. The difficulty in comparing these data sets is openly debated in the article by Lastovica *et al*. 2000. The high prevalence of *C. concisus* may be age related as Engberg *et al.* (2000) noted that *C. concisus* can be isolated from healthy and diarrheic patients in nearly equivalent proportions but maybe associated with gastroenteritis when isolated from small children and infants (Engberg *et al*. Author's reply in Lastovica *et al*. 2000). Furthermore, it has been suggested that *C. concisus* should be considered as a commensal organism of the human gut rather than as a primary pathogen of gastroenteritis (Engberg *et al.* 2000, Van Etterijck *et al.* 1996). Importantly, what the South African study highlights, and has been noted by other researchers, is that the true incidence and species distribution of *Campylobacter* infections is largely unknown as most isolation methods are directed at identifying *C. jejuni* and *C. coli* by the use of selective methods that exclude other *Campylobacter* species and related organisms (Engberg *et al.* 2000, On 1996, Corry *et al.* 1995).

Campylobacter jejuni subspecies *doylei* is rarely isolated in developed countries. An area reporting a relatively high frequency of isolates is Australian aboriginal communities (Albert *et al.* 1992, Steele and Owen 1988). Although these bacteria hydrolyse hippurate like *C. jejuni* subsp. *jejuni*, they are unable to reduce nitrate to nitrite. Furthermore, they grow slowly at 37°C and generally fail to grow at 42°C and are more susceptible to the antimicrobials cephalothin and polymixin (Steele and Owen 1988).

Table 1: Frequency of *Campylobacter* isolation from the diarrhetic stools of paediatric patients in South Africa

Campylobacter species	Percentage positive
C. jejuni subsp. *jejuni*	31.3
C. jejuni subsp. *doylei*	9.2
C. coli	2.9
C. lari	0.1
C. upsaliensis	23.0
C. concisus	23.6
C. hyointestinalis	1.3

1.1.4 Survival of *Campylobacter*

1.1.4.1 Survival in food matrices

The thermotolerant campylobacters have a reputation for fastidious culture requirements (Park 2002). They are microaerophilic organisms requiring 5-10% oxygen, 10% carbon dioxide and 80% nitrogen and/or hydrogen. Isolation methods for *Campylobacter* usually require substances that reduce the oxygen tension and neutralise the toxic compounds that form in the presence of light and oxygen (Barros-Velázquez *et al.* 1999, Corry *et al.* 1995). Therefore they are unlikely foodborne pathogens since they do not, in general, grow in aerobic conditions, nor do they grow below 30°C, both of which should restrict their proliferation in food. Furthermore, they are readily destroyed during cooking as temperatures of 60°C for 15 minutes or 57.5°C for 30 minutes result in death of these microrganisms (Park 2002, Barros-Velázquez *et al.* 1999). In contrast to most microbes, which show a gradual decline in growth towards their minimum growth temperature, the campylobacters show a rapid growth decline and yet are still observed to be metabolically active at temperatures as low as 4°C (Park 2002, Hazeleger *et al.* 1998, Blaser *et al.* 1980). *Campylobacter* is motile at these low temperatures and displays chemotaxis enabling the bacterium to move towards more favourable environments (Hazeleger *et al.* 1998). These observations highlight the need to distinguish between growth of micro-organisms and their viable environmental persistence, especially in food.

Monitoring of the survival of 1 x 10^6 cfu g^{-1} of *C. jejuni* inoculated into a variety of cooked and raw foods and maintained over a range of temperatures (2-20°C), consistently revealed that *C. jejuni* survived best at 2°C. *C. jejuni* could still be detected (>50 cfu g^{-1}) in cooked minced beef up to 49 days at 2°C, compared with 13 days at 10°C and 6 days at 20°C. Detection of

C. jejuni in raw chicken and raw minced beef at 2°C was limited to 24 and 27 days respectively (Curtis *et al.* 1995). Viable *Campylobacter* has been isolated from frozen foodstuffs, although at much lower levels compared to fresh food (Jørgensen *et al*. 2002, Dufrenne *et al*. 2001, Humphrey and Cruickshank 1985). The effect of freezing on *Campylobacter* will be discussed in regard to chicken carcasses in Chapter Three.

In addition to temperature, campylobacters are sensitive to other environmental stresses. Their optimum pH is 6-8 and they are unable to multiply below pH 4.9 with high mortality below this pH (Park 2002, Barros-Velázquez *et al.* 1999, Blaser *et al.* 1980). The pH of meat has been shown to be critical to the survival of the organism at refrigeration temperatures, with significantly higher survival in beef at pH 6.4 than beef's normal pH of 5.8 (Gill and Harris 1982). Campylobacters are unable to grow in sodium chloride above a concentration of 2.0% (Doyle and Roman 1982a), suggesting sensitivity to osmotic stress. They have demonstrated sensitivity to drying (Kusumaningrum *et al.* 2003, Doyle and Roman 1982b), which is relevant to the contamination of food preparation surfaces, with Humphrey *et al.* (1995) proposing that *Campylobacter* surveillance of catering facilities should concentrate on moist areas.

1.1.4.2 Survival in water

A study of the survival of *C. jejuni* in drinking water evaluated 19 different strains isolated from human clinical cases, two reference strains, poultry, bovine and water sources (Cools *et al.* 2003). The strains were suspended in sterilised drinking water at a concentration of $\geq 10^6$ colony forming units per ml (cfu ml^{-1}) and incubated in the dark at 4°C. Survival was determined by culture on both a selective and non-selective agar and isolates showed longer survival on the non-selective medium. The *C. jejuni* isolates from poultry remained culturable over the longest time period (30-52 days). This compared with the water and human clinical isolates which became non-culturable after 29 days. The prolonged survival of poultry isolates in drinking water has implications for the transmission of campylobacters and their persistence in the environment. A study of *Campylobacter* survival in water by Obiri-Danso *et al.* (2001) found a 1 log reduction in numbers of campylobacters (cfu ml^{-1}) in natural populations from river water after approximately 100 hours incubated at 4°C in the dark. For river water temperatures of 10°C and 20°C, the time taken for a 1 log reduction in the number of campylobacters (cfu ml^{-1}) was 90 hours and less than 12 hours respectively. Survival was somewhat better in seawater than river water, with natural populations surviving for up to 24 hours at 20°C and 37°C, but

persisting up to 120 hours at 4°C and 10°C. Natural populations of *Campylobacter* were below the level of detection within 30 minutes when exposed to sunlight (equivalent to an English June day) and held at 17-20^{o}C. These results are consistent with observations that *Campylobacter* tend to be more frequently isolated from water in the winter months, as this is the time when the water temperature and exposure to UV will be lower (Carter *et al.* 1987).

1.1.4.3 Survival in faeces

Campylobacter has been reported to survive in sheep faeces for up to four days when left outside at field temperatures (Jones *et al.* 1999). *Campylobacter* inside cow pats would be protected from drying and UV radiation and could be expected to survive for long periods. Unpublished New Zealand data shows good survival (one month) under moist, cool conditions (personal communication, Andrew Hudson, ESR).

Data provided by Keith Jones (Lancaster University) shows a maximum of seven days survival of *Campylobacter* in gull faeces deposited at a rubbish tip. As there are limited data available on *Campylobacter* survival in duck faeces, for this study, I am assuming that the survival time of *Campylobacter* in duck faeces will approximate that of its survival in gull faeces.

1.1.5 Viable but non-culturable bacteria

The proposal that some bacteria can enter a survival stage termed viable but non-culturable (VBNC) has generated considerable controversy over the last two decades. In the 1980s Professor Rita Colwell and her associates while investigating gram negative bacteria, particularly enteric pathogens, noted that when subjected to stress such as encountered in aquatic environments, a large proportion of culturable cells became non-culturable (Grimes *et al.* 1986). Subsequently, a proportion of these non-culturable cells could be resuscitated under specific conditions such as addition of nutrients (Roszak *et al.* 1984). These VBNC cells would not be detected by conventional culture techniques and thus could be overlooked as a health risk. A discrepancy has also been noted between the viable counts and counts observed by microscopy (Cappelier *et al.* 2000, Kogure *et al.* 1978). Pathogenic bacteria that have been reported to enter the VBNC state include *E. coli* (Pommepuy *et al.* 1996), *Salmonella* (Roszak *et al.* 1984), *Vibrio cholerae* (Binsztein *et al.* 2004) and *C. jejuni* (Rollins and Colwell 1986).

Opponents of the VBNC theory suggest that the VBNC phenomenon can be most readily explained by the well characterised mechanisms of cell injury and death (Barer and Harwood 1999, Kell *et al.* 1998). They suggest that the process of cell death is not immediate but a gradual decline in metabolic function, the rate of which is dependent on the type of stress encountered, e.g. UV irradiation and temperature shifts. At some point in this metabolic decline a bacterium goes beyond the point of resuscitation, and cell death and lysis follows. Routine techniques to resuscitate injured cells in the laboratory include initial incubation at lower temperatures and delayed addition of antibiotics to an enrichment broth used for selection of the target microbe. Employing multiple types of media may also aid recovery of bacteria that are inactive on a particular medium. It is also recognised that some cells subjected to sunlight irradiation are able to repair injury from UV light even when incubated on selective media (Sinton *et al.* 2002).

As the *Campylobacter* cell ages it has been noted that it undergoes morphological changes. During exponential growth the cell is the well recognised curved rod shape but when the bacterium encounters stress or enters the stationary phase morphology changes to a coccoid form, which is associated with reduced viability (Ng *et al.* 1985). This shape change has been cautiously associated with the putative viable but non-culturable state (Rollins and Colwell 1986). Other evidence suggests, however, that the coccoid cells are degenerate, forming as a result of stress and/or the ageing process and therefore are undergoing degradation leading to their non-culturability and eventual non-viability (Hazeleger *et al.* 1995, Moran and Upton 1986). Furthermore, Hazeleger *et al*. (1995) has shown that the formation of cocci at different temperatures affects their physical composition with cocci formed at 4°C having a similar membrane fatty acid composition as exponential curved rods. Potentially, this could suggest that cocci formed at lower temperatures may still be pathogenic and play a role in the transmission of campylobacters through the environment. It has also been shown that cells of *C. coli,* which showed reduced ability to form coccoid cells, were not disadvantaged in their rate of survival under non-growth conditions (Kelly *et al.* 2001). The latter researchers have also presented evidence of the occurrence of spontaneously arising mutants in the stationary growth phase that then overtake the growth of the original population (Kelly *et al.* 2003).

The demonstration of the VBNC state requires employing methods to determine the metabolic state of cells and their (apparent) capacity to regain culturability. These attributes include cell integrity e.g. an intact cell membrane and the possession of some form of measurable metabolic

activity, such as, ATP levels, protein synthesis (Cappelier *et al.* 2000, Hazeleger *et al.* 1995) and cellular respiration which can be determined by using a redox dye: 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). CTC is reduced by an electron transport chain to an insoluble red fluorescent CTC formazan salt. Intracellular accumulation of these red CTC crystals in the bacterial cells is indicative of cellular respiration (Cappelier *et al.* 1997). The main premise of these tests is that they differentiate between actively growing cells and dead cells and this has been one of the criticisms that such tests are, of necessity, calibrated by comparison between culturable and dead cells. It cannot, therefore, be reliably demonstrated that they identify VBNC cells. Interestingly, proteome analysis comparing the protein synthesis between VBNC cells and starved cells of the gram positive bacterium *Enterococcus faecalis* has revealed a distinct protein profile for VBNC cells (Heim *et al.* 2002). The protein profile of VBNC cells differed from both starved and exponentially growing cells, which the authors suggest demonstrates a distinct metabolic phase for *E. faecalis* that differs from normal growth and from the starvation phase where the cells are defined as remaining culturable with an inability to divide.

The question of how relevant the VBNC stage is to human pathogenesis depends on the ability of the VBNC cell to be resuscitated during passage through an animal or human. In all VBNC experiments where the researcher is trying to demonstrate recovery of VBNC cells after passage through an animal model, the main difficulty is the verification that the initial inoculum does not contain a single viable bacterium which could initiate cell division. Dilution is considered the best way to ensure that the culturable cells are absent from the inoculum (Cappelier *et al.* 1999b).

The resuscitation of VBNC *C. jejuni* cells has been demonstrated after passage through rats (Saha *et al.* 1991), mice, and one day old chicks (Cappelier *et al.* 1999a), and recovery from inoculation into embryonated eggs (Cappelier *et al.* 1999b). The *Campylobacter* VBNC strains recovered from the embryonated eggs were also shown to have regained their ability to attach to HeLa cells (tissue culture cell lines derived from human cervical cancer cells), which suggests that they have maintained their pathogenicity and could pose a threat to public health. The *C. jejuni* strains passaged through rats were also demonstrated to have retained their ability to produce toxins. In contrast to these findings, other researchers have been unable to recover VBNC induced *C. jejuni* from animal models, which included chicks, mice and embryonated eggs (van de Giessen *et al.* 1996, Medema *et al.* 1992). It has been suggested that some of these differences could be attributed to the methods of VBNC induction of viable *C. jejuni* cells and

in the case of eggs the site of inoculation was suggested to be critical for providing an environment suitable for growth of campylobacters (Cappelier *et al.* 1999b).

The debate continues between scientists who recognise a survival stage for bacteria termed the VBNC and those who view the recovery of VBNC cells as an outgrowth from injured cells or a remnant of undetected culturable cells. The relevance to research on *C. jejuni* is that it is possible that *Campylobacter* might enter a viable but non-culturable stage when encountering a stressful environment. This has relevance to this study as will be discussed in Chapter Two.

1.1.6 Antibiotic resistance of *Campylobacter* strains

Antibiotic therapy is infrequently used in the treatment of campylobacteriosis. When required the antibiotic of choice is erythromycin (Nachamkin 2003, Blaser and Wang 1980), to which most *C. jejuni* strains causing human infection are susceptible (Harrow *et al.* 2004, Gaudreau and Gilbert 1998, Aarestrup *et al.* 1997, Reina *et al.* 1994). *C. coli*, however, has been shown to have a higher level of resistance (up to 35% of strains isolated from human faeces) to erythromycin compared to *C. jejuni*, which has a maximum of 5.5% of strains from human cases showing resistance (Goodchild *et al.* 2001, Saenz *et al.* 2000, Reina *et al.* 1992).

The antibiotic resistance of *C. jejuni* and *C. coli* isolated from ten different environmental sources including human and animal faeces, river water and meat products was determined in a study conducted in South Canterbury, New Zealand (Harrow *et al.* 2004). Overall, it was concluded that most of the *Campylobacter* isolates had low resistance to the clinically relevant antibiotics: erythromycin (e.g. resistance of 2% in *C. jejuni* and 11.8% in *C. coli*), nalidixic acid, ciprofloxacin and tetracycline. Five of the isolates from pig offal, however, were identified as being highly erythromycin resistant with their minimum inhibitory concentration (MIC) determined as ≥ 256 μg ml^{-1}. These five isolates were all genetically unique as established by PFGE analysis and represented four strains of *C. coli* and one *C. jejuni* strain. The authors suggest that the use of macrolides in the prophylactic administration of antibiotics to pigs in New Zealand may account for the appearance of erythromycin resistant strains of campylobacters. A study of *C. coli* isolated from a farm environment in the United Kingdom also revealed a low level of resistance to antibiotics in samples taken from cattle, sheep, water, birds and soil (Leatherbarrow *et al.* 2004).

It has been noted that there is an increasing number of *C. jejuni* strains that are resistant to fluoroquinolones (e.g. ciprofloxacin), which are antibiotics used in a wide range of infections including those of the respiratory tract (Hooper 2001, Saenz *et al.* 2000, Reina *et al.* 1992).

New Zealand studies of clinical isolates of *C. jejuni* and *C. coli* reported an increase in resistance to erythromycin and ciprofloxacin from 1.5% to 5.5% and 2.5 to 5.5%, respectively (Goodchild *et al.* 2001, Dowling *et al.* 1998). The survey conducted in 2001 noted a statistically significant higher level of resistance to both erythromycin and ciprofloxacin in non-*C. jejuni* compared with *C. jejuni* isolates. Furthermore, a study in Auckland, New Zealand administered a quinolone antibiotic (lomefloxacin) to patients reporting with diarrhoea. Eighty-five percent of these diarrhoeal cases were attributed to *Campylobacter* infection (Ellis-Pegler *et al.* 1995). The lomefoxacin was administered over a five day period as part of a double blind trial that included pacebos. Within the five day period, 28% of the *Campylobacter* isolates rapidly developed resistance to the quinolone.

These trends of increasing bacterial resistance in New Zealand are being mirrored by international studies. A study in the U.S.A. investigating the increase in fluoroquinolone resistance reported a rise in the proportion of *C. jejuni* human isolates from 1.3% in 1992 to 10.2% in 1998 (Smith *et al.* 1999). While a significant number of the resistant strains from humans were acquired during foreign travel, it was also noted that 14% of isolates from local chicken products (n = 91) were ciprofloxacin resistant. Molecular subtyping of the resistant strains isolated from chicken products also showed a correlation with domestically acquired cases of campylobacteriosis. It has been suggested that the prophylactic feeding of antibiotics (including fluoroquinolones) to chickens and other farm animals is leading to an increase in antibiotic resistant strains of pathogenic bacteria (Saenz *et al.* 2000, Reina *et al.* 1992).

A Canadian study of 203 *C. jejuni* human clinical isolates over a three year period identified 50% of isolates as being resistant to a minimum of 64 μg of tetracycline ml^{-1} (Gibreel *et al.* 2004). This resistance to tetracycline had increased from 8% of isolates to 50% over a 20 year period. Of concern, was the finding that 37% of the tetracycline resistant *C. jejuni* isolates were resistant to MIC of 256 to 512 μg ml^{-1} This resistance level is higher than reported in previous Canadian studies which identified 128 μg ml^{-1} as the highest tetracycline MIC in the early 1980s. In general, antibiotic resistance rates to other antibiotics were determined to be low with no isolates being resistant to chloramphenicol or erythromycin. Kanamycin resistance was

identified in 2.9% of isolates and 2.5 % were resistant to nalidixic acid. Four of the five nalidixic resistant isolates were also resistant to ciprofloxacin. This is expected as resistance to both antibiotics requires mutations in the gyrA subunit of the DNA gyrase enzyme (Wang *et al.* 1993). Antibiotic resistance to cephalothin and susceptibility to nalidixic acid are criteria used in the phenotypic assignment of *Campylobacter* isolates to the species *C. jejuni* and *C. coli* (Nachamkin 2003).

The debate about the use of antibiotics in food animals continues with a review by Phillips *et al.* (2004) concluding that there is only a small added danger to human health from the spread of antibiotic resistant bacteria derived from farmed animals. They suggest that the "precautionary principle" of banning the use of antibiotics in animal husbandry is based on a lack of scientific study and requires a full quantitative risk assessment of the facts.

1.2 Epidemiological aspects of *Campylobacter* infection

1.2.1 Incidence of campylobacteriosis

Campylobacteriosis is New Zealand's most frequently notified disease (Table 2) with an incidence in 2004 of 12,213 cases (326.8 per 100,000 population) which represents 53.2% of all notifiable cases (Anonymous 2005). There has been a sustained increase in the number of reported cases in New Zealand since the disease became notifiable in 1980 (Figure 1). Although the number of cases dropped in 2004 (Figure 1), case numbers rose again in 2005 with the unofficial figures being 370 cases per 100,000 (personnel communication Lisa Lopez, EpiSurv, ESR). It is well recognised that the true number of cases presenting to general practitioners is much higher than the notification rate, probably in the order of five to ten times (Withington and Chambers 1997). In addition, the number of campylobacteriosis cases in New Zealand far exceeds the numbers in other developed countries (Table 3). There is an approximately four fold difference in case numbers between New Zealand and Australia, and New Zealand and the United Kingdom, and approximately a sixteen fold difference to the United States.

Table 2: Rates of selected notified enteric disease in New Zealand

Notifiable Disease	Rate per 100,000 population			
	2004	2003	2002	2001
Campylobacteriosis	326.8	395.6	334.3	271.5
Cryptosporidiosis	16.4	21.9	26.1	32.3
Giardiasis	40.5	42.0	41.4	42.9
Salmonellosis	28.9	37.5	50.3	64.7
VTEC/STEC infection	2.4	2.8	2.0	2.0

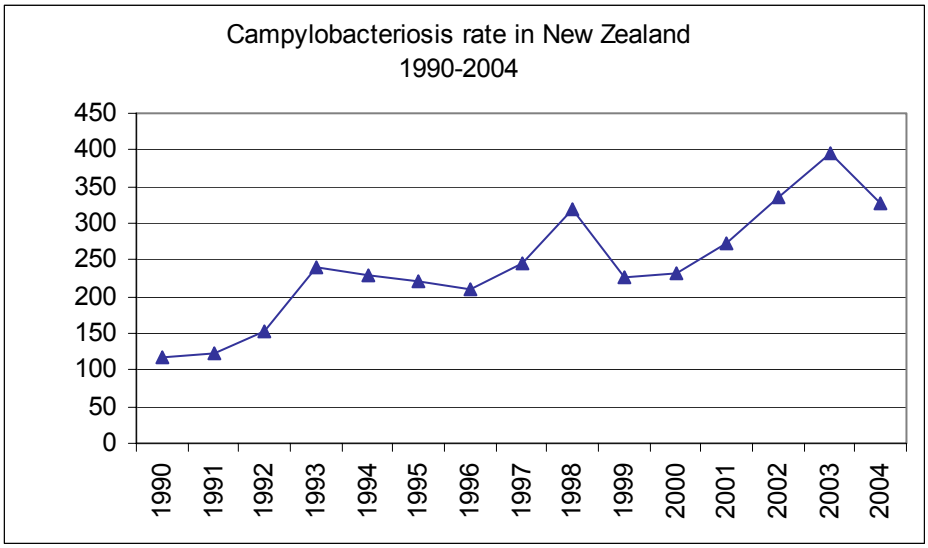

Figure 1: Rates of campylobacteriosis in New Zealand since 1990

Table 3: Rates of campylobacteriosis in selected developed countries

Country	Period	Rate per 100,000	Reference
New Zealand	2004	327	Anonymous (2005)
USA	2001	13.8	Centers for Disease control and Prevention (2001)
United Kingdom	1999	103.7	Gillespie *et al*. (2002)
Canada	1986-1998	39-54	Health Canada (2001)
Denmark	2003	66	Anonymous (2002)
Australia*	2005	105	Communicable Diseases Australia (2005)

*Figures are for the period April to June 2005 and do not include New South Wales which only notify cases associated with foodborne incidents.

1.2.2 Symptoms of campylobacteriosis

The incubation period of *Campylobacter* is usually between one and three days but can be as long as ten (Koenraad *et al.* 1997, Faoagali 1984). The symptoms of human campylobacteriosis include an initial period of fever, headaches and malaise that lasts for up to 24 hours. This is then followed by diarrhoea and in most cases severe abdominal pain. The fever persists, but nausea and vomiting are less common features of the infection occurring in approximately 40% of cases (Gillespie *et al.* 2002, Koenraad *et al.* 1997). The patient may excrete *Campylobacter* organisms for up to three weeks post-infection, with the *Campylobacter* count in faeces from infected humans in the range of 10^6 to 10^8 bacteria per gram (Taylor *et al.* 1993).

1.2.3 Complications associated with campylobacteriosis

Most cases of campylobacteriosis are self-limiting, however infections arising from the direct spread of *Campylobacter* from the gastrointestinal tract can include cholecystitis, pancreatitis, peritonitis and massive gastrointestinal haemorrhage (Allos 2001). Although rare, the following illnesses can occur as extraintestinal manifestations of *Campylobacter*: meningitis, endocarditis, septic arthritis, osteomyelitis and neonatal sepsis. Bacteraemia is detected in less than 1% of cases of campylobacteriosis and occurs predominantly in the very young or old or the immunocompromised (Allos 2001). A study of the short and long term effects of mortality associated with foodborne illness identified a higher risk of mortality up to 30 days post-

infection compared with control cases (Helms *et al.* 2003). This finding was significant even after the factor of co-morbidity with other existing illnesses was taken into account. Analysis also showed that *Campylobacter* was associated with increased long term mortality up to one year after infection. This research suggests that the mortality rate from campylobacteriosis may be underestimated.

1.2.3.1 Campylobacter cases associated with Guillian–Barré syndrome and the related Miller-Fisher syndrome

Two well recognised complications arising from infection by *C. jejuni* are Guillian–Barré syndrome (GBS) (Endtz *et al.* 2000) and the related Miller-Fisher syndrome (Salloway *et al.* 1996). These syndromes are neuro-paralytic, autoimmune disorders that affect the peripheral nervous system and have led to fatal respiratory paralysis in 5% of GBS cases. Typical symptoms include progressive weakness beginning in the legs and moving upwards to the arms and cranial nerves (Hadden and Gregson 2001). Patients may experience numbness, pain and difficulty swallowing. GBS follows in 0.1% of campylobacteriosis cases (Nachamkin *et al.* 1998). New Zealand has reported a high incidence of GBS, for example, eight cases in the province of Canterbury in 1995 (Withington and Chambers, 1997) which was consistent with case numbers from previous years. These figures are not unexpected based on the high incidence of *Campylobacter* infection in New Zealand.

It is difficult to be conclusive about the relationship between GBS and campylobacteriosis because there is a lag of one to three weeks post-infection before the onset of paralysis. As a consequence, tests for campylobacters in a patient's stool may be negative. Serologic studies, however, have reported a high prevalence of *C. jejuni* antibodies in the serum of GBS patients. Case control serologic studies have consistently reported higher numbers of GBS patients who were seropositive for *Campylobacter* in comparison to controls (Nachamkin *et al.* 1998). It has been suggested that GBS developed after infection by *Campylobacter* may result in a more severe and prolonged paralysis in comparison to other suspected causes of GBS. Confirmation of these observations will require larger patient numbers with and without prior *Campylobacter* infection (Nachamkin *et al.* 1998).

An unusual discovery was made during the sequencing of the *C. jejuni* genome. Sequence analysis identified three sets of the genes involved in sialic acid biosynthesis (Parkhill *et al.*

2000). Sialic acid is a rare component of lipopolysaccharides (LPS) on the cell surface of *C. jejuni* that mimics the structure of certain human gangliosides. Through the process of molecular mimicry the sialic acid may be important in evasion of the host's immune system but also confer an autoimmune disease on the host in the post-infection phase (Endtz *et al*. 2000, Salloway *et al*. 1996).

Some studies have concluded that certain Penner serotypes of *C. jejuni* are associated with GBS e.g. HS:19 which occurs infrequently (6%, n = 554) in uncomplicated cases of *Campylobacter* infection but was identified in 67% of GBS cases related to enteritis (Takahashi *et al.* 2005, Engberg *et al.* 2001). In addition, a study of *Campylobacter* clinical isolates from Japan noted an association between *C. jejuni* serotype HS:2 and cases of Miller-Fischer syndrome (Takahashi *et al.* 2005). Other studies, however, have identified serotypes that are isolated more frequently from uncomplicated cases and are also isolated from patients who develop GBS (Dingle *et al.* 2001b, Endtz *et al.* 2000, Nachamkin *et al.* 1998). Furthermore, studies using DNA microarray analysis noted high genomic heterogeneity among isolates associated with GBS and did not identify any genes or regions specific to GBS for those isolates (Leonard *et al.* 2004). Therefore, it is unknown as to whether certain biotypes of *Campylobacter* result in a higher incidence of GBS or Miller-Fischer syndrome associated with campylobacteriosis. In addition, some studies have indicated that the structure of the LPS is only partly responsible for the autoimmune response and that host susceptibility is also likely to play a role (Ang *et al.* 2002).

Another recognised complication of *Campylobacter infection* is reactive arthritis which occurs in 1-3% of campylobacteriosis cases (Colmegna *et al.* 2004, Altekruse *et al.* 1999). Reactive arthritis (ReA) has been described as a sterile synovitis (inflammation of the sinovial membrane of a joint) that develops after an infection of the gastrointestinal or genitourinary tracts by micro-organisms including *Chlamydia, Yersinia, Salmonella, Shigella* and *Campylobacter*. Recent evidence, however, has shown the detection of culturable bacteria or bacterial components (e.g. *Campylobacter* RNA and LPS) from the affected joints. This has led to ReA being redefined as an immune-mediated synovitis resulting from slow bacterial infections and showing the presence of bacterial antigens present in the joint. These antigens are thought to have been synthesized by bacteria residing either in the joint and/or elsewhere in the body (Colmegna *et al.* 2004). ReA is characterised by pain in multiple joints, in particular the knee joints, and symptoms may become chronic (Altekruse *et al.* 1999). Two independent studies

have concluded that the treatment of the gastrointestinal infection with antibiotics does not appear to reduce the subsequent incidence of arthritis (Colmegna *et al.* 2004).

1.2.4 Seasonality of *Campylobacter*

The incidence of human campylobacteriosis in New Zealand and the United Kingdom appears to follow the seasonal trend of being highest in summer and lowest in winter (Owen *et al.* 1997, Brieseman 1990). Early autumn is another time of year in which a large number of cases is reported in developed countries (Barros-Velázquez *et al.* 1999, Skirrow 1990). A comparison of seasonal trends between European countries and New Zealand noted steady seasonal patterns in Britain and continental countries, with the peak number of human cases consistently occurring in distinct weeks in spring/summer (Nylen *et al.* 2002, Owen *et al.* 1997). Interestingly, the data for New Zealand revealed less consistency as the peak week varied from year to year and the summer increase of cases was more prolonged than in the European countries. A study, which combined serotyping and PFGE data on strains of *C. jejuni* isolated from three geographically distinct areas in England over a one year period, noted that there was no particular HS serotype associated with the seasonal peak (Owen *et al.* 1997). Moreover, the increase in numbers during the peak could be attributed to an increase in the less common serotypes, in comparison to an increase in the numbers of normally dominant serotypes. The reasons for these peaks in reported cases are unknown. A typing study conducted in New Zealand also noted a lack of overlap between *Campylobacter* subtypes isolated in autumn and summer from a range of matrices including human faeces (Hudson *et al.* 1999).

A New Zealand study examined the regional and seasonal variation in reported cases of campylobacteriosis and noted distinct differences between the seasonality of the North and South Islands (Hearnden *et al.* 2003). Furthermore, much of the rural North Island displayed low variation between seasons and a low summer incidence of campylobacteriosis, whereas the large urban areas in the upper North Island and their environs exhibited higher summer incidence and greater seasonality than the rural areas. The highest seasonality and highest summer incidence was observed in the lower North Island centres of Wellington and Lower Hutt and in the greater part of the South Island, including the two main urban areas of Christchurch and Dunedin. The authors suggest that these seasonal variations may indicate differences in transmission routes between these regions.

1.2.5 Age and gender distribution

In New Zealand and other developed countries campylobacteriosis can affect all age groups, with the highest incidence usually occurring in infants and up to four years of age. There is also a pronounced secondary peak of incidence in young adults, particularly between the ages of 20 and 28 years of age with a slight increase of males over females (Anonymous 2005, Tam 2001, Engberg *et al.* 2000, Tauxe 1992, Skirrow 1987). In New Zealand for the year 2004, the rate of infection was 355/100,000 population (6467 cases) for males and 287/100,000 (5495 cases) for females (Anonymous 2005).

In developing countries there is a different age pattern with high rates of repeated infection in young children up to 100 times that of developed nations. The number of infections, however, is significantly reduced in the older population. Furthermore, studies have shown the carriage of *Campylobacter* spp. by individuals (14.8%) who do not show signs of diarrhoea (Mathan and Rajan 1986, Rajan and Mathan 1982). It has been suggested that the acquisition of immunity reduces the number of infections and adults are likely to be asymptomatic in the older population (Taylor *et al.* 1993, Taylor 1992b). This is in contrast to developed countries where the carriage of *Campylobacter* by healthy adults has not been observed.

1.2.6 Ethnicity

As for previous years in New Zealand, cases of reported campylobacteriosis in 2004 were highest in those of European descent (326/100,000 population) with Pacific Peoples reporting the lowest rate at 63/100,000 (Anonymous 2005). These results are supported by a case control study conducted in New Zealand where people of European descent made up the majority (>90%) of cases and Maori and Pacific Islanders made up 5.3% and 0.8% respectively of cases (Eberhart-Phillips *et al.* 1997).

1.2.7 Estimating the economic burden of campylobacteriosis

New Zealand cases of campylobacteriosis caused by foodborne transmission have been estimated to cost $40,136,000 annually. This comprises almost 73% of the total cost of foodborne infectious intestinal disease in New Zealand (Scott *et al*. 2000). This cost includes all medical costs, the value of productive days lost and the statistical value of mortality. A similar figure of $40 million was quoted by Withington and Chambers (1997) when an estimate of those cases not presenting to a general practitioner was included and calculations based on 1995 data. Hospitalisation arising from *Campylobacter* infection was reported to be 7.6% of cases in New Zealand in 2004, although this figure was only calculated from cases who answered the question relating to hospitalisation (53.6% of cases) (Anonymous 2005). The value of lost quality of life has not been factored into this estimation but the acute phase of the illness is very debilitating and unpleasant as the outline of symptoms in Section 1.2.2 suggests.

Estimates of the annual cost of campylobacteriosis in the United States of America based on medical and productivity losses were in the order of 1.2 to 6.6 billion dollars ($US). This represented 6 to 19% of total foodborne illness costs when applied to a selected group of aetiological agents: *Campylobacter*, *Clostridium perfringens*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella* (non-typhoid), *Staphylococcus aureus* and *Toxoplasma* (Buzby and Roberts 1997).

The annual USA costs of GBS-associated campylobacteriosis have been estimated as US$0.2 - $1.8 billion. These figures are based on calculations of the economic burden due to resources spent on medical care and lost productivity resulting from illness or death (Buzby *et al.* 1997). These annual costs from GBS-associated campylobacteriosis are in addition to the economic costs quoted in Buzby and Roberts (1997).

In 2000, the Economic Research Service (ERS) released new estimates of the cost of *Campylobacter* infection that included the chronic complication of GBS. The estimated annual cost was based on 1.9 million cases resulting in 10,539 hospitalisations and 99 deaths and was calculated at $1.2 billion US dollars (Economic Research Service 2004). The estimates took into account medical costs, productivity losses and the cost of premature death, using an age-adjusted approach. For example, it was assumed that the cost of the death of a child less than one year of age was five fold higher in comparison to individuals over 84 years of age. They

note that these estimates are not directly comparable to earlier ERS estimates (Buzby and Roberts 1997, Buzby *et al.* 1997) which were based on earlier data and methodologies for valuing costs.

A study conducted in the Netherlands endeavoured to establish the health burden of *Campylobacter* on the population (Havelaar *et al.* 2000). This analysis used the Disability Adjusted Life Year (DALY) methodology to integrate all of the outcomes of *Campylobacter* infection based on epidemiological and clinical data in an attempt to factor in the effects of disease on the quality of life. The DALYs are an estimation of the sum of the "Years of Life Lost" by premature mortality and the years "Lived with Disability". Severity of illness is integrated into the calculation by factoring in a weighted value between zero and one. Their results for *Campylobacter* infection estimated a total health burden of approximately 1400 DALY per year, with a range of 900 to 2000 DALY per year. The large range is indicative of the incomplete nature of the data available for analysis. The authors suggest that this lack of data requires implementation of an active surveillance for pathogens causing gastroenteritis, in comparison to the present situation of a passive flow of information based on clinical reports. To place the calculation of the DALY in perspective, disorders such as meningitis, sepsis, upper respiratory infections, ulcers and accidental drowning are estimated to be in the range of 3000-10,000 DALY per year.

1.3 The pathogenesis of *Campylobacter*

In a review of *C. jejuni* pathogenesis Konkel *et al.* (2001) proposed a model for infection based on current knowledge and from observations of biopsy specimens taken from piglets used as an animal model of campylobacteriosis because of their anatomical similarity to humans. It has been difficult to do invasion studies of *Campylobacter* in animal models because they rarely develop the severe symptoms associated with human infection. Infected piglets, however, develop similar symptoms to human infection, such as bloody diarrhoea, epithelial cell degeneration and production of inflammatory cells in the intestine. The exact *in vivo* target site for colonisation of the host intestinal cells is not yet known.

When *C. jejuni* cells enter the small intestine they migrate toward the mucus-filled crypts of the intestinal wall. This is where motility involving the flagella and chemotaxis are thought to play an important role (Konkel *et al.* 2001, Wassenaar *et al.* 1995, Wallis 1994, Wassenaar *et al.* 1993, Wassenaar *et al.* 1991).

In the microenvironment of the mucus-filled crypts *C. jejuni* cells undergo an adaptive response by synthesising various proteins that allow their interaction with host target cells. It is postulated that adhesins play a significant part in *C. jejuni* binding to specific host cell receptors. Putative adhesins include CadF, an outer membrane protein required for the binding of campylobacters to fibronectin on the host intestinal epithelial cells (Konkel *et al.* 1999a). The flagellum, lipopolysaccharide and the major outer membrane protein (MOMP) are also postulated to be involved in adherence of *Campylobacter* to host cells (Konkel *et al.* 2001, Fry *et al.* 2000, Wallis 1994)

Binding is followed by internalisation into the host cell, which is aided by proteins including *Campylobacter* invasion antigens (Cia). Mutagenic studies have revealed that the absence of Cia protein secretion results in a significant reduction in the number of *C. jejuni* cells internalised by host epithelial cells (Konkel *et al.* 1999b).

When bacteria become internalised in host cells they must be able to survive reactive oxygen species such as hydrogen peroxide and nitric oxide. *C. jejuni* employs several mechanisms to neutralise the effects of oxygen radicals. Hydrogen peroxide is inactivated by the catalase enzyme encoded by the *katA* gene (Day *et al.* 2000). Superoxide dismutase (SOD) inactivates the destructive oxygen species, superoxide and is another potential mechanism for neutralising toxic oxygen species (Konkel *et al.* 2001).

Most pathogenic bacteria have developed mechanisms for acquiring iron. Free iron is a scarce resource in the host because it is strongly complexed with transferrin and lactoferrin and thus not readily available. The bioavailability of iron in the mammalian host is limited to 10^{-24} M, which is well below the minimum requirement of 10^{-7} M for bacterial growth (Braun and Hantke 2002). *C. jejuni* has multiple iron-uptake systems for ferric and ferrous ions including, the enterochelin (*ceu*BDCE) and the ferrichome (*fhu*ABD) uptake systems, the hemin uptake operon, (*Chu*ABCD), and the TonB system which facilitates the transport of iron sequestering molecules across the outer membrane (Konkel *et al.* 2001, Houng *et al.* 2001).

From biopsy specimens one of the clinical signs of infection is atrophy of the villi and one hypothesis suggests that this is due to one or more bacterial toxins, such as cytolethal distending toxin (CDT) (Konkel *et al.* 2001, Wassenaar 1997). There is an intense inflammatory response

to *C. jejuni* infection resulting from the overproduction of cytokines. It is this inflammatory response that probably intensifies the symptoms of campylobacteriosis.

In support of the pathogenic role of certain genes, the prevalence of seven putative virulence and toxin genes was investigated in *C. jejuni* and *C. coli* isolates from Danish pigs and cattle. A high prevalence of genes associated with adhesion (*cad*F), iron acquisition, (*ceu*E), motility and adhesion (*fla*A) and toxin production (*cdt*B) were identified, with the cytolethal distending toxin (CDT) gene cluster occurring in greater than 80% of isolates (n = 40) (Bang *et al.* 2003).

1.4 Infective dose of *Campylobacter*

Dose response data provides the link between exposure to an infectious agent and the probability of developing an infection and subsequent illness. Not all people who become infected/colonised by an intestinal microorganism will develop symptoms and be classified as ill; some will remain asymptomatic until the microorganism is cleared from their system. It is assumed that the longer a person is infected, the greater the probability of the person becoming ill (Teunis *et al.* 1999).

Researchers studying the dose response of *Campylobacter*, administered *C. jejuni* to a total of 111 volunteer immunocompetent young adults (Black *et al.* 1988). The doses of *Campylobacter* ranged from 8×10^2 to 2×10^9 organisms. Of the ten subjects given the lowest dose of 800 CFU, five people tested positive for *C. jejuni* in their stool samples and one reported illness with diarrhoea and fever. Another study involved a volunteer who ingested a clinical isolate of *Campylobacter* and reported illness at 500 *C. jejuni* (Robinson 1981). In the Black *et al*. (1988) study, all subjects at the higher doses tested positive for the excretion of *C. jejuni* in their faeces and produced an antibody response to *C. jejuni*, but without exhibiting any of the symptoms of illness. Overall, although infection occurred in subjects who ingested a higher dose, the risk of developing illness appeared to decrease with increasing doses. Only 22% of infected volunteers developed symptoms of illness and the highest ratio of illness to infection occurred at the intermediary dose of 9×10^4 *C. jejuni* (Medema *et al.* 1996). The doses where infection and illness occur for *Campylobacter* are much lower than for other bacterial agents of gastroenteritis such as *Salmonella*, *Shigella* and *Vibrio cholerae* (Rose and Gerba 1991). There is speculation that this low infective dose may be one of the explanations why *Campylobacter* is the most frequently notified enteropathogen in developed countries (Medema *et al.* 1996).

The results of models developed to assess the dose response of *Campylobacter* (Teunis *et al.* 1999, Medema *et al.* 1996) appear to be in conflict with data collected from two recent outbreaks of campylobacteriosis in primary school children who had consumed unpasteurised milk (Teunis *et al.* 2005). Both outbreaks show a clear (exponential) relationship between the amount of milk consumed and the ensuing rate of illness, however the actual concentration of *Campylobacter* in the milk post-infection was not able to be determined. Using new information from the outbreaks and combining it with data from the Black *et al*. (1988) study, Teunis *et al*. (2005) produced an updated dose-response relation for *C. jejuni* infection. This new model shows increased infectivity at low doses by approximately 36 times in comparison to previous estimations, which were based solely on the human feeding study (Teunis and Havelaar 2000, Teunis *et al.* 1999). This also means that at low doses the probability of infection is directly proportional with dose. Although the dose-response relation is seen to rise exponentially at low doses, it flattens out and only at very high doses reaches near 100% response. This suggests that a small number of people will not develop an infection even when exposed to the microorganism at high doses.

Two strains of *C. jejuni* were used in the Black *et al*. (1988) study with one showing greater virulence as shown by the severity of illness (e.g. increased volume of diarrhoea). This signifies that different strains of a bacterium can produce very different dose responses and disease outcomes, and therefore, a dose response model can only be regarded as indicative.

Teunis *et al*. (1999) concluded that the host plays a major role in controlling levels of intestinal species in the gut as species type and concentrations differ between individuals. Immunocompromised people have a higher probability of infection causing illness and different dose responses have been observed between young children and adults ingesting milk contaminated with *Campylobacter* (Teunis *et al*. 2005). The latter is thought to be related to an acquired immune response in the adult population which protects them from subsequent *Campylobacter* infection. Although the data are lacking on this assumption, it is supported by the volunteer studies where people who had developed an infection and illness from *C. jejuni* after consuming contaminated milk were rechallenged with the same strain of *C. jejuni* one month after initial recovery. These people did not develop further symptoms of illness, however, control subjects challenged at the same time with the same strain did exhibit illness (Black *et al.* 1988). Furthermore, the high levels of *Campylobacter* infection that occur in

developing countries decrease with increasing age and this may be related to acquired immunity as *C. jejuni*-specific serum antibodies are significantly higher in children in the developing world compared to the United States (Taylor *et al.* 1993, Blaser *et al.* 1985).

1.4.1.1 An evaluation of the 1988 dose response data from a 2004 perspective

An evaluation of the data used for dose response models concluded that the models have negated the effect of strain variation within a species of bacteria (Coleman *et al.* 2004) and discusses the limitations of the trial, which may have led to the unexpected finding that the risk of illness does not increase with increasing dose of *C. jejuni*.

Results of the feeding study of Black *et al*. (1988) showed that strain 81-176 caused the most severe diarrhoea and showed a higher invasion potential compared with strain A3249. Subsequent investigation demonstrated that in comparison to A3249, strain 81-176 has a significantly higher invasive potential in four human cell lines (Oelschlaeger *et al.* 1993). Further characterisation of strain 81-176 revealed the presence of a 45-kb plasmid that confers tetracycline resistance and a 37-kb virulence plasmid (Poly *et al.* 2005, Bacon *et al.* 2002, Bacon *et al.* 2000).

Black *et al*. (1988) noted the presence of two colony morphologies for the 'less virulent' strain A3249. The non-spreading colony represented *Campylobacter* cells that did not produce flagella. In comparison, the spreading colonies represented cells that were flagellated. In preparing the inoculum for the dose of A3249, the researchers prepared two distinct inocula from each of the colony types, which were combined in the final inoculum to represent equal proportions. Stool cultures of volunteers infected with A3249 were observed to contain only colonies of the spreading type (flagellated). It has been suggested that *in vivo* passage may select for flagellated bacteria, and this concurs with the theory that flagella in *C. jejuni* may be an important virulence factor (Konkel *et al.* 2004, Carrillo *et al.* 2004). It is difficult, therefore, to be sure of the effective dose when it consists of a mixture of putative virulent and avirulent cells.

The methods employed in the 1988 study may also have contributed to the results. For example, the tendency of *Campylobacter* cells to cluster together (autoagglutination) raises difficulties for enumeration of campylobacters by plate count methods (Miller *et al.* 2000), and the specific

growth phase of the *Campylobacter* cells at the time of administration was unknown. In addition, differences in host variability between the adult volunteers may have contributed to the observed anomalies (Coleman *et al*. 2004).

1.5 Risk factors associated with campylobacteriosis

Case-control studies have determined the risk factors associated with sporadic cases of campylobacteriosis. Although chicken consumption regularly features as a major risk factor (Rosenquist *et al.* 2003, Eberhart-Phillips *et al.* 1997, Ikram *et al.* 1994, Kapperud *et al.* 1992, Harris *et al.* 1986), other independent factors are also identified as being statistically significant. They include recent travel outside the country of origin; contact with farm animals and pets; consumption of shellfish/fish, any undercooked or barbequed meat and activities associated with recreational waters (Gillespie *et al.* 2002, Kapperud *et al.* 1992, Harris *et al.* 1986). New Zealand case-control studies have noted similar risk factors (Eberhart-Phillips *et al.* 1997, Ikram *et al.* 1994), although Eberhart-Phillips *et al.* (1997) did not identify a risk associated with meat consumption other than poultry. Additional factors associated with increased risk of campylobacteriosis in the New Zealand studies included rainwater as a source of drinking water and consumption of unpasteurised dairy products.

Gillespie *et al*. (2002) have also suggested that it may be important to identify risk factors at the *Campylobacter* species level. For example, it was noted that people reporting illness associated with *C. coli* infection were more likely to have consumed bottled water and eaten specific meat types such as pâté in the two weeks before illness developed. In comparison, those infected with *C. jejuni* were more likely to have reported contact with animals prior to infection. These differences between species could be significant when investigating transmission routes for *Campylobacter*.

1.5.1 Cases of human campylobacteriosis associated with chicken consumption

A Danish risk assessment of cases of human campylobacteriosis associated with the consumption of chicken estimated that one case of *Campylobacter* infection arose from 14,300 servings of chicken (Rosenquist *et al.* 2003). As an example, they estimated that the number of *Campylobacter* cases arising from chicken consumption in private kitchens was 14,000 per year (95% CI; 7753 – 20,942 cases), based on a figure of 201 million servings of chicken per year

ingested in Denmark. Although only 4386 human cases of campylobacteriosis were reported to the authorities in 2000, it is expected that the actual number of cases is from 7.6 times to 100 times higher than the number of notified cases, suggesting that in the year 2000 the number of cases would have been in the range between 30,000-440,000 people (Rosenquist *et al.* 2003). These figures suggest a true frequency of campylobacteriosis in the range of 600-8300 cases per 100,000 population in Denmark and support the estimate of 14,000 cases attributable to chicken consumption associated with preparation in private households.

A case-control study that determined the risk factors for human campylobacteriosis was undertaken in Christchurch, New Zealand and identified the recent consumption of chicken in 80% of clinical cases (Ikram *et al.* 1994). Chicken consumption was also identified as a major risk factor by another New Zealand case-control study (Eberhart-Phillips *et al.* 1997) with an increased risk associated with eating chicken in restaurants. In contrast, a protective factor was noted for chicken that had been roasted, baked or frozen prior to cooking. A Norwegian case-control study determined the home consumption of chicken either purchased raw or frozen (Odds Ratio = 3.20; $P = 0.024$) to be one of the risk factors associated with sporadic cases of campylobacteriosis (Kapperud *et al.* 1992). Cross-contamination from raw chicken to other food products has also been noted as a risk factor with a decrease in incidence associated with washing of cutting boards with soap between food preparations (Rosenquist *et al.* 2003, Harris *et al.* 1986). The New Zealand case-control study of Eberhart-Phillips *et al.* 1997 did not identify food preparation practices in the kitchen as being a risk factor.

The incidence of campylobacteriosis in Iceland peaked at 116 per 100,000 population in 1999 and dropped to 33/100,000 the following year (Stern *et al.* 2003). This reduction coincided with a drop in prevalence of *Campylobacter*-positive chicken carcasses from 62% in 1999 to 15% in 2000. No single factor could be identified as a range of interventions were implemented at the same time, which included freezing of chicken products from *Campylobacter*-positive flocks, public education and biosecurity procedures on farms. The implications, however, were that interventions in poultry husbandry and consumer awareness of the hazards associated with poultry pathogens led to a reduction in cases. Another incident that occurred in Belgium highlighted the impact that the consumption of poultry may have on campylobacteriosis cases. In June, 1999, a dioxin crisis attributed to contamination of feed components, resulted in the withdrawal of all poultry from the Belgium market (Vellinga and Van Loock 2002). Subsequent

analysis of *Campylobacter* cases for the same period suggested a significant drop in incidence leading to an estimation of 40% of cases associated with poultry consumption.

1.5.2 Outbreaks of campylobacteriosis

Tenover *et al.* (1995) defined outbreaks as the "increased incidence of an infectious disease in a specific place during a given period that is above the baseline rate for that place and time frame". Outbreaks of campylobacteriosis have most often been associated with the consumption of poultry (Pearson *et al.* 2000, Rosenfield *et al.* 1985); drinking unpasteurised milk (Evans *et al.* 1996, Fahey *et al.* 1995, Brieseman 1984) or poorly/untreated water and contact with untreated surface waters (Inkson 2002, Miettinen *et al.* 2001, Stehr-Green *et al.* 1991, Brieseman 1987). Outbreaks associated with water are discussed in further detail in Chapter Three. Outbreaks associated with contamination by a food handler have also occurred on rare occasions (Olsen *et al.* 2001). Outbreaks, in general, are infrequent relative to the large number of sporadic cases of campylobacteriosis and this is partly attributed to the high genetic diversity observed in *Campylobacter* subtypes, which makes it difficult to identify common sources of infection (Friedman *et al.* 2004, Hedberg *et al*. 2001). Further discussion of this aspect of *Campylobacter* epidemiology is presented in Chapter Five.

1.6 Research Model

1.6.1 Objectives

The main objective of this study was to develop a robust and sensitive method to detect viable *Campylobacter* in a wide range of matrices having different PCR inhibitory properties and varying concentrations of contaminating campylobacters. This objective was designed to facilitate the investigation of environmental reservoirs that may harbour subtypes of *Campylobacter* pathogenic to humans. The choice of a single optimal enrichment broth allows for greater efficacy in the laboratory setting when dealing with large sample numbers of multiple matrices. This consideration was important when developing methods for large surveys intended to elucidate the transmission routes of a bacterium through the environment. It also fulfills the need for consistent methodology to aid the epidemiological study of transmission of campylobacters from the environment to humans.

To fulfil the above objective it was important to ascertain if the method could determine the carriage rate of *C. jejuni* and *C. coli* in the matrices under investigation. Further validation determined whether the method could identify a wide range of *Campylobacter* subtypes in the environment to negate the possibility that certain subtypes would predominate due to the selective nature and other intrinsic properties of the method. The latter was achieved by validating the method on a matrix known to carry multiple subtypes of the same *Campylobacter* species. River water passing through farmland was chosen as being likely to receive multiple inputs of *Campylobacter* from varied sources including sheep, dairy cows, cattle, wild animals and birds. This validation also confirmed that the method was able to detect multiple subtypes present in an individual sample.

This last validation was relevant because studies have recognised the importance of identifying multiple isolates from a single sample in order to identify both dominant and minor subtypes (Schouls *et al.* 2003, Schlager *et al.* 2002, Kramer *et al.* 2000). These researchers caution that basing a study on the isolation of dominant subtypes of a bacterial species may exclude significant information, especially where the study is examining the frequency of virulence factors in a bacterial population or tracking the source of an outbreak. In addition, identification of minor subtypes from environmental matrices may contribute to the knowledge about

potential host specificity of *C. jejuni* subtypes and whether all subtypes identified in a matrix contribute to human infections.

This requirement for identifying all subtypes in an individual sample is highlighted by an outbreak case of *E. coli* O157:H7 where the epidemiological evidence strongly suggested that the cases were all linked (Proctor *et al.* 2002). However, only six of the isolates from nine cases were identical to one of the two isolates identified in the meat linked with the outbreak. The second isolate from the meat sample was unrelated to any of the four subtypes identified in the human faecal specimens. If this second subtype had been the only isolate identified from the meat sample then the importance of the ground beef as the source of the outbreak would have remained unconfirmed.

Chicken meat has been implicated as a major route of infection for human campylobacteriosis. Cases associated with chicken consumption are reported to account for up to 70% of all *Campylobacter* infections (Stern 1992). Supporting this association is the high prevalence of the pathogenic campylobacters *C. jejuni* and *C. coli* harboured by broiler flocks and associated chicken products (Kramer *et al.* 2000). Furthermore, indistinguishable subtypes of *Campylobacter* have been identified in chicken meat and human clinical specimens suggesting either another unidentified common source or the transfer of infection between the two matrices (Karenlampi *et al.* 2003, Hänninen *et al.* 2000, Kramer *et al.* 2000).

Based on the importance of chicken as a potential reservoir of *Campylobacter,* the second major objective of this thesis was to establish if chicken carcasses harboured more than one subtype of a pathogenic *Campylobacter* species. If more than one subtype was present, the aim was to determine whether the recognition of multiple subtypes increased the likelihood of establishing an epidemiological relationship between consumption of chicken meat and *Campylobacter* infection in humans. Assessment of the correlation between *C. jejuni* subtypes isolated from human faeces and chicken meat was based on identifying indistinguishable or related genotypes in both matrices.

1.6.2 Hypotheses

In fulfilment of the outlined objectives, three testable hypotheses were developed.

1. A robust enrichment-PCR assay can be developed to detect and identify pathogenic *Campylobacter* spp. from a range of environmental matrices.

Reliable recovery and identification of campylobacters is time consuming due to their fastidious requirements for cultivation and the few biochemical tests that can be employed in their identification. Improved and validated methodology is needed to facilitate meaningful epidemiological studies. An enrichment-PCR method was to be developed as a tool to test a wide range of environmental matrices for the presence of two major pathogenic *Campylobacter* species, *C. jejuni* and *C. coli*, and ensure detection of target campylobacters at low levels. The matrices identified as potential reservoirs or transmission routes in this study were: human, dairy and beef cattle, sheep, chicken, duck, possum and rabbit faeces; meat products from cattle, sheep, pigs and chickens; and river water. The method was designed to simplify the cultivation process for large-scale surveys in routine laboratories. Identification of matrices that act as reservoirs or transmission routes of this bacterium through the environment to humans will greatly enhance the understanding of sources of campylobacteriosis. This information could lead to appropriate intervention measures and assessment of risk management techniques to reduce the likelihood of contracting campylobacteriosis.

2. Chicken carcasses carry multiple subtypes of *C. jejuni*.

Investigation of individual samples for the presence of pathogenic *Campylobacter* species has focussed on identifying isolates to the species level. This research aims to investigate another layer of complexity by determining if multiple subtypes of the same species are present in an individual chicken meat sample. *C. jejuni* is known to cause 80-90% of the cases of campylobacteriosis and therefore it was the *Campylobacter* species chosen as the focus for determining the prevalence of multiple subtypes. The carriage of multiple subtypes of *C. jejuni* will be determined by the typing method of pulsed-field gel electrophoresis (PFGE) using two restriction enzymes. Previous research in a chicken abattoir suggested that not all subtypes of *C. jejuni* have the same survival capabilities and showed that the carriage of *C. jejuni* subtypes

by a chicken carcass can alter during its course through the processing plant (Newell *et al.* 2001). This information can be used to manage the critical points during processing from the farm to the consumer where there is potential for contamination by pathogenic campylobacters.

3. All subtypes of *C. jejuni* identified on chicken carcasses are also identified in human faecal specimens.

There is a correlation between the subtypes of pathogenic *Campylobacter* identified in chicken carcasses and those identified from human faecal specimens. Previous international comparisons of the subtypes isolated from chicken carcasses and human faecal specimens have identified unique subtypes in both matrices. This information is relevant because potentially not all subtypes are pathogenic to humans. Characterisation of those subtypes that occur in both matrices allows for a comparison with subtypes unique to one of the two matrices. This could lead to the investigation of potential virulence factors that confer an advantage associated with the multiple matrix isolates.

1.6.3 Possible outcomes

For a given environmental matrix to act as a reservoir or transmission route it must harbour *Campylobacter* organisms. Therefore, the first possible outcome is that the *Campylobacter* organism can be recovered from the following environmental matrices: river water; animal, bird and human faeces; meat and chicken products. Developing a methodology that enables the detection of *Campylobacter* organisms from these matrices can test this prediction.

To establish the presence of indistinguishable or related subtypes of *C. jejuni* in multiple matrices it is important to determine if there are multiple subtypes present in an individual sample. Therefore, the second possible outcome is that multiple subtypes can be identified in samples from water and chicken carcasses.

For a *C. jejuni* subtype to be identified as a potential infectious agent in human campylobacteriosis it must first be identified in human faeces. Hence, the third possible outcome is that *C. jejuni* subtypes identified in chicken meat products will also be identified in human faecal specimens from patients suffering from campylobacteriosis.

1.7 Format of the thesis

Chapter One is a general introduction to *Campylobacter* bacteriology and epidemiology with the scientific relevance and background to the research detailed in subsequent chapters. In addition, Chapter One contains the research model for this study and outlines the specific objectives related to the three hypotheses that form the basis of this study. Chapters Two to Five each follow the generalized format of introduction, materials and methods, results and discussion.

Chapter Two presents the development and testing of the detection limits of the enrichment-PCR method of campylobacters in all of the environmental matrices under investigation. Chapter Three presents the application of the enrichment-PCR method to the environmental isolation of *Campylobacter* from chicken carcasses and water. It also contains the validation study of the ability of the enrichment-PCR method to isolate multiple subtypes of *C. jejuni* from an individual sample. This was achieved by the pulsed-field gel electrophoresis (PFGE) analysis of multiple subtypes isolated from a grab sample of river water. Chapter Four presents the results of PFGE molecular typing of multiple isolates of *C. jejuni* from chicken carcasses. Chapter Five discusses the correlation of indistinguishable and related subtypes isolated from human and chicken matrices. Chapter Six is a discussion of all the results obtained in this thesis and includes possibilities for future research.

2 Method development of enrichment PCR protocol

2.1 Introduction

Reliable recovery and identification of campylobacters from environmental matrices is challenging. Epidemiological studies to determine routes of campylobacter infection require improved and validated methodologies to enhance the ability to detect campylobacters from a wide range of environmental matrices. The large surveys required to establish a database of *Campylobacter* subtypes for comparison with clinical isolates necessitate rapid, routine laboratory methods that are robust and allow isolation of the target bacterium for analysis by subtyping technologies. Difficulties encountered when developing a method, which reliably detects *Campylobacter* from a range of environmental sources, include the low numbers present in/on matrices such as foodstuffs and waterways, and the presence of inhibitors intrinsic to a particular matrix such as faeces. These issues are examined in the following discussion.

2.1.1 *Campylobacter* isolation from environmental matrices

At present, enrichment is followed by conventional plating to obtain single colonies of *Campylobacter* for identification. Confirmation of the identity of bacterial species by conventional phenotypic tests requires the purification of individual colonies prior to identification by biochemical tests. This entails a 48 hour incubation of two consecutive subcultures to ensure the purity of target colonies. Some of the biochemical tests include antibiotic susceptibility assays which require further incubation for 48 hours. This can lead to a period of up to ten days for identification by the conventional method. Futhermore, campylobacters are not readily identified as there are few biochemical tests that can be exploited for their classification and they require fastidious conditions for cultivation. These factors limit the available phenotypic tests for differentiation at the species level.

The main biochemical test used to distinguish *C. jejuni* from *C. coli* is based on the presence of an enzyme which breaks down hippurate. It is now known that approximately 5% of *C. jejuni* isolates are misidentified as they do not express this enzyme even though the gene is present in the genome (Linton *et al.* 1997). A study, which compared phenotypic and genotypic methods of identifying campylobacters (Waino *et al.* 2003), concluded that phenotypic identification schemes needed to be supplemented by genotypic methods such as species specific PCR. One

example of misidentification by phenotypic testing included 29% of 309 putative *C. coli* isolates identified on the basis of a negative hippurate test being re-identified as hippurate variable/negative *C. jejuni* cultures by genotypic tests.

Employing techniques, such as the Polymerase Chain Reaction (PCR) that detect the DNA in an organism can reduce the time required for identification, as multiple species are able to be detected simultaneously in a sample by a rapid and cost effective test.

PCR has been used extensively for detecting pathogenic microbes, including thermotolerant campylobacters, in many different matrices (Rudi *et al.* 2004, Dedieu *et al.* 2004, Inglis and Kalischuk 2003, Hong *et al.* 2003, Sails *et al.* 2002, O'Sullivan *et al.* 2000, Konkel *et al.* 1999a, van Doorn *et al.* 1999, Vanniasinkam *et al.* 1999, Denis *et al.* 1999, Waage *et al.* 1999, Harmon *et al.* 1997, Linton *et al.* 1997, Ng *et al.* 1997, Rasmussen *et al.* 1996, Waegel and Nachamkin 1996, Ayling *et al.* 1996, Kirk and Rowe 1994). Rarely have these assays been developed for and evaluated over a wide range of matrices for the purpose of establishing a protocol to determine transmission routes and reservoirs of campylobacters to facilitate epidemiological studies.

PCRs have been developed for the direct detection of *C. jejuni* in human faecal samples (Linton *et al.* 1997, Ng *et al.* 1997, Waegel and Nachamkin 1996) and chicken faeces (Rudi *et al.* 2004). These assays have been designed to accommodate the respective substrates human and chicken faeces which are known to have a *Campylobacter* count in the range of 10^6 to 10^8 (Taylor *et al.* 1993) and 10^5 to 10^7 (Rudi *et al.* 2004) colony forming units per gram (cfu g^{-1}) of faeces. This is in comparison to the significantly lower numbers of 10^2 to 10^3 cfu g^{-1} in dairy cows and 10^2 to10^4 cfu g^{-1} in sheep (Hutchison *et al.* 2004, Stanley *et al.* 1998c, 1998b). The generally low numbers of campylobacters in most environmental matrices necessitates the use of enrichment techniques to overcome environmental stresses and facilitate growth of the target organisms. Direct plating of cattle and sheep faeces onto agar media has been reported to produce lower recoveries in comparison to enrichment techniques (Madden *et al.* 2000, Stanley *et al.* 1998c, 1998b, Atabay and Corry 1998). Atabay and Corry (1998) also suggested that after enrichment, plating onto at least two different agar types was required to maximise detection of positive samples. Waage *et al.* (1999) noted that for foods analysed without enrichment, the PCR results were variable. They recommended the use of an enrichment step to increase the sensitivity of the PCR method and overcome the non-reproducibility of the results.

Maher *et al.* (2003) conducted an evaluation of methods for *Campylobacter* detection which compared direct plating on a *Campylobacter* selective medium with enrichment prior to plating on a selective medium. They concluded that there was a 30% increase in detection of *Campylobacter* from human faeces when samples were enriched in broth prior to plating onto the selective agar.

When trying to develop a PCR assay for detection of thermolerant campylobacters in a wide range of matrices it is important to recognise the inhibitory effects inherent to a particular matrix. Humic substances in water and components in food and faecal samples, such as complex polysaccharides, as well as blood in enrichment media can all contribute to inhibition of the Polymerase Chain Reaction (Maher *et al.* 2003, Waage *et al.* 1999, Waegel and Nachamkin 1996, Rossen *et al.* 1992). Enrichment broths can aid dilution of inhibitors and washing of enriched cells prior to PCR can remove remaining inhibitory compounds.

Competition from other microflora, usually present in higher numbers, can be partially overcome by employing the selective temperature of 42°C which is optimal for thermotolerant campylobacters (Griffiths and Park 1990). Antimicrobials in the enrichment medium also reduce competition from fungi and other bacteria, and the addition of compounds which reduce the toxic effects of oxygen benefits the stressed microaerophilic *Campylobacter* cells (Baylis *et al.* 2000). It should be noted, however, that the addition of antibiotics to a medium may inhibit the growth of injured campylobacters (Mason *et al.* 1999, Humphrey and Cruickshank 1985). Isolation from water was enhanced by delayed addition of antibiotics to the enrichment medium for 4-8 hours, but for poultry carcasses it was determined that the isolation rate was improved by addition of anitibiotic to the initial enrichment (Mason *et al.* 1999). A pre-incubation period of four hours at 37°C has been shown to be efficacious in the recovery of injured *Campylobacter* cells (Waage *et al.* 1999, Humphrey 1986).

Taking into account all of the above parameters, selection of a suitable enrichment medium is difficult and as noted by Madden *et al*. (2000) choice of broth will take into account overall rate of recovery and ability to enrich different species and subtypes of a *Campylobacter* species of interest. The method reported in this study attempted to design an experimental system which would identify viable *C. jejuni* and *C. coli* in a range of matrices expected to have a wide variation of *Campylobacter* numbers present. Also, as noted by Scates *et al*. (2003),

identification of campylobacters throughout the food chain requires employment of the same method to each matrix to allow for genuine comparisons to aid epidemiological studies.

2.1.2 *Campylobacter* prevalence in environmental matrices

Previous studies in Christchurch examined the case histories of human campylobacteriosis cases (Eberhart-Phillips *et al.* 1997, Ikram *et al.* 1994) and concluded that a likely source of campylobacteriosis was the consumption of undercooked chicken or the contamination of other foods by uncooked chicken meat. This is supported by a study performed in Finland where subtyping analyses identified 34% of *C. jejuni* human isolates with indistinguishable serotype and genotype combinations to *C. jejuni* strains isolated from chickens prior to slaughter (Karenlampi *et al.* 2003). With the application of temporal data to make an association between the date of chicken faecal sampling and faecal sampling from infected humans, the percentage of indistinguishable subtypes was reduced to 21%. Ikram *et al*. (1994) also suggested that there are other, as yet, unknown environmental sources which are acting as transmission routes or reservoirs of *Campylobacter*.

A Canadian study centered on Eastern townships in Quebec combined a case-control study of campylobacteriosis with a study of the prevalence of *Campylobacter* spp. in retail chickens. From August to October, a seasonal increase in chicken *Campylobacter* isolates was noted, whereas the human cases of campylobacteriosis peaked a month earlier in July and decreased ten fold for the months of September and October (Michaud *et al.* 2004). Furthermore, less than fifty percent of cases could be associated with the risk factors of eating undercooked poultry, consuming raw milk and its products and eating poultry in a restaurant. Other environmental sources of *Campylobacter*, such as drinking water, were suggested as being important in the transmission of *Campylobacter* to humans (Michaud *et al.* 2005, 2004).

A reservoir is defined as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population" (Haydon *et al.* 2002). In this study, the term, reservoir is applied to a matrix which harbours *Campylobacter* and supports its replication. A transmission route is defined as a vehicle of bacterial transmission within which campylobacters are unable to multiply. Animal reservoirs, besides poultry, known to harbour campylobacters include cattle, dairy cows, sheep and wild birds (Savill *et al.* 2003, Giacoboni *et al.* 1993,

Kakoyiannis *et al.* 1988). Campylobacters are not able to multiply in aqueous environments but water can act as a transmission route for their distribution. Rivers receive input from many faecal sources, in particular, where high-density farming occurs alongside waterways. Other important transmission routes could be birds as their mobility allows them to act as intermediary hosts between warm-blooded animals (Jones *et al.* 1999). The following section provides a summary of the environmental matrices from which campylobacters have been isolated. These matrices, along with human faeces were to be included as substrates in the development of the enrichment-PCR method.

2.1.2.1 Chicken produce

Surveys have shown that 30-100% of poultry harbour *Campylobacter* as normal commensal flora of their intestinal tract (O'Sullivan *et al.* 2000). The *Campylobacter* intestinal contents of chickens at the time of slaughter are reported to be present in numbers up to 10^7 cfu g^{-1} (Stern 1994). The prevalence of *C. jejuni* in chicken meat is also reported to be high, with 48 to 98% of commercial broiler carcasses testing positive (Bryan and Doyle 1995, Stern 1992, Park *et al.* 1991), although one study from the Netherlands yielded a low prevalence of 16% *C. jejuni* (Stern 1992). Surveys in the United Kingdom have reported prevalences of *C. jejuni* as 77% (n = 198) (Kramer *et al.* 2000) and 52% in chicken pieces (Sails *et al.* 2003a) and *C. coli* from 6.6% of the samples (Kramer *et al.* 2000). A Canadian study of campylobacters in whole retail chickens identified 41 (23%) positive samples from a total of 177 samples. *C. jejuni* isolates accounted for 90% of the positive cultures with *C. coli* identified in the remaining positive samples (Michaud *et al.* 2004). Prevalence of *C. jejuni* in fresh chicken in previous New Zealand studies was determined to be 54% (n = 50 whole chickens), 57% (n = 137 whole chickens) and 56.6% (n = 113 chicken pieces) (Anonymous *et al.* 1999, Campbell and Gilbert 1995, Hudson *et al.* 1999) respectively.

2.1.2.2 Ruminant animals

Although there is a widely held assumption that poultry products are responsible for most sporadic cases of campylobacteriosis (Hänninen *et al.* 2001, Federighi *et al.* 1999), data from many studies suggest the implication of other animal reservoirs (Devane *et al.* 2005, Brown *et al.* 2004, Leatherbarrow *et al.* 2004, Acha *et al.* 2004, Stanley and Jones 2003, Petersen *et al.* 2001a, Corry and Atabay 2001, Nielsen *et al.* 2000, On *et al.* 1998). It is known that ruminant

animals can shed *Campylobacter* in their faeces (Bailey *et al.* 2003, Meanger and Marshall 1989). With the large number of ruminant animals in New Zealand these observations raise questions as to the potential for domesticated mammals to act as environmental reservoirs of *Campylobacter* and the significance of their role in the transmission of campylobacters through the environment to humans, either directly or via food, water or other transmission routes.

In a UK study of thermotolerant *Campylobacter* isolates from sheep, the main species isolated was *C. jejuni* (90%), followed by *C. coli* (8%) and *C. lari* (2%) (Jones *et al.* 1999). This same pattern was found with the isolation of *Campylobacter* from sheep intestines at the time of slaughter (Stanley *et al.* 1998c). Over a one-year sampling period there were consistently high carriage rates of *Campylobacter* detected in the intestines of sheep at slaughter. Shedding of *Campylobacter* in faeces, however, was found to vary depending on feed and the season, with high numbers of *Campylobacter* isolated during the lambing season and low numbers during the winter period. *C. jejuni* was found to survive in sheep faeces left in the outside environment for up to four days. The numbers of campylobacters found in sheep faeces were consistently lower than the numbers found in their intestines. Jones *et al.* (1999) postulated that a sheep may shed up to 7×10^7 *Campylobacter* per day (figures from late summer sampling) which would contribute to the bacterial loading of runoff into streams and rivers.

The seasonal variation of thermotolerant campylobacters observed in sheep also seems to be the case for beef and dairy cattle in the UK (Stanley *et al.* 1998b). The peak periods for both beef and dairy cattle occurred in spring and autumn. A New Zealand study by Meanger and Marshall (1989) found the peak period of infection to be autumn (31%) closely followed by summer (24%). It also demonstrated that the same genotypes of *C. jejuni* and *C. coli* were found in sheep and dairy cows on the same farm, which suggests cross infection between the two animal species.

In overseas studies the intestinal cell density of *Campylobacter* in beef cattle, as determined by the Most Probable Number (MPN) technique at the time of slaughter, was 6.1×10^2 MPN g^{-1} fresh weight of intestinal contents (MPN gfw^{-1}) (Stanley *et al.* 1998b). In the same study the average number of *Campylobacter* present in adult dairy cattle was found to be 70 MPN gfw^{-1} and 3.3×10^4 MPN gfw^{-1} in calves. A more recent study, which used real-time quantitative PCR to determine *C. jejuni* cell density in beef cattle, identified 27% of samples (n = 299) as containing the maximum population of between 10^4 and 5×10^5 cells per gram of faeces (Inglis

et al. 2004). A range of faecal carriage rates for *C. jejuni* in dairy cows has been reported (Table 4).

Table 4: *C. jejuni* carriage rates in bovine animals

Percentage carriage	Number of animals	References
37.7	2,085 dairy cows	Wesley *et al.* 2000
7.0	136 dairy cows and calves	Atabay and Corry, 1998
54.0	24 calves	Grau 1988
12.5	96 adult dairy	Grau 1988
0-10.0	720 dairy cows (prevalence based on population per farm)	Harvey *et al.* 2004
38.0	382 beef cattle	Inglis *et al.* 2003

As expected because of the presence of *Campylobacter* in dairy herds, outbreaks of campylobacteriosis associated with unpasteurised milk have long been reported (Galbraith *et al.* 1982). Cases are mostly reported due to a failure in pasteurisation processes (Fahey *et al.* 1995) or visits to farms where unpasteurised milk is consumed (Evans *et al.* 1996). Investigation of an outbreak associated with consumption of raw milk in Austria confirmed that isolates from clinical samples and the suspected dairy cows had indistinguishable subtypes as determined by pulsed-field gel electrophoresis analysis utilising two restriction enzymes (Lehner *et al.* 2000).

A study explored the presence and survivability of *Campylobacter* in dairy slurries (Stanley *et al.* 1998d). Thermotolerant *Campylobacter* were readily isolated from stored slurries and from slurries disposed onto land during the winter. The campylobacters could be detected in the slurry for up to 20 days after application. This has implications for runoff of campylobacters into waterways, resulting in contamination of recreational aquatic environments.

Although farm animals are born free of *Campylobacter*, various studies have demonstrated the transfer of campylobacters from mothers and the immediate farm environment to lambs (Jones *et al.* 1999), calves (Stanley *et al.* 1998b) and pigs (Weijtens *et al.* 1997). The higher *Campylobacter* numbers found in the offspring of farm animals decreases as they reach maturity as their intestinal tracts become fully developed. The *Campylobacter* species most commonly isolated from pigs is *C. coli* (Christensen and Sorenson, 1999). The prevalence of *C. coli* in pig

faeces has been reported as 58% (n = 203) (Munroe *et al.* 1983). A study by Weijtens *et al.* (1997) reported that *Campylobacter* counts in pig faeces ranged from 10^2 to 10^4 cfu g^{-1}.

2.1.2.3 Meat Products

The ubiquitous presence of campylobacters in the intestines of cattle, sheep and pigs suggests that they would be common on eviscerated carcasses. This has been found to be the case but their numbers decline rapidly, presumably due to the sensitivity of campylobacters to drying (Park *et al.* 1991). A survey of abattoirs in Northern Ireland revealed no isolates of *Campylobacter* in 100 lamb and 100 beef carcasses (Madden *et al.* 1998). The same study also detected no *Campylobacter* species in 50 retail packs of beef and 50 packs of pork. A more recent Irish study of retail meat still found low prevalences of *Campylobacter* in beef (3.2%, n = 221) and lamb (11.8%, n = 262) (Whyte *et al.* 2004). This concurs with a Japanese study (Ono and Yamamoto 1999) which failed to detect *C. jejuni* in beef and pork. Madden *et al.* (1998) suggested that this low prevalence could be taken as an indicator of good slaughterhouse hygiene practices.

A report for the Danish Meat Research Institute (Christensen and Sorenson 1999) discusses the problems of *Campylobacter* contamination during the slaughter process. *Campylobacter* were found on 43-85% of pig carcasses before the tunnel chilling process. After chilling, the prevalence of *Campylobacter* had dropped to 11-18% of the carcasses. Almost all of the *Campylobacter* isolates were *C. coli*. An Irish study also reported a low prevalence of 5.1% of *Campylobacter* in retail pork (n = 197) (Whyte *et al.* 2004).

Offal may be more highly contaminated by *Campylobacter* because of its moist nature which, given that the product is chilled, will enhance the survival of the organism (Park *et al.* 1991). In a survey of offal from pigs, beef, chickens and sheep the percentage isolation of *C. jejuni* was reported as 27% (n = 25) (Sails *et al.* 2003a). The individual prevalence of campylobacters identified in offal is presented in Table 5.

Table 5: Prevalence of *Campylobacter* contamination in offal

Matrix	*Campylobacter*	Percentage positive	Reference
Lamb Liver	*C. jejuni*	75.0 (n = 96)	Kramer *et al*. (2000)
		66.2 (n = 272)	Cornelius *et al*. (2005)
	C. coli	13.5 (n = 96)	Kramer *et al.* (2000)
Ox liver	*C. jejuni*	49.0 (n = 96)	Kramer *et al*. (2000)
	C. coli	2.1 (n = 96)	Kramer *et al*. (2000)
Pig liver	*C. jejuni*	34.3 (n = 99)	Kramer *et al*. (2000)
		4.8 (n = 400)	Moore and Madden (1998)
	C. coli	42.4 (n = 99)	Kramer *et al.* (2000)
		9.8 (n = 400)	Moore and Madden (1998)
	C. lari	0.5 (n = 400)	Moore and Madden (1998)

2.1.2.4 Wild birds

The mobility of wild birds and their internal temperature of 42°C makes them ideal candidates for aiding the transmission of *Campylobacter* through the environment (Jones 2001, Skirrow 1990). Studies have highlighted the potential role of birds as vehicles for *Campylobacter* transmission from farm animal faeces (Brown *et al.* 2004, Adhikari *et al.* 2004). The mode of transmission was demonstrated to be the transfer of campylobacters by birds pecking cowpats (Skirrow 1994) and the same transmission route has been suggested for sheep (Jones *et al.* 1999). The prevalence of *C. jejuni* isolated from mallard ducks has been reported as 34% (n = 243) (Luechtefeld *et al.* 1980) and 40% (n = 82) (Fallacara *et al.* 2001).

2.1.2.5 Water

Thermotolerant *Campylobacter* are widespread in the environment and subsequently in waterways (Kemp *et al.* 2005, Devane *et al.* 2005, Savill *et al.* 2001) where their presence is a sign of recent contamination with animal and bird faeces, farm run-off or sewage (Jones 2001). Contamination of waterways by *Campylobacter* follows seasonal trends. The prevalence of *Campylobacter* in water increases in winter when water temperatures are lowest (Brennhovd *et al.* 1992, Carter *et al.* 1987). During the summer when there is an increase in ultraviolet radiation and the water temperature rises the prevalence of *Campylobacter* falls (Obiri-Danso *et al.* 2001). *Campylobacter* do not multiply in water because of their high minimum growth

temperature (*circa* 30°C). Instead, water acts as a transmission route between warm-blooded hosts. The finding of *Campylobacter* in groundwater during an investigation of a polluted spring (Stanley *et al.* 1998a) is significant for those who derive their drinking water supply from such aquifers. The *C. jejuni* biotypes isolated from the groundwater were indistinguishable from biotypes isolated from cows on a dairy farm situated within the hydrological catchment of the polluted spring. These results support the role of water as a transmission route for campylobacters through the environment to humans. Cases of campylobacteriosis associated with water will be discussed further in Chapter Three.

2.1.2.6 Feral Animals

The Australian brushtail possum (*Trichosurus vulpecula*) is a serious pest to the agricultural and forestry industries of New Zealand. The European rabbit (*Oryctolagus cuniculus*) causes serious damage to agricultural pasture lands. There are approximately 70 million Australian brushtail possums in New Zealand (www.maf.govt.nz/MAFnet/, accessed January 2006), and the possum is a significant source of infection for new cases of bovine tuberculosis in New Zealand cattle (Kao and Roberts 1999). The European rabbit is a laboratory model for studies of *Campylobacter* infection (Walker *et al.* 1992) suggesting that they may be capable of harbouring *Campylobacter* in the wild. Until recently, there have been few reports of *Campylobacter* isolation from rabbits. One study of the spatial distribution of *Campylobacter* in a defined area of farmland identified a *C. coli* isolate from rabbit faeces (Leatherbarrow *et al.* 2004). A similar study identified *C. jejuni* isolates from 11% of non-avian wildlife. The 271 non-avian wildlife samples were characterised as being derived from "mainly rabbits and badgers" (Brown *et al.* 2004).

2.1.2.7 Other potential reservoirs/transmission routes of Campylobacter

2.1.2.7.1 Pets as reservoirs of campylobacters

All four thermotolerant campylobacters have been identified in dog faeces including 26% of samples which showed multiple infection by different *Campylobacter* species (Koene *et al.* 2004). In two of these samples, three species of *Campylobacter* were identified in each sample. *C. upsaliensis* seems to be the most frequently isolated *Campylobacter* from dog and cat faeces (Table 6) (Burnens *et al.* 1992).

A Danish study investigated 45 cases of campylobacteriosis where the patients were living with pet dogs or cats (Damborg *et al.* 2004). Overall campylobacters were recovered from eleven pets and *C. jejuni* was identified in four dogs and four cats, two pets carried *C. lari* and one carried *C. coli.* Dogs showing signs of gastroenteritis were all one year or less in age. Genotyping of isolates from pets and patients revealed only one case among the 45 studied where an indistinguishable *C. jejuni* strain was identified in a patient and pet living in the same household. As part of the same study, a cluster analysis of other canine and human *C. jejuni* isolates from different Danish counties showed greater than 95% similarity between genotypes, suggesting the occurrence of closely related strains between these two matrices.

A longitudinal study of the excretion of thermotolerant campylobacters by young pet dogs monitored the puppies from a few months old until they reached two years of age (Hald *et al.* 2004a). From nine to fifteen months of age all of the puppies were excreting campylobacters constantly. This had reduced to 67% of the dogs carrying campylobacters at two years of age. Distribution of *Campylobacter* species in the dogs over the entire study period was: *C. upsaliensis*, 75.0%; *C. jejuni* 19.4%; *C. lari*, 2.1%; *C. coli* 0.7%. Unidentified *Campylobacter* species made up 2.8% of the isolates. Genotyping of *C. upsaliensis* isolates revealed a high degree of clonality of strains within individual dogs. In comparison, there was much higher diversity with the *C. jejuni* isolates, with a discrete subtype seen in most samples from each dog. As observed in other studies, dogs less than one year old were more likely to carry *C. jejuni* than older dogs.

Table 6: Prevalence of *Campylobacter* species in pets

Animal species	*C. jejuni*	*C. coli*	*C. upsaliensis*	*C. lari*	References
Dogs (n = 289)	20	6	98	ND	Baker *et al.* (1999)
(n = 30)	12		16	3	Koene *et al.* (2004)
Cats (n = 195)	8	ND	22		Baker *et al.* (1999)

2.1.2.7.2 Flies

Flies and other insects such as beetles carry *Campylobacter* and have been suggested as significant sources of contamination (Hald *et al.* 2004b, Adhikari *et al.* 2004, Berndtson *et al.* 1996, Jacobs-Reitsma *et al.* 1995). Insects harbour campylobacters on hairs on their body and feet or in their alimentary tract (Rosef and Kapperud 1983). Flies have the potential to transmit *C. jejuni* to humans via the indirect route of food. A study of flies trapped outside a broiler house in Denmark identified 8.2% of the flies as carriers of *C. jejuni* (Hald *et al.* 2004b) and 9% of flies trapped on a New Zealand dairy farm were carriers of *C. jejuni* (Adhikari *et al.* 2004). Furthermore, an interesting observation has been made relating the seasonal summer increase in *Campylobacter* cases to the proliferation of flies at the same time of year (Nichols 2005). It is postulated that although most infections are sporadic they often still maintain a geographic and temporal distribution of subtypes and that transmission by flies could be one source of the "random" distribution of *Campylobacter* infections.

2.1.3 Objectives

- To aid epidemiological investigation of *Campylobacter* transmission by the development of a robust, efficient and sensitive method for the detection and identification of *Campylobacter* in a wide range of environmental matrices. The matrices include animal and human faeces, water and food.
- To design a Polymerase Chain Reaction for the identification of *C. jejuni* and *C. coli.*
- To determine the optimal broth for enrichment of the target campylobacters in each of the selected matrices.
- To determine the detection levels of *C. jejuni* and *C. coli* in each of the matrices by use of artificially seeded water, food and faecal matrices, and compare results with the levels obtained by the conventional plating method.

2.2 Materials and methods

2.2.1 Media and reagents

Media and Reagents used in this research were prepared as described in Appendices I and II (respectively). Unless otherwise stated, the chemicals used in this methods section were obtained from Sigma (Castle Hill, New South Wales, Australia).

2.2.2 Bacterial strains and culture conditions

Campylobacter and non-*Campylobacter* bacterial cultures listed in Table 7 were used to validate the PCR oligonucleotide primers to determine the specificity of the PCR reaction. Thermotolerant *Campylobacter* spp. and subspecies of *C. sputorum* and *C. fetus* were cultured on Columbia Blood Agar (CBA) Base (Merck, Darmstedt, Germany) supplemented with 5% defibrinated sheep's blood. Campylobacters were incubated at 42°C for thermotolerant species and 37°C for *C. sputorum* and *C. fetus* spp., under microaerophilic conditions generated by the Oxoid (Basingstoke, UK) CampyGen^{TM} system. *C. gracilis, C. showae, C. concisus* and *C. curvus* were grown on Tryptic Soy BHI Vitamin K Yeast extract (TSBKY) medium at 37^{o}C under anaerobic conditions, generated by the Oxoid AnaeroGen^{TM} system.

Non-*Campylobacter* species, with the exception of *B. ureolyticus*, were cultured overnight in Brain Heart Infusion (BHI; Difco, Detroit, MI, USA) broth at 35°C. *B. ureolyticus* was cultured on CBA with 5% defibrinated sheep's blood at 37^{o}C under anaerobic conditions.

Table 7: Micro-organisms used to assess the specificity of the PCR oligonucleotide primers

Organism	Source
Arcobacter butzleri	CCUG 30485, ATCC 49616
Arcobacter cryaerophilus	CCUG 17801, ATCC 43158
Bacillus cereus	NCTC 8035
Bacillus subtilis	NCTC 3610
Bacteroides ureolyticus	NZRM 2009
Campylobacter coli	NZRM 2607
Campylobacter coli	ERL 97/454
Campylobacter concisus	ATCC 33237
Campylobacter curvus	ATCC 35224
Campylobacter fetus subsp. *fetus*	NZRM 2398, NCTC 10842
Campylobacter fetus subsp. *venerealis*	NCTC 10354
Campylobacter gracilis	ATCC 33236
Campylobacter hyoilei	CCUG 33450, ATCC 51729
Campylobacter jejuni	NCTC 11351
Campylobacter jejuni subsp. *doylei*	NCTC 11951
Campylobacter jejuni	Environmental isolate F38011
Campylobacter jejuni	Environmental isolate 4135
Campylobacter jejuni	Environmental isolate ERL 96 3376
Campylobacter jejuni	Environmental isolate ERL 96 3377
Campylobacter lari	NZRM 2622
Campylobacter lari	960786 (from Massey University, Palmerston North, New Zealand)
Campylobacter showae	ATCC 51146
Campylobacter sputorum subsp. *sputorum*	ATCC 33562
Campylobacter upsaliensis	NZRM 3675, ATCC 43954
Enterobacter aerogenes	NCTC 10006
Enterococcus faecalis	NCTC 775
Escherichia coli	ATCC 25922
Helicobacter pylori	NZRM 2925
Klebsiella pneumoniae	NCTC 9633
Listeria innocua	NCTC 11288
Listeria ivanovii	Isolate from Ruakura Agricutlural Research Centre, Hamilton, New Zealand
Listeria monocytogenes	NCTC 7973
Morganelli morganii	NCTC 235
Proteus vulgaris	ATCC 13315
Pseudomonas aeruginosa	NCTC 10662
Saccharomyces cerevisae	NCTC 10716
Salmonella Menston	NCTC 7836
Shigella flexneri	NCTC 5
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 12228
Streptococcus bovis	NCTC 8177

Abbreviations for Table 7

ATCC	American Type Culture Collection
CCUG	Culture Collection, University of Göteborg
ERL	Enteric Reference Laboratory (ESR, New Zealand)
NCTC	National Collection of Type Cultures
NZRM	New Zealand Reference Culture Collection, Medical Section

2.2.3 Development of the multiplex PCR assay

2.2.3.1 Primer Design.

Thermotolerant *Campylobacter* species were detected via DNA amplification of a portion of the 23S ribosomal RNA (rRNA) gene. Species-specific identification was achieved using primers targeting the *lpx*A gene of *C. jejuni* and the *ceu*E gene of *C. coli.* The sequences for each of these genes were obtained from EMBL (http://www.embl-heidelberg.de) and Genbank (http://www.ncbi.nlm.nih.gov/Genbank) and aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). Primer Express (Applied Biosystems, Foster City, California) was used to design the candidate primers. The primers were screened using BLAST (http://www.ncbi.nih.gov/blast/blast/cgi) for non-specific cross-reactivity. The 23S rRNA primers were modifications of primers designed by (Eyers *et al.* 1994, 1993) with the primer pair being extended at the 5' end to increase the melting temperature to match that of the other primers used. An annealing temperature of 60^{o}C was chosen to increase the specificity of the reaction. The 5' to 3' primers were Therm 1M Forward AAA TTG GTT AAT ATT CCA ATA CCA ACA TTA G and Therm 2M Reverse GGT TTA CGG TAC GGG CAA CAT TAG for the detection of thermotolerant campylobacters, LpxA Forward CCG AGC TTA AAG CTA TGA TAG TGG AT and LpxA Reverse TCT ACT ACA ACA TCG TCA CCA AGT TGT for the detection of *C. jejuni*, and CeuE Forward CAT GCC CTA AGA CTT AAC GAT AAA GTT and CeuE Reverse GAT TCT AAG CCA TTG CCA CTT GCT AG for the detection of *C. coli*. Primers were purchased from Invitrogen, (Carlsbad, CA, USA).

2.2.3.2 Multiplex PCR Conditions.

PCR amplifications were performed in a total volume of 50 µl using PCR buffer (0.050 mol l^{-1} KCl, 0.010 mol l^{-1} Tris, pH 8.3) (Applied Biosystems), 5 pmol of primers LpxA forward and reverse, and Therm 1M forward and Therm 2M reverse, 10 pmol each of CeuE forward and

reverse, 1.25 Units of AmpliTaq, (Applied Biosystems) 250 µM each dNTP (Invitrogen), 1-100 ng DNA, 4.0 mmol l^{-1} $MgCl_2$. Bovine serum albumin was added (0.2 mg ml^{-1}) to prevent inhibition of amplification. Thermal cycling conditions for the Perkin Elmer Thermal Cycler 9700 (Applied Biosystems) were: an initial denaturing cycle at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, 60°C annealing for 1 minute, 74°C extension for 1 minute, with a final 8 minute extension step at 74°C. For each PCR run, positive (10 µl of a mixture of *C. jejuni* and *C. coli*, equivalent to 100 ng of DNA each) and negative (10 µl of molecular biology grade water) controls were included.

2.2.3.3 Visualisation of PCR products.

PCR products were resolved by subjecting them to 2% (w/v) agarose gel electrophoresis (SeaKem LE, Cambrex Bio Science, Baltimore) for approximately 75 minutes at 100 V cm^{-1} in TBE (Tris borate EDTA) buffer (0.09 mol l^{-1} Trizma base, 0.09 mol l^{-1} boric acid, 0.02 mol l^{-1} EDTA, pH 8.0) containing 0.5 µl ml^{-1} ethidium bromide to enable visualisation of PCR products by UV transillumination. Molecular weight markers were included at both ends of each gel (1kb plus DNA ladder, Invitrogen, Carlsbad, California) to ensure the products were of the correct size and establish consistent interpretation of samples in the gel. Polaroid photographs were taken of the fluorescently stained PCR products.

2.2.4 PCR template preparation

2.2.4.1 DNA extraction of bacterial standards

Each bacterial reference culture was grown in the appropriate enrichment medium as outlined in Section 2.2.2. Purified DNA was extracted from bacterial cultures by the following method. A large loopful of fresh bacterial culture was harvested from an agar plate and suspended in 300 µl of extraction buffer (0.025 mol l^{-1} Trizma base, 0.010 mol l^{-1} EDTA and 0.050 mol l^{-1} glucose). Alternatively if grown in broth, ten ml of each culture was harvested by centrifugation at 3,300 x g for 20 minutes, the supernatant removed and 300 µl of extraction buffer added. Lysis was achieved by the addition of 20 µl of lysozyme (50 mg ml^{-1} in ddH_2O) followed by incubation at room temperature for 5 minutes. Subsequently, 12 µl of 20% sodium dodecyl sulphate (SDS) and 4 µl of proteinase K (10 mg ml^{-1} in ddH_2O) were added and further incubated at 37°C for 30 minutes. After lysis, DNA was extracted and purified by the Phenol/Chloroform method (Sambrook *et al.* 1989). DNA was dissolved in 20 µl of ddH_2O and

stored at -20°C. The concentration and purity of DNA was determined by spectrophotometric analysis (λ = 260, 280) and the sample diluted to a final concentration of approximately 100 ng μl^{-1}. A 10 µl volume of each bacterial DNA standard was added to the PCR to determine *Campylobacter* primer specificity.

2.2.4.2 Colony Identification

Identification of bacterial colonies by PCR was achieved by removal of a portion of a single isolated colony and resuspension in 27.0 µl of ddH_2O in a 0.5 ml thin-walled PCR tube. Within 15 minutes from the time of colony resuspension, the tube was heated for 3 minutes at 100°C and then cooled to 4°C. Prepared premix was added to the PCR tube to obtain a final volume of 50 µl. PCR analysis was performed as outlined above in Section 2.2.3.2.

2.2.4.3 Enrichment Broth cultures.

Cells grown in enrichment broths for PCR identification were harvested by centrifugation of 1 ml at 4,000 x g for 20 minutes (4ºC) to pellet the cells. The supernatant was discarded and the pellet washed three times in sterile phosphate buffered saline (PBS) (BR14, Oxoid) before final resuspension in 400 µl of PBS. The washed cells were lysed by heating at 100°C for 12 minutes, and the sample DNA separated from cellular debris by centrifugation at 12,000 x g for 10 minutes at 4ºC prior to transfer of 10 µl of supernatant to the PCR premix.

2.2.5 Determination of the sensitivity of the PCR

Reference isolates of *C. jejuni* (ERL96 3376) and *C. coli* (ERL 97/454) were grown microaerophilically in m-Exeter broth (described below in Section 2.2.6.1) at 42°C for 24 hours. The initial concentration of cells in the broths were determined by preparing a decimal dilution series in 0.1% peptone water (Fort Richard, New Zealand) for each species and spread plating 100 µl volumes of each dilution (in triplicate) onto CBA plates and incubating microaerophilically, as described above, prior to enumeration.

To determine the lowest number of *C. jejuni* and *C. coli* cells required for a positive PCR test, a decimal dilution series of a cocktail of these species was prepared in m-Exeter broth and the

cells from each (non-enriched) dilution harvested and washed (as described above) prior to heat lysis and testing by PCR.

2.2.6 Determination of the optimal enrichment broth for growth of *C. coli* and *C. jejuni* in a range of food, faecal and water matrices.

An overview of the enrichment-PCR method is provided in Figure 2.

2.2.6.1 Preparation of enrichment broths

Broths tested for their ability to enrich *Campylobacter* spp. were Nutrient Broth #2 (Oxoid CM 67), Brucella broth (Difco 0495-17-3, Fort Richard), Tryptic Soy (Difco 0370), Preston Enrichment broth (Bolton and Robertson 1982), Exeter broth (Humphrey *et al.* 1995). Nutrient broth #2, Brucella and Tryptic Soy broths were made in accordance with the manufacturer's directions and after autoclaving 2 vials of Oxoid antibiotic supplement SR117E (reconstituted in 50:50 acetone:sterile distilled water) were added to the broths. Preston broth contained 25 g of Nutrient Broth #2, 950 ml of distilled water, 0.25 g ferrous sulphate, 0.25 g sodium metabisulphite (Bolton *et al.* 1982). After autoclaving the following ingredients were added: 50 ml of lysed horse blood, two ml of 0.25 g ml^{-1} sodium pyruvate (filter sterilised), and 2 vials of Oxoid antibiotic supplement SR117E.

Modified Exeter (m-Exeter) (modified from Humphrey *et al*. 1995) contained 25 g Nutrient Broth #2 dissolved in 950 ml of distilled water. After autoclaving the following supplements were added: 50 ml lysed horse blood, 5 ml filter-sterilised solution containing 4% sodium metabisulphite, 4% sodium pyruvate and 10% $FeSO_4.7H_20$ solution (stored frozen); 15 mg cefaperazone, and 2 vials of Oxoid antibiotic supplements SR117E or SR204E (containing 2500 iu polymixin B, 5 mg rifampicin, 5 mg trimethoprim and 50 mg actidione [SR117E] or 5 mg amphotericin B as a replacement for actidione [SR204E]). M-Exeter broth varies from the Exeter formulation of Humphrey *et al*. (1995) with the inclusion of an antifungal agent, but provides the convenience of the commercial availability of the antibiotic supplement. The Oxoid antibiotic supplement SR117E was discontinued due to problems associated with the availability of the antifungal agent actidione (cycloheximide). Concerns have been raised about the toxicity of actidione for mammalian cells (Martin *et al.* 2002) resulting in a decrease in its availability and a subsequent increase in cost. Consequently, after the enrichment trials, all m-

Exeter broth contained Oxoid antibiotic supplement SR204E. Comparative laboratory testing of the two antifungal agents confirmed that amphotericin B is a suitable replacement for actidione in *Campylobacter* selective media (Martin *et al.* 2002).

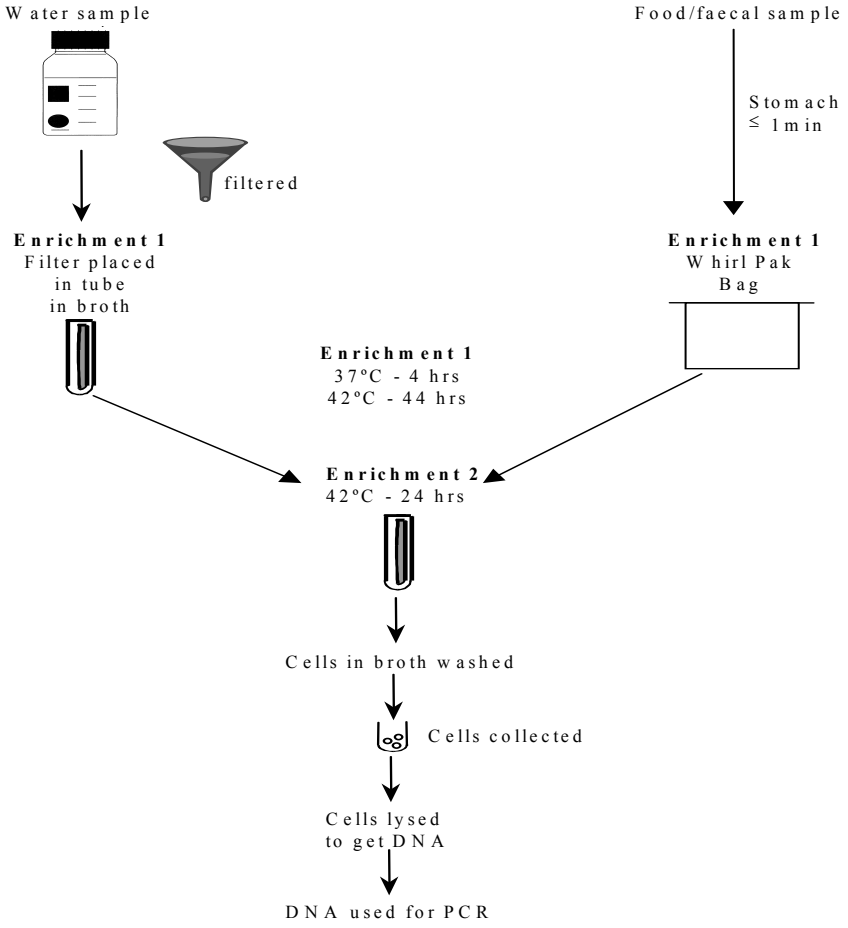

Figure 2: Flow diagram of the enrichment-PCR method for detection of *Campylobacter*

2.2.6.2 *Preparation of matrices*

Faecal matrices tested were sourced from human, sheep, cattle, dairy cow, duck, chicken, possum and rabbit. Meat matrices tested were lamb, beef, pork and chicken, sheep kidneys and livers, while water was obtained from a river.

For all faecal samples, except chicken faeces, 2.5 g of each were weighed into a sterile Whirl-Pak Bag (Nasco, Fort Atkinson, WI, USA) and the enrichment broth under test was added to a final weight of 50 g. A Colworth Stomacher 400 (A.J. Seward, London, UK) was used to mix

samples for 15 seconds. For chicken faeces the weight of faecal material added to the Whirl Pak bag was 1.0 g in a final volume of 50 ml of broth.

For meat and offal samples 10 g of diced meat was weighed into a sterile Whirl Pak bag and 90 ml of the appropriate enrichment broth was added. The sample was mixed in the stomacher for 1 minute. For the swabbing of meat samples a 10 cm^2 portion of the surface of each meat sample was swabbed in multiple directions with a sterile cotton bud which had been pre-soaked in BPW. The cotton bud was then aseptically put into 25 ml of the appropriate enrichment broth by breaking the cotton bud into two portions and disposing of the non-sterile end.

Whole chicken carcasses were aseptically transferred into a sterile plastic bag with 250 ml of sterile buffered peptone water (pH 7.2) (BPW) (1.07228, Merck). After massaging the carcass with the BPW, 10 ml of the chicken rinse was transferred to a sterile Whirl Pak bag. Ninety ml of trial enrichment broth was added and the sample was stomached for 15 seconds.

River water samples (100 ml) were aseptically filtered through HA 0.45 μm filter membranes (HAWG047SI, Millipore, Bedford, MA, USA). At the completion of filtering, the funnel was rinsed with 30 ml of BPW and the membrane aseptically transferred to a sterile bottle containing 25 ml of trial enrichment broth.

2.2.6.3 Incubation conditions for enrichment broths

For each matrix the primary enrichment broth was incubated at 37°C for a minimum of 4 hours under microaerophilic conditions generated by the Oxoid CampyGen™ system. This initial 4 hours at 37°C aided the recovery of injured cells. After this recovery period enrichments were transferred to an incubator operating at 42°C and incubation continued up to a total of 48 hours in a microaerophilic atmosphere. After 48 hour incubation, 0.1 ml of the primary enrichment broth was transferred into a 10 ml m-Exeter secondary enrichment broth and incubated in a microaerophilic atmosphere at 42°C for 24 hours prior to PCR analysis. This second enrichment was to reduce the possibility of detecting non-viable cells by PCR (Savill *et al.* 2001).

Controls included for all enrichment PCR experiments were positive controls of *C. jejuni* (NCTC 11351^T) and *C. coli* (NZRM 2607^T) and negative controls of *E. coli* (ATCC 25922) and uninoculated broth. Enrichment broths designated as controls were inoculated with a 24 hour

culture of the appropriate bacterium and incubated in a microaerophilic atmosphere in the temperature conditions outlined above. The negative control of uninoculated broth followed the same regime of incubation prior to PCR analysis.

2.2.6.4 Determination of the optimal enrichment broth

To determine which of the five broths consistently detected both bacterial species to the lowest level for all matrices, the five trial enrichment broths in combination with all of the matrices were inoculated with a decimal dilution series composed of clinical isolates of human pathogenic strains of *C. jejuni* (ERL 96 3376) and *C. coli* (ERL 97/454). The mixed dilution series was prepared in peptone water and 100 µl volume of each dilution was inoculated into the primary enrichment broths containing the various matrices. Primary and secondary enrichment were then performed as described above prior to detection of campylobacters by PCR. The numbers of *C. jejuni* and *C. coli* cells present in the inoculum were determined by separate serial dilution of each bacterial species, plating on blood agar in triplicate, followed by incubation at 42°C for 48 hours under microaerophilic conditions prior to counting.

2.2.6.5 Determination of the detection limit of Campylobacter cells by the enrichment-PCR method for all matrices, and comparison with the technique of conventional plating

2.2.6.5.1 Procedure for determining the detection limit of the enrichment-PCR method

Once the optimal broth (modified-Exeter) had been chosen, it was necessary to determine how many bacterial cells in the original matrix could be detected using the enrichment-PCR. It was, therefore, required that the matrices be free of viable *Campylobacter*. Food and faecal matrices were sterilised by gamma irradiation (dosage: 10 kilograys) by Schering Plough Animal Health, Upper Hutt. The river water was sterilised by autoclaving 100 ml volumes at 121^{o}C, 103.4 kPa for 15 minutes. Food matrices tested for the detection limit assays were limited to the chicken carcass rinse and offal samples from beef, pig and sheep, as these matrices had consistently shown the lowest number of bacterial cells detectable during the enrichment broth trials, in comparison to the irreproducible results obtained with red meat samples. Uninoculated controls containing the sterilised matrix plus m-Exeter enrichment broth were included for all experiments. All experiments for the determination of sensitivity levels were performed in duplicate.

M-Exeter broth in combination with each of the matrices, was inoculated with decimal dilutions composed of the human clinical isolates *C. jejuni* (ERL963376) and *C. coli* (ERL97/454) prior to enrichment. The bacterial suspensions were diluted to obtain cell numbers which ranged from approximately 2000 cells to one cell for both *C. coli* and *C. jejuni* in the primary enrichment. After incubation of the secondary enrichment the cells were harvested and washed prior to PCR testing as described above.

2.2.6.5.2 Procedure for determining the detection limit of the conventional plating method

The procedure for determining the detection limit of the conventional plating method followed the same setup as for the enrichment-PCR method, in that m-Exeter broth in combination with each of the sterilised matrices, was inoculated with decimal dilutions composed of the human clinical isolates *C. jejuni* (ERL963376) and *C. coli* (ERL97/454). The bacterial suspensions were diluted to obtain cell numbers which ranged from approximately 2000 cells to one cell for both *C. coli* and *C. jejuni* in the primary enrichment. The broth was incubated in a microaerophilic environment for four hours at 37^{o}C and then incubation continued at 42^{o}C up to a total of 48 hours. After 48 hours, 100 µl volumes from the highest dilutions (10^{-6} to 10^{-10}) of the primary enrichments for each matrix were spread-plated onto m-Exeter plates and incubated in a microaerophilic environment for 48 hours at 42^{o}C. Ten colonies from each plate showing bacterial growth were isolated. A hippurate hydrolysis test (On and Holmes 1992) was performed on each of the ten isolates to differentiate between *C. jejuni* and *C. coli*. No other biochemical tests were performed as all bacterial cells present in the primary enrichment were either *C. jejuni* or *C. coli*.

This procedure follows the standard method for detection of *Campylobacter* by conventional selective plating procedures with one exception. In the standard method two to five colonies are usually tested. By testing ten colonies we increased the probability of detecting both *Campylobacter* species on the same plate.

2.2.6.5.3 Determination of the detection limits of rabbit and possum faeces

Possum and rabbit faeces were not included in the initial trials. The development of the methodology for these matrices was different based on the knowledge gained from the experiments conducted on the other matrices. Changes to the methods for the possum and rabbit faeces comprised:

- M-Exeter broth was chosen as the enrichment broth without trialling the other broths.
- In contrast to using sterile matrices, possum and rabbit faeces were tested by PCR to ensure that they were negative for target *Campylobacter* species.
- Identification of *Campylobacter* species for determination of the detection levels of the conventional plating method was based on a PCR assay as described below, rather than on hippurate analysis.

All of the colonies present on the plate inoculated with the primary enrichment were collected and subcultured into m-Exeter broth for 48 hours. This broth was subcultured into a second m-Exeter enrichment for a further 24 hour incubation to remove the possibility of detecting only DNA from dead cells on the plates. Both enrichments were incubated in a microaerophilic environment at 42^{o}C. The cells were harvested and washed and a PCR test performed on the heat lysed cells as outlined previously.

2.3 Results

2.3.1 Characteristics of the PCR developed

A specific and sensitive PCR method was developed for the simultaneous identification of *C. jejuni* and *C. coli* in enrichment cultures. All four thermotolerant *Campylobacter* species (*C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*) tested produced a 246 bp amplicon with the multiplex primers. This amplicon was the expected size for a fragment of the 23S rRNA-DNA region of thermotolerant campylobacters, as determined from published nucleotide sequences. Additionally, *C. jejuni* and *C. coli,* the species of primary interest in this study, yielded the expected PCR products (Figure 3) with the species-specific primers; the *lpx*A product of 99 bp (*C. jejuni*) or the *ceuE* product of 695 bp (*C. coli*). A positive identification of either target species required the simultaneous presence of two products, the thermotolerant *Campylobacter* and the species-specific amplicons. *Campylobacter hyoilei* yielded products characteristic of *C. coli* and this is consistent with the report that *C. hyoilei* is synonymous with *C. coli* (Vandamme *et al.* 1997). In addition, sixteen stock cultures of *C. jejuni* and three cultures of *C. coli* that were isolated from environmental sources in Canterbury, New Zealand by J.D. Klena were also used to evaluate the PCR method. These environmental isolates had previously been confirmed as *C. jejuni* and *C. coli* by conventional biochemical testing based on gram stain, motility, colony morphology, hippurate analysis and nalidixic acid susceptibility testing. No amplicons were detected using the DNA of other species of *Campylobacter* and non-campylobacters as template. The concentration and purity of the DNA template of all bacterial species was confirmed by spectrophotometric analysis.

Determination of the sensitivity of the PCR assay showed that it simultaneously detected *C. jejuni* and *C. coli* in broth culture down to a dilution which equated to 57 cells of *C. jejuni* and 69 cells of *C. coli* per PCR reaction.

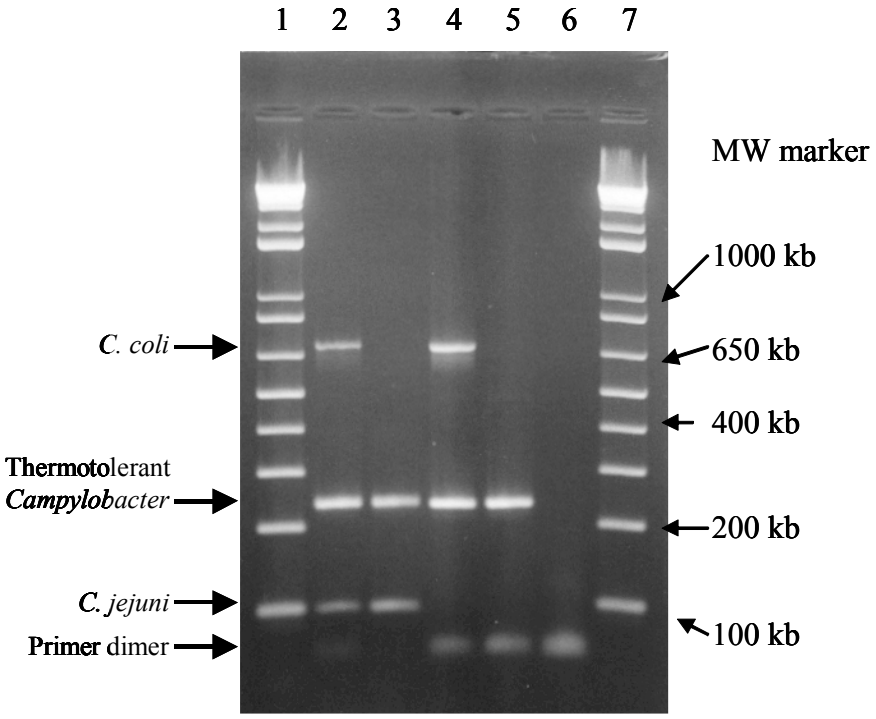

Figure 3: Gel electrophoresis of multiplex PCR products from the amplification of purified thermotolerant *Campylobacter* DNA

Lane 1 and 7: Molecular weight (MW) marker, 1 Kb Plus DNA, Invitrogen; Lane 2: *C. jejuni* and *C. coli*; Lane 3: *C. jejuni*; Lane 4: *C. coli*; Lane 5: Thermotolerant *Campylobacter* (*C. lari*); Lane 6: negative control

2.3.2 The optimal enrichment broth

The broths from the enrichment trials are presented in Table 8. The concentration of *C. jejuni* added to the broths under trial ranged from 3.5×10^5 to 1.0×10^0 cells and that of *C. coli* ranged from 1.4×10^6 to 2.0×10^0 cells. The matrices were not sterile prior to addition of the *C. jejuni* and *C. coli* inocula. Table 8, therefore, represents a relative comparison between the broths to allow the identification of the broth(s) that could consistently detect each bacterial species at low numbers.

In some matrices such as water, cattle and sheep faeces all of the broths allowed detection of target campylobacters to the same low level. Overall, campylobacters in all matrices tested

could be enriched in m-Exeter broth and consistently detected at low numbers. M-Exeter broth was, therefore, selected as the optimal enrichment broth.

Inhibition of the PCR by chicken faeces was overcome by increasing the volume of m-Exeter broth from 25 ml to 50 ml for the primary enrichment and doubling the amount of Taq Polymerase (from 1.25 Units to 2.5 Units) added to the PCR premix (Table 8). It has been suggested that the high urea concentrations found in chicken faeces may contribute to inhibition of the PCR (Claveau *et al.* 2004). Lamb and beef meats posed technical problems because the results were not reproducible. In particular, *C. coli* was often not detected in the dilution series or could only be detected in the lower dilutions thus requiring 10^4 *C. coli* cells to be present in the initial inoculum, e.g. refer to beef and beef swab in Table 8. Dilution of the enrichment broths after the secondary enrichment and prior to the washing of cells improved the detection of campylobacters, which suggested that inhibitors in the beef and lamb enrichments were affecting the PCR assay. In contrast to the lamb and beef matrices, the offal matrices did not exhibit inhibition of the PCR and have been reported to have a higher prevalence of campylobacters compared with meat samples (Kramer *et al.* 2000). Therefore, based on the determination of the detection limits of campylobacters in sheep kidney and liver samples enriched in m-Exeter broth and Preston medium (Table 8), offal was chosen as a replacement for the meat matrices as it provided a more consistently sensitive alternative to beef, lamb and pig meats for the detection of *C. jejuni* and *C. coli*. Although m-Exeter allowed detection of low levels of campylobacters in diced chicken meat and chicken swabs it was decided that the whole chicken carcass rinse would be more likely to detect campylobacters as the diluent rinse method washes all surfaces of the chicken including the gut cavity.

Table 8: Lowest number of bacterial cells detected in each matrix in combination with the trial broths

Matrix	Bacteria	Lowest number of bacterial cells detected in matrix				
		Nutrient broth # 2	Brucella	Tryptic Soy Broth	M-Exeter Medium	Preston Medium
Water	*C. jejuni*	4	4	419	4	4
	C. coli	2	2	148	2	2
Sheep faeces	*C. jejuni*	35	35	35	35	35
	C. coli	128	128	128	128	128
Cattle faeces	*C. jejuni*	35	35	35	35	35
	C. coli	128	128	128	128	128
Chicken faeces (1g/25 ml)	*C. jejuni*	35×10^4	NPD	35×10^4	35×10^4	35×10^4
	C. coli	128	128	128	1.28×10^4	128
Chicken faeces (1g/50 ml)*	*C. jejuni*	NT	NT	NT	26	26
	C. coli	NT	NT	NT	144	144
Dairy faeces	*C. jejuni*	4	4	4	4	4
	C. coli	2	2	2	2	2
Duck faeces	*C. jejuni*	4	NPD	NPD	4	419
	C. coli	2	NPD	NPD	2	148
Human faeces	*C. jejuni*	35	3.5×10^3	35	35	35
	C. coli	128	128	1.28×10^4	128	128
Lamb	*C. jejuni*	92	92	92	92	92
	C. coli	NPD	NPD	NPD	92×10^4	NPD
Lamb swab	*C. jejuni*	92	9.2×10^5	9.2×10^5	92	92
	C. coli	7	9.2×10^5	9.2×10^5	7	702
Beef	*C. jejuni*	92	92	92	92	92
	C. coli	7	NPD	NPD	7	NPD
Beef swab	*C. jejuni*	92	92	9.2×10^3	92	92
	C. coli	7.0×10^4	7.0×10^4	7.0×10^4	7	702
Chicken	*C. jejuni*	92	92	92	92	92
	C. coli	7	7	NPD	7	702
Chicken swab	*C. jejuni*	92	92	9.2×10^3	92	92
	C. coli	NPD	NPD	NPD	7	7
Chicken carcass	*C. jejuni*	NT	NT	NT	26	NT
	C. coli	NT	NT	NT	144	NT
Pork	*C. jejuni*	92	9.2×10^5	92	92	9.2×10^5
	C. coli	7.0×10^2	7.0×10^4	NPD	7	7.0×10^4
Pork swab	*C. jejuni*	92	92	92	92	92
	C. coli	NPD	7.0×10^4	NPD	7	7
Sheep kidney	*C. jejuni*	NT	NT	NT	1	1
	C. coli	NT	NT	NT	12	12
Sheep liver	*C. jejuni*	NT	NT	NT	1	1
	C. coli	NT	NT	NT	12	12

NT = not tested

NPD = no PCR product detected

*To overcome inhibition the volume of enrichment broth was increased and 2.5 Units of Taq Polymerase added to the PCR reaction.

2.3.3 Determination of the detection limit of *Campylobacter* in the enriched matrices

A comparison of the conventional and PCR-based methods for the detection of *C. jejuni* and *C. coli* in m-Exeter broth is presented in Table 9. This study demonstrated a good correlation between the two methods. All uninoculated controls in the study were negative by enrichment PCR and conventional plating methods (data not shown). The results in Table 9 show that the enrichment PCR assay can detect less than ten viable cells per sample of either *C. jejuni* or *C. coli* in each matrix tested. The exception was rabbit faeces where the lowest detection level of *C. coli* was 32 cells in one sample and 3.2 cells in a replicate. As all findings are based on duplicate tests, a result was not reported unless it was replicated, which at these extremely low inoculum levels becomes more difficult and requires multiple samples to be tested.

Conventional plating, with the exception of *C. jejuni* in duck faeces and *C. coli* in rabbit faeces, detected less than 10 viable cells per sample of either *C. jejuni* or *C. coli* in each matrix tested. The detection limit for *C. jejuni* in duck faeces was 15 viable cells per sample. The detection limit for *C. coli* in rabbit faeces was the same in both methods i.e. 32 viable cells per sample. Of the 13 matrices tested, 11 (85%) gave the same result for the two detection methods. Examination of the data for the 13 matrices showed that all samples positive by culture were positive by PCR, but PCR detected lower levels of *C. jejuni* in one of the matrices (duck faeces) and lower levels of *C. coli* in one of the matrices (chicken carcass) in comparison with conventional plating.

Table 9: Detection levels of *C. jejuni* and *C. coli* in enrichment-PCR assay compared with conventional plating method

Matrix	PCR method detection level of viable cells		Conventional method detection level of viable cells	
	C. jejuni	*C. coli*	*C. jejuni*	*C. coli*
Beef liver (per 10 grams)	1	1	1	1
Pig liver per 10 grams)	1	1	1	1
Sheep liver (per 10 grams)	1	1	1	1
Chicken carcass (per 10 ml washing)	1	1	1	7
River water (per 100 ml)	1	1	1	1
Duck faeces (per 2.5 grams)	1	1	15	1
Chicken faeces (per 1.0 gram)	1	2	1	2
Human faeces (per 2.5 grams)	2	8	2	8
Cattle faeces (per 2.5 grams)	2	8	2	8
Dairy faeces (per 2.5 grams)	1	1	1	1
Sheep faeces (per 2.5 grams)	2	7	2	7
Possum faeces (per 2.5 grams)	2	3	2	3
Rabbit faeces (per 2.5 grams)	1	32	1	32

2.4 Discussion

2.4.1 Development of the enrichment-PCR method

A multiplex enrichment PCR assay was developed which can simultaneously detect *C. jejuni* and *C. coli*. The confirmation of identity for either species required the presence of two amplicons, one based on the thermotolerant group of campylobacters and the second being specific for either *C. jejuni* or *C. coli*. This allowed for a double confirmation of identity. No other *Campylobacter* species or non-*Campylobacter* species produced the target or non-specific amplicons of other molecular sizes.

On and Jordan (2003) evaluated eleven PCR assays developed to identify *C. jejuni* and *C. coli* and concluded that no single assay was 100% sensitive and/or specific for *C. jejuni*. In contrast, four of five assays were specific and sensitive for the *C. coli* strains tested. Amplified fragment length polymorphic studies (AFLP) of *C. jejuni* concluded that the genome of *C. jejuni* is very diverse, contributing to the complexity in developing PCR-based identification systems (Duim *et al.* 1999). The same study found *C. coli* to contain a relatively homogeneous genome in comparison to *C. jejuni* and this was supported by the above finding that the PCR assays of *C. coli* were specific and sensitive (On and Jordan 2003). The authors concluded that a polyphasic approach to PCR identification was required.

The assay developed in this study provides a double confirmation by utilising two genes to confirm identity of each species. Furthermore, the method allows for culture from the enrichment broth to confirm the phenotypic identity of campylobacters detected by PCR and to facilitate the typing of *Campylobacter* isolates for epidemiological studies.

Four of the seven *C. jejuni*-specific PCRs examined by On and Jordan (2003) were able to identify less than 40% of the test strains (n = 5) of *C. jejuni* subsp. *doylei*, and only one PCR gave 100% correct identification. *C. jejuni* subsp. *doylei* is recognised as an infrequent cause of gastroenteritis in humans (On 1996) and it is seldom identified in communities of the developed world (Steele and Owen 1988). The PCR assay developed for this study was shown to produce the appropriate amplicons for *C. jejuni* subsp. *doylei* which would identify it as *C. jejuni*. It should also be noted, however, that *C. jejuni* subsp. *doylei* has an optimum temperature of 37°C

(Steele and Owen 1988), and therefore the selective temperature may need to be reduced to 37°C during enrichment if this subspecies is the target of interest.

It was noted during the optimisation experiments that all negative controls had primer-dimer formation (Figure 3). The formation of primer-dimers has been used in PCR as an internal control for amplification of the PCR (Rasmussen *et al.* 1996). Therefore primer-dimers were a useful internal control whose absence suggested that there was either an inhibition problem in the sample or a reagent failure of the PCR. Thus the primer-dimer formation reduces the likelihood of reporting a false negative result.

2.4.1.1 Determination of the optimal broth

In this study, modified-Exeter (m-Exeter) broth was determined to be the optimal enrichment broth for the enrichment and detection of the target *Campylobacter* species by multiplex PCR. M-Exeter broth contains blood and the supplements ferrous sulphate, sodium pyruvate and sodium metabisulphite which are known to act as oxygen quenching or detoxifying agents (Bolton *et al.* 1984). These supplements assist oxygen sensitive *Campylobacter* during isolation procedures when they are exposed to aerobic atmospheres. Addition of antibacterial and antifungal agents in the m-Exeter medium aids the suppression of competing microflora. The choice of a single optimal enrichment broth allows for greater efficacy in the laboratory setting when dealing with large sample numbers of multiple matrices. This consideration is important when developing methods for large surveys intended to elucidate the transmission routes of a bacterium through the environment and fulfils the need for consistent methodology to aid the epidemiological study of campylobacters in the environment (Scates *et al.* 2003).

2.4.1.1.1 Viable but non-culturable bacteria

There is debate between scientists who recognise a survival stage for stressed bacteria termed the viable but non-culturable (VBNC) and those who view the recovery of "VBNC" cells as an outgrowth from injured cells or a remnant of undetected culturable cells (for more detail, refer to Chapter One). The relevance to this research is the possibility that *Campylobacter* could be an organism that is able to enter a VBNC stage when encountering a stressful environment, such as waterways. This theory was relevant to the development of a method for determining the prevalence of thermotolerant campylobacters in various matrices. Taking into account the

VBNC theory was one of the factors contributing to the decision to use an enrichment method rather than direct PCR, which could have led to the detection of dead cells and those in a degenerate (possibly VBNC) form. The double enrichment, therefore, was employed in the enrichment-PCR method to reduce the prospect of detecting non-viable cells by the PCR. It had been previously determined that the number of non-viable cells that would need to be present in a 100 ml water sample in order to produce a false positive result in a secondary enrichment was at least 7.5 x10^6 cells (Savill *et al.* 2001). It was considered that it was highly unlikely that this level of non-viable cells would be present in a 100 ml water sample, reducing the likelihood of a false positive. This level of cells, however, maybe detected on a chicken carcass, albeit infrequently (Stern and Robach 2003) and after processing the cells may be dead or in the VBNC state, therefore viability should be confirmed by conventional plating.

Recent innovations may negate the need to employ a double enrichment to exclude the inclusion of non-viable/dead cells from detection by PCR. Addition of ethidium monoazide (EMA) to samples was shown to differentiate between viable and dead cells because the EMA covalently binds to DNA in dead cells and prevents PCR amplification of their DNA (Rudi *et al.* 2005). Selective amplification of DNA in viable cells occurs because the EMA can only penetrate cells that have damaged cell membranes. This system has been evaluated with *C. jejuni* in mixed bacterial populations and complex samples that included food and faeces, and the effects of disinfection and antibiotic treatments were also tested (Rudi *et al.* 2005). Another interesting technique being employed in Real-time PCR is the novel method of flotation which is based on variations in buoyant densities between different bacterial species and allows separation of the target organism (Wolffs *et al.* 2005). Furthermore, it has been shown to reduce PCR inhibition due to removal of inhibitors present in environmental matrices such as chicken rinse samples. Viable cells of *Campylobacter* could be separated from dead and VBNC cells by the flotation treatment thereby eliminating false positive results due to detection of DNA from non-viable cells. Further validation of these promising techniques will verify their use for routine PCR applications.

2.4.1.1.2 Effect of incubation temperature

A comparative study of the effect of incubation temperature on the recovery and genotypes found in chicken pieces and lamb livers reported that the incubation temperature (37°C versus 42°C) for enrichment in Preston broth had no significant effect on the number of positive

samples obtained or on the *Campylobacter* species isolated (Scates *et al.* 2003). A significant difference was noted, however, in the diversity of genotypes isolated at the two incubation temperatures. Although, the diversity of genotypes was higher at 42°C, the authors strongly suggest that when conducting epidemiological surveys of *Campylobacter* genotypes the use of 37°C and 42°C will yield a higher diversity of genotypes. This could improve the assessment of the importance of transmission routes and reservoirs of campylobacters.

2.4.1.2 Choice of matrix and preparation method

Studies of pork, lamb and beef offal have indicated a high prevalence of *Campylobacter* (Kramer *et al.* 2000, Bolton *et al.* 1985). Offal was, therefore, chosen as the matrix of choice over pork, lamb and beef meat which produced inconsistent results for all trial enrichment broths. Whole chicken carcasses were chosen instead of chicken swabs or 10 g subsamples of chicken meat as the rinse method allows detection of *Campylobacter* from all surfaces of the chicken, including the gut cavity. Jørgensen *et al.* (2002) identified higher numbers (one order of magnitude) of campylobacters from carcass rinse samples with and without entire skin compared with *Campylobacter* numbers from neck skin samples. Averages of *Campylobacter* ranged from $\log_{10}$ 4.1 to $\log_{10}$ 5.1 cfu per chicken.

2.4.1.3 Sensitivity tests for the enrichment-PCR method

The results of the sensitivity tests for detection of *C. jejuni* and *C. coli* in m-Exeter broth showed that in most matrices less than ten cells per sample of either *C. jejuni* or *C. coli* could be detected in the original sample by multiplex PCR. The highest level of detection was reported for *C. coli* in rabbit faeces where the range of detection was 3-32 cells in the original sample. These limits of detection correspond to the sensitivity reported for other *Campylobacter* PCR assays (Waage *et al.* 1999, Hernandez *et al.* 1995, Eyers *et al.* 1994, 1993, Oyofo *et al.* 1992). The low levels of detection validated the simultaneous enrichment and PCR detection of the two *Campylobacter* species in the same broth. It also confirmed that PCR detection was not compromised by inhibition by blood in the enrichment medium, or components of faeces or food products which have demonstrated inhibitory effects in previous studies (Wilson 1997, Rossen *et al.* 1992). Determination of the sensitivity levels in non-sterile possum and rabbit faeces indicates that even in the presence of high levels of background microflora, the enrichment-PCR assay can detect low levels of *Campylobacter*.

2.4.1.4 Comparative study between conventional plating and enrichment-PCR methods

The comparison of the developed enrichment-PCR method with the conventional plating method produced few anomalies. The PCR assay had the same level of sensitivity as the conventional plating method, except for chicken carcasses and duck faeces. In these two matrices, the number of cells per sample as determined by PCR assay was lower than the conventional plating method. The poorer detection by the conventional plating method may be due to the smaller proportion of broth (approximately 10-20 µl) used to inoculate an agar plate in comparison with the 1 ml of enrichment broth taken for PCR detection. Therefore, if a smaller inoculum size is used for analysis where a calculation of the dilution suggests only one cell is present, the likelihood of detection of that one cell is reduced (Johnson *et al.* 1998).

With the exception of the possum and rabbit faeces, the sensitivity levels for the conventional plating method in this study are based on the results of testing ten colonies from each plate by the hippurate reaction. The hippurate hydrolysis test identified the lowest numbers of *C. jejuni* which could be detected on the plates from the primary enrichment. The chicken carcass and duck faecal enrichments were examples where the standard laboratory practice of testing less than five colonies from the plate of the primary enrichment could show an absence of *C. jejuni*. This was in comparison to the PCR test which showed the presence of *C. jejuni* and *C. coli* down to a level of one viable cell in the same sample. In the case of the duck faeces this led to the sensitivity for detecting *C. jejuni* by the conventional plating method being one order of magnitude higher, (15 cells per sample) compared with the PCR test. This shows the advantage of detection by PCR over conventional plating where a higher proportion of cells present in the enrichment are represented in the sample. Although this research only reports on artificially contaminated matrices for determination of sensitivity levels, subsequently this method has been further validated by a comparison with conventional plating during a survey of naturally occurring thermotolerant *Campylobacter* contaminating the surfaces of poultry packs (Wong *et al.* 2004). Sensitivity of the enrichment-PCR method was similar to conventional plating. Furthermore, in combination with the Most Probable Number technique (MPN), the enrichment-PCR method detected *Campylobacter* to a level of 6MPN/100 ml chicken rinse.

Interestingly, Kramer *et al*. (2000) and Sails *et al*. (2002) noted the preferential enrichment of *C. coli* over *C. jejuni*, whereas direct plating favoured the isolation of *C. jejuni*. In subsequent

studies (personal communication, Paula Scholes, ESR) the enrichment-PCR method has also shown that where *C. jejuni* and *C. coli* are identified by PCR in the same enrichment, subsequent isolation of *C. jejuni* from the enrichment broth onto agar plates proved difficult due to the high numbers of *C. coli* on the plate. This suggests the benefits of PCR detection over conventional plating, where the multiplex PCR is able to identify multiple target species in a sample. In the case of *Campylobacter* species, the colony morphology of multiple target bacteria is difficult to differentiate. This would suggest that many more than five colonies per plate may need to be characterised by biochemical tests to be confident that all *Campylobacter* species have been identified.

2.4.1.5 Direct PCR and Real Time PCR methods for the detection of Campylobacter.

Direct PCR for the detection of *Campylobacter* in human faeces (Linton *et al.* 1997) has been developed by various researchers. The low concentration of *C. jejuni* and *C. coli* has, however, hindered attempts to develop direct PCR from bovine faeces. A PCR has been developed for the direct detection of campylobacters, including *C. jejuni* and *C. coli*, in bovine faeces (Inglis and Kalischuk 2003). This PCR used an internal control to detect PCR inhibition by faecal components such as bile salts and complex polysaccharides (Monteiro *et al.* 1997). A two-step nested PCR was employed for *C. coli* and *C. jejuni* detection to increase the sensitivity of the method. This resulted in detection of 100% of faecal samples inoculated with 10^4 cfu g^{-1} of either *C. jejuni* or *C. coli*. At inoculation levels of 10^3 cfu g^{-1}, 83% of *C. coli* samples were positive compared to 67% of *C. jejuni* samples. *C. coli* was not detected at seeded levels of 10^2 cfu g^{-1}, whereas 17% of *C. jejuni* samples inoculated at 10^2 cfu g^{-1} produced a PCR amplicon. Stanley *et al.* (1998b) noted that the average concentration of campylobacters in bovine faeces was 10^2 cfu g^{-1} for adults and 10^4 cfu g^{-1} for calves. This would suggest that the lower detection limit for this PCR is at the average *Campylobacter* concentration for adult cattle and consequently some samples would report false negative results if tested. This is confirmed by the isolation of *C. jejuni* and *C. coli* by conventional plating on various media designed for these target organisms (e.g. (blood-free) charcoal-cefoperazone-deoxycholate agar, CCDA) which showed greater sensitivity than the PCR assay in 13% of the bovine faecal samples.

Inglis and Kalischuk (2003) used the QIAamp DNA Stool minikit for DNA extraction from bovine faeces, because it is designed to remove PCR inhibitors derived from faeces. The amount of faeces used per sample was 200 mg in comparison to the 10 grams for enrichment

used in this study. Because little is known about the spatial distribution of the low levels of campylobacters in cowpats, it may be necessary to increase the number of samples analysed from a single cowpat if adequate detection of campylobacters is to be achieved by direct PCR analysis using the minikit system.

Subsequent research by this team resulted in the development of a real-time quantitative PCR (RTQ-PCR) for the detection of *C. jejuni* in bovine faeces (Inglis and Kalischuk 2004). RTQ-PCR has the advantage of greater specificity compared with gel-based PCR due to the inclusion of a probe which recognises sequence internal to the primer set (Josefsen *et al.* 2004). It is also faster as no electrophoresis is performed and contamination is reduced in the closed tube assembly, with elimination of contamination carryover by incorporation of dUTP in the reaction. In comparison to direct PCR, the RTQ-PCR method of Inglis and Kalischuk (2004) did not employ a nested PCR as it did not improve the sensitivity for *C. jejuni* which was 3 x 10^3 cfu g^{-1} of faeces. The results of detection by RTQ-PCR in naturally contaminated bovine faeces were comparable with the results of conventional plating methods.

An RTQ-PCR has also been developed for detection of *C. jejuni* in foods (Sails *et al.* 2003a). This method required enrichment at 37°C for 24 hours, followed by incubation at 42°C for another 24 hours prior to RTQ-PCR. The limit of detection was approximately 12 genome equivalents and a comparative assay with detection by culture reported 57 positive samples by plating, in comparison to 63 positive samples using the RTQ-PCR method. Another RTQ-PCR developed for assaying *C. jejuni* in water did not use enrichment prior to PCR and discrepancies between culture negative and RTQ-PCR positive samples were tentatively attributed to non-viable or dead cells (Yang *et al.* 2003).

2.4.2 Conclusions

In conclusion, an enrichment-PCR assay was developed for the detection of *C. jejuni* and *C. coli* from 13 different environmental matrices including animal faeces and meat products, river water and human faeces. The specificity of the PCR assay was confirmed by the lack of cross reactivity when the multiplex PCR was tested against a range of other *Campylobacter* species and other bacteria.

Sensitivity tests of the assay showed that in most matrices less than ten cells per sample of either *C. jejuni* or *C. coli* could be detected in the original sample. Rabbit faeces was the only matrix where sensitivity was greater than ten cells (range 3-32 cells of *C. coli*) but this was comparable with the range determined by the conventional method for *C. coli* cells in the same matrix. The enrichment-PCR method has similar sensitivity levels to the conventional plating method for identifying *Campylobacter* but has the advantage of reducing the time required for identification from a maximum of ten days by the conventional method to 4-5 days. This time period is still longer than the rapid methods of direct PCR and RT-PCR. Also, since samples were enriched to increase sensitivity, this negates possible quantification of the initial concentration of *Campylobacter* in the sample. The ability of the enrichment PCR method to allow the growth of a wide range of *Campylobacter* subtypes of the same species is described in Chapter Three.

In food laboratories, due to a limitation of resources, only a few colonies (approximately two to five) per plate are tested for confirmation by phenotypic techniques. The comparative assay performed in this study showed the advantages of PCR detection where a higher proportion of cells present in the enrichment are represented in the sample, allowing increased detection of both *C. jejuni* and *C. coli* in the same sample.

The main benefit of this study resides in the employment of a single enrichment broth for the low level detection of *C. jejuni* and *C. coli* in 13 environmental matrices. In contrast, most assays have been validated against a small range of matrices, such as poultry carcasses (Hong *et al.* 2003), bovine faeces (Inglis and Kalischuk 2004, 2003), water (Kirk and Rowe 1994) and human faeces (Maher *et al.* 2003). Yang *et al.* (2003) have developed a RT-PCR method for the

detection of *C. jejuni* in poultry, milk and environmental waters which is one of the few assays developed for detection in a multiple range of matrices.

The enrichment-PCR assay, therefore, allows the efficient identification of pathogenic campylobacters in a wide range of environmental matrices. Employment of a single enrichment broth for 13 matrices will assist the large surveys required to better understand the transmission of *Campylobacter* through the environment to humans. The use of a single method to identify *Campylobacter* in these matrices will facilitate epidemiological studies in investigating an infection source.

3 Application of the enrichment-PCR method to field studies

3.1 Introduction

The enrichment-PCR method was applied in field trials to determine if it could detect the prevalence of *C. jejuni* and *C. coli* in whole chicken carcasses and to ascertain if the method was able to identify multiple subtypes of a single species of *Campylobacter* in a water sample.

3.1.1 Methodology for the detection of *Campylobacter*

Campylobacter species are unusual foodborne pathogens in that they have strict requirements for a microaerophilic atmosphere (5% oxygen and 10% carbon dioxide) and a restricted temperature range for growth (Corry *et al.* 1995). They will, generally, not grow below 30°C and therefore, in comparison, to other more robust food pathogens they are not expected to multiply in food or during food storage (Park 2002). The specific growth conditions required in the laboratory to maximise recovery of stressed campylobacters from environmental matrices, such as food, has led to a large number of different formulations for enrichment broths and agars (Corry *et al.* 1995). These include media that contain blood and/or other oxygen quenchers and antimicrobials which counteract competition from other microflora. Comparative studies of methods used to isolate campylobacters from environmental matrices have identified differences in the prevalence of campylobacters in an individual matrix, which could be attributed to features of the methods employed (Tangvatcharin *et al.* 2005, Baserisalehi *et al.* 2004, Jørgensen *et al.* 2002, Moore 2000, Baylis *et al.* 2000, Scotter *et al.* 1993).

There is always a balance between selectivity of a method to reduce competition from other microflora and the recovery and growth of the target micro-organisms. The enrichment-PCR method uses an incubation temperature of 42°C in m-Exeter broth to enrich for *Campylobacter* followed by plating onto m-Exeter agar. The temperature of 42°C is selective for thermotolerant campylobacters (Griffiths and Park 1990), which include *C. jejuni* and *C. coli,* the two major pathogenic species of interest to this study. Also, the m-Exeter broth contains antibiotics that are selective for the target campylobacters (Humphrey *et al.* 1995). It is known that some of the antibiotics in m-Exeter, such as rifampicin and polymixin B, can be inhibitory to both injured

campylobacters and certain subtypes of *Campylobacter* species (Baylis *et al.* 2000, Humphrey 1990).

The discrepancies in prevalences reported by different methods may be partially ascribed to the differences between strains within a *Campylobacter* species (Murphy *et al.* 2005, Baylis *et al.* 2000). The identification of a high diversity of *C. jejuni* subtypes in environmental matrices as determined by subtyping techniques is well documented (Stern *et al.* 2004, Hopkins *et al.* 2004, Siemer *et al.* 2004, Dickins *et al.* 2002). This high diversity may be attributed to the heterogeneous nature of its genome as recognised when the DNA sequence of *C. jejuni* was first published by Parkhill *et al.* (2000). It has been suggested that the plasticity of the genome of *C. jejuni* contributes to its ability to adapt to new and hostile environments (de Boer *et al.* 2002) which makes it a successful pathogen despite its supposed physiological fragility (Park 2002). This high diversity contributes to the difficulty of establishing a method which identifies all *Campylobacter* subtypes relevant to the aetiology of human campylobacteriosis.

It is probable that given the selective nature of the enrichment-PCR method the growth characteristics of each environmental *Campylobacter* subtype in the enrichment broth will vary. The degree of this variation is unknown and consequently if the initial sample contained equal numbers of two subtypes it is possible that one may grow faster in the selective conditions and be identified by subtyping because it is the dominant strain present on the plate (Dickins *et al.* 2002). In reality, it is probable that the concentrations of each subtype in the water sample will be variable. Therefore, if the proportion of each subtype on the agar plate is dependent on its initial concentration in the water sample and subsequent growth rate, it is important to ascertain if the method will detect only a limited number of subtypes.

3.1.2 *Campylobacter* prevalence in live chickens and chicken meat products

It was important to ascertain if the enrichment-PCR method was able to detect campylobacters in the matrices under investigation. Chicken carcasses were chosen to assess the prevalence of *Campylobacter* contamination as estimated using the enrichment-PCR method. Surveys have shown that 30-100% of poultry harbour *Campylobacter* as normal commensal flora of their intestinal tract where they colonise the caecum and colon (Newell 2002, O'Sullivan *et al.* 2000). The *Campylobacter* intestinal contents of chickens at the time of slaughter are reported to be present in numbers up to 10^7 cfu g^{-1} (Newell 2002, Stern *et al.* 1984). A survey of the caecal

contents of 2,325 broiler chickens in Quebec, Canada (Nadeau *et al.* 2002) reported a high prevalence of campylobacters with a majority (67%) of the farms testing positive for campylobacters and *C. jejuni* being identified in 95.2% of the positive birds, followed by 4.8% *C. coli.*

The high prevalence of campylobacters identified in live chickens is reflected in the prevalence of *Campylobacter* in chicken products. For example, a 2001 UK-wide survey found an average of 50% of commercial fresh and frozen chicken products positive for *Campylobacter* (www.foodstandards.gov.uk/news/chickensum.htm). The 4881 samples tested included fresh, frozen, chicken portions and whole chickens in proportion to the market share. Fresh chicken had a prevalence of 63% while a lower prevalence of 33% was found in frozen chicken. This was similar to the 50% prevalence of *Campylobacter* identified in 444 retail chickens in Ireland (Whyte *et al.* 2004). Another United Kingdom study identified 83% of 241 fresh and frozen chicken carcasses as harbouring *Campylobacter* (Jørgensen *et al.* 2002). *C. jejuni* comprised 98% of the isolates from this UK study with the remaining isolates being identified as *C. coli*.

3.1.3 Counts per chicken carcass

A study undertaken in the United Kingdom found that average *Campylobacter* counts were $\log_{10}$ 4.9 and $\log_{10}$ 5.1 cfu per chicken (n = 241) for carcass rinse and carcass rinse plus whole skin samples, respectively (Jørgensen *et al.* 2002). Approximately 20% of fresh chicken carcasses carried between $\log_{10}$ 5.0 and $\log_{10}$ 6.99 *Campylobacter* species per carcass, with one carcass containing >$\log_{10}$ 9.0 cfu. In the same study, frozen chickens (n = 4) had a range of 2.8-4.4 $\log_{10}$ cfu campylobacters per carcass. A Dutch study determined the levels of *Campylobacter* contamination on fresh retail chickens (n = 45) and identified 18% of the chickens as having >5500 *Campylobacter* cells per carcass (Dufrenne *et al.* 2001). Another 18% of the fresh retail chickens had less than ten campylobacters per carcass. Over half (57%) of the frozen chickens (n = 44) contained less than ten campylobacters per carcass. A larger Danish study reported a range of <0.4 to 400 cfu g^{-1}campylobacters in frozen chicken products (n = 474), with 80% negative (<0.4 cfu g^{-1}). In comparison, the range for fresh chicken products (n = 558) was <0.4 to >4000 cfu g^{-1} with approximately 55% of fresh chicken products negative (<0.4 cfu g^{1}) for campylobacters (Anonymous, 2002). These findings support the theory that freezing of chicken carcasses reduces the levels of *Campylobacter* contamination.

3.1.4 Water as a transmission route for *Campylobacter*

Reported cases of campylobacteriosis in New Zealand attributed to water, include an outbreak in the township of Ashburton (Brieseman 1987). In this incident, contamination of the town water supply after heavy rains was implicated as the likely source of infection. The water for the town supply is derived from the Ashburton River which is surrounded by sheep and beef farms. Prior to this contamination event, chlorination of the supply only occurred at times of heavy rainfall and the delay in beginning chlorination after the onset of rain may have allowed *Campylobacter* into the town supply. Other water-related *Campylobacter* outbreaks are a camp and convention centre in Christchurch (Stehr-Green *et al.* 1991) and a College, where a malfunctioning UV treatment light may have caused the influx of *Campylobacter* into the water supply (Inkson 2002).

The Canadian Walkerton Inquiry (Hrudey *et al.* 2003, Clark *et al.* 2003) highlights the dangers of waterborne transmission of pathogens. Seven people died and over 2,300 became ill when Walkerton Town's water supply became contaminated with *Campylobacter* and *E. coli* O157:H7. It was presumed that the contamination arose from farm animal run-off into a shallow well, from which the water supply was taken as subtyping of *Campylobacter* and *E. coli* O157:H7 isolates revealed indistinguishable molecular subtypes and phage types for the majority of isolates from clinical specimens and farm animal manure collected around one of the contaminated wells.

Numerous waterborne outbreaks of gastroenteritis have been reported in Finland (Hänninen *et al.* 2003, Miettinen *et al.* 2001) with several being linked with *Campylobacter* infection. Three of these outbreaks, which were associated with *Campylobacter,* were investigated using Penner serotyping and PFGE analysis (Hänninen *et al.* 2003). In only one of the outbreaks did analysis of water samples and clinical samples identify indistinguishable subtypes of *C. coli*, confirming the source of the outbreak as contaminated drinking water from groundwater. Although campylobacters were identified in the drinking water samples from another outbreak they were not identical to the patients' clinical isolates and no campylobacters were isolated from environmental samples in the third outbreak. These outbreaks illustrate the difficulty of determining the sources of an outbreak due to the delay between exposure and recognition of a potential transmission route. Campylobacters were identified in the wells and surrounding water

sources in one of the outbreak incidences suggesting a constant flow of campylobacters through the drinking water system. At the time of sampling, however, none of these environmental isolates matched the clinical isolates and therefore a direct link to the groundwater wells could not be confirmed.

The case of a large waterborne outbreak involving *Campylobacter* and *E. coli* O157:H7 occurred in the United States at a county fair (Bopp *et al.* 2003). *E. coli* O157:H7 was isolated from the stools of 128 people who attended the fair and suffered a gastrointestinal illness. A very high proportion (91.5%) of these 128 isolates were confirmed as being related subtypes by PFGE typing analyses. The same *E. coli* O157:H7 subtypes were isolated from samples taken from the water distribution system at the fairground suggesting that water was the transmission route for this organism. At the time of this outbreak the potable water was obtained from six shallow wells, four of which were not chlorinated. *C. jejuni* was isolated from the stools of 44 who reported a diarrhoeal illness after attending the same county fair. Only one case of co-infection with *C. jejuni* and *E. coli* O157:H7 was confirmed. PFGE results for the *C. jejuni* isolates revealed that 29 of the 35 human isolates that were subtyped had indistinguishable PFGE patterns. *C. jejuni* was not identified in water or septic tanks sampled during this investigation and therefore the cases of campylobacteriosis could not be confirmed as a waterborne outbreak. As the authors noted the high proportion of a single strain type is suggestive of a large contamination from a single source.

A recent study investigated the ability of the freshwater crustacean *Daphnia carinata* to reduce populations of *C. jejuni* in simulated natural aqueous conditions (Schallenberg *et al.* 2005). *Daphnia* is recognised as an efficient grazer of aquatic bacteria. The study showed that ingestion and passage through *Daphnia* reduced the population of *C. jejuni* and lack of survival of *C. jejuni* after passage through *Daphnia* was confirmed by determining the culturability of *C. jejuni* in the faecal material of the crustacean. The authors suggest that this finding could have implications for the water industry in terms of the potential role of *Daphnia* in the biocontrol of human bacterial pathogens in recreational waters and drinking water reservoirs.

3.1.5 Subtyping methodologies

The characterisation of *Campylobacter* subtypes from various sources and a determination of their relative contribution to human infection is a prerequisite for the investigation of the

transmission routes of a pathogen. It also allows the detection of changes in infectious disease aetiology.

The high diversity of *Campylobacter* strains identified in the environment necessitates a validation of the enrichment-PCR method to ensure that it is capable of detecting a wide range of *Campylobacter* subtypes in a matrix and multiple subtypes within an individual sample.

Numerous methods for the subtyping of *Campylobacter* have been described. The international literature was reviewed by the author to determine the optimal genotypic approach for this project. No single ideal method has been identified as suitable for all research studies (Nielsen *et. al.* 2000, McKay *et al.* 2001). Therefore each typing method was evaluated according to the criteria of discrimination, typeability, reproducibility and cost effectiveness (Wassenaar and Newell, 2000). A short description of each phenotypic and genotypic typing system mentioned in this thesis is presented in Appendix IV.

The advantages and disadvantages of Amplified Fragment Length Polymorphism (AFLP) and Multi Locus Sequence Typing (MLST), and the more commonly used Pulsed Field Electrophoresis (PFGE) are considered below. These are the typing systems receiving the greatest international recognition because of the advent of computer assisted software technologies which has simplified data interpretation and increased accessibility for laboratories reporting on disease surveillance.

3.1.5.1 Amplified Fragment Length Polymorphism (AFLP)

AFLP fingerprinting is used for strain identification and differentiation of genetically related bacteria. Automation of the technique allows for high throughput and rapid sample analysis for epidemiological investigations (Wassenaar and Newell 2000). The method can be standardised to allow interlaboratory comparisons. The discriminatory power of AFLP is generated by the choice of restriction enzymes and selective primers. The first step involves digestion of the total genome with two restriction enzymes. Subsequent PCR amplification targets only those digested fragments that are flanked by both restriction enzyme sites. The large number of bands generated can be reduced by incorporating one or more specific nucleotides adjacent to the restriction site. This additional step allows selective amplification of those fragments containing the specific nucleotide(s) flanking the restriction site. The PCR primers are fluorescently

labelled and analysis of the amplicons is performed on denaturing polyacrylamide gels (Wassenaar and Newell 2000).

AFLP generates between 50 and 80 bands. The high number of multiple small bands makes it less likely that this technique is susceptible to genomic instability (Wassenaar and Newell, 2000) and is highly discriminatory between isolates (Lindstedt *et al.* 2000). A comparison of molecular genotyping methods identified AFLP as the most discriminatory method, and subdivided 50 *Campylobacter* strains derived from poultry into 41 distinct genotypes (de Boer *et al.* 2000). The next most discriminatory method was PFGE using *Sma*I digestion, which identified 38 genotypes, followed by *fla*A RFLP (31 genotypes) and ribotyping (26 genotypes). During a survey of campylobacters isolated from chicken faeces Wittwer *et al*. (2005) also found AFLP typing to be more discriminatory than RFLP typing of the flagellin gene and antibiotic resistance typing.

Disadvantages of the AFLP method are that an automated DNA sequencer and computer assisted analysis are essential for identification and therefore a major capital investment is required (Duim *et al.* 1999). A simpler version of AFLP has been trialled which allows its use in more laboratories. It is called single-enzyme-amplified fragment length polymorphism (SAFLP) and uses a single restriction enzyme for digestion of the DNA followed by ligation of digested fragments with an adapter sequence. PCR amplification of the adapter–tagged fragments is performed with a single primer which is complementary to the adapter sequence (Gibson *et al.* 1998). Electrophoretic separation of the 8-10 amplicons generated by SAFLP of the *Campylobacter* genome is achieved on a 1.5 % agarose gel using ethidium bromide for visualisation of the banding pattern (Champion *et al.* 2002). A comparison of PFGE and SAFLP techniques for distinguishing outbreaks of campylobacteriosis showed that SAFLP was as discriminatory as PFGE and less labour intensive (Champion *et al.* 2002). Further research may lead to the validation of SAFLP as a less expensive alternative to PFGE for outbreak identification.

3.1.5.2 Multi Locus Sequence Typing (MLST)

MLST is another typing technique able to distinguish between closely related strains as it is based on sequence polymorphisms within seven to ten conserved housekeeping genes. Analysis

of the number of unique alleles per gene allows the generation of different combinations (sequence types) so that all strains can be distinguished from each other.

MLST population studies of *C. jejuni* have revealed a low overall degree of sequence diversity (Suerbaum *et al.* 2001). *C. jejuni*, however, has high rates of intraspecies recombination which creates many different combinations of alleles suitable for generating a large number of discriminatory sequence subtypes (Suerbaum *et al.* 2001).

MLST is a highly reproducible method that generates data that is readily comparable between laboratories but it has a requirement for high capital investment, similar to AFLP, and requires complex data analysis. These techniques may be useful as non-routine methods for high resolution genotyping where further discrimination between isolates is required for the determination of genetic lineages.

3.1.5.3 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is the more widely used genotypic method for routine subtyping. The basic premise of PFGE is that the total DNA content (chromosomal and plasmid DNA) present in a bacterial cell is subjected to digestion by a restriction enzyme which cleaves the DNA infrequently (Wassenaar and Newell, 2000). The digested DNA fragments are separated on the basis of molecular size by agarose gel electrophoresis using a technique involving switching the orientation of the electric field over a 120°C angle in a pulsed manner. This successfully separates the large DNA fragments and the resultant patterns of migration of DNA are recorded as an isolate's PFGE "fingerprint". In practice, the interpretation of DNA fragments is restricted to those that fall within the size range of 25-700 kb (Michaud *et al.* 2001). This is designed to exclude plasmid DNA (<25 kb) and fragments above 700 kb which are not well resolved on the PFGE gel as they tend to co-migrate.

In a comparative commentary on molecular methods of genotyping PFGE was described as being the "gold standard" for DNA-based subtyping (Olive and Bean 1999). PFGE was depicted as having high discrimination power between strains but with moderate set up costs in comparison to AFLP and DNA sequencing techniques, such as MLST.

An evaluation of subtyping methods used to distinguish between *C. jejuni* isolates associated with a campylobacteriosis outbreak and those isolates linked to sporadic illness (Fitzgerald *et al.* 2001a) concluded that PFGE was the most discriminatory subtyping method. The other methods evaluated were Penner serotyping, restriction fragment length polymorphisms (RFLP) of the flagellin (*fla*A) gene, sequencing a region of the *fla*A gene (582 bp) and sequencing the entire *fla*A gene. The PFGE, serotyping and sequencing of the 582-bp region all separated the outbreak from sporadic cases.

The discriminatory power of PFGE is attributed to its ability to determine polymorphisms derived from the entire bacterial genome rather than relying on differences within one or two genes (or gene products), as is the case with the other subtyping schemes tested by Fitzgerald *et al.* (2001a). This conclusion was supported by a comparative study of six typing methods which found that the genotypic techniques of PFGE and random amplified polymorphic DNA (RAPD) were highly discriminatory (Nielsen *et al.* 2000). In comparison, RFLP of the flagellin gene (RFLP *fla*A), denaturing gradient gel electrophoresis of the flagellin gene (*fla*-DGGE) and riboprinting were found to be less discriminatory.

A study of plasmids and their affect on typing systems was undertaken for the bacterium *Enterococcus faecium* (Werner *et al.* 2003). Twenty-four transconjugants from a genetically isogenic strain collection, differing in their possession of non-identical resistance-conferring plasmids, were typed by RAPD, AFLP and PFGE (*Sma*I). RAPD was the least discriminatory and AFLP the most discriminatory. The authors concluded that PFGE could still be recommended as the gold standard based on its ability to distinguish between highly related strains where 22 of the 24 transconjugants differed by less than three bands in comparison to the wild-type (parent) strain.

3.1.5.3.1 Choice of restriction enzyme for PFGE

A comparative study investigated the utility of using *Sma*I as the restriction enzyme (RE) of choice for PFGE analysis (On *et al.* 1998). They compared the profile groups obtained with *Sma*I and three other restriction enzymes and concluded that *Sma*I is a “generally robust means of accurately determining *C. jejuni* strain relationships”. This finding was supported by Fizgerald *et al.* (2001) who employed PFGE analysis using two enzymes: *Sma*I and *Sal*I to determine the source of an outbreak attributed to a foodhandler. The *Sma*I digest was shown to

be more discriminatory than the *Sal*I RE digestion. On *et al.* (1998), however, also noted that some isolates giving the same profile for *Sma*I digestion could be further subdivided by the use of a second RE. Tenover *et al.* (1995) suggested that the use of two restriction enzymes is potentially useful in cases where isolates are collected over an extended period of time.

For the work described in this study, PFGE using *Sma*I was determined to be the most appropriate method for the validation of the enrichment-PCR method to identify multiple subtypes. This was based on its high discriminatory power and low set up cost and the ability to standardise the method between laboratories, which allows for interlaboratory comparisons at the international level, as discussed in Chapter Four.

3.1.6 Objectives

- To establish the ability of the enrichment-PCR method to determine prevalence of *Campylobacter* in environmental matrices and test the ability of the enrichment-PCR to detect multiple subtypes in a single sample.
- To evaluate the prevalence of *Campylobacter* in chicken products at the point of purchase.
- To determine whether the method is able to detect a wide range of *Campylobacter* subtypes and that certain strains do not predominate using the enrichment-PCR method. River water flowing through farmland is expected to have multiple inputs of campylobacters from varied sources and is chosen as a suitable matrix for this validation. The subtyping method employed is pulsed-field gel electrophoresis (PFGE) using *Sma*I as the restriction enzyme.

3.2 Materials and methods

3.2.1 Media and reagents

Media and Reagents used in this research were prepared as described in Appendices I and II (respectively). Unless otherwise stated, the chemicals used in this methods section were obtained from Sigma (Castle Hill, New South Wales, Australia).

3.2.2 Isolation and identification of campylobacters from whole chicken carcasses

All chicken products were purchased from retail stores as fresh whole chickens over a one year period from retail outlets in a rural town. Six chicken carcasses were sampled fortnightly for the first four months of the project and this number was increased to nine chickens for the remaining eight months. This resulted in 204 chicken carcasses being tested. The chicken carcasses were transported to the laboratory under refrigeration at 4°C and processed by the enrichment-PCR method within 24 hours of purchase. The enrichment-PCR method for chicken carcasses is described in Chapter Two. PCR identification was performed on one purified isolate per chicken carcass.

3.2.3 Collection and processing of river water samples

3.2.3.1 Survey of Campylobacter in river water

The prevalence of *Campylobacter* in river water was determined over a one year period. Three sites along the Ashburton River were sampled. The site below the infiltration gallery (Region A) was sampled every week and two sites (Region B and C) on the Upper Branches of the Ashburton River which were adjacent to farm land were sampled every fortnight (Table 10).

Table 10: Sampling frequency for water from the Ashburton River

Ashburton River Site	Sampling frequency	Number of samples collected	Sampling plan
Region A	Weekly	4	Collected on same day 2 in morning, 2 in afternoon
Region B	Fortnightly	2	Collected on same day 1 in morning, 1 in afternoon
Region C	Fortnightly	2	Collected on same day 1 in morning, 1 in afternoon

The samples were transported to the laboratory under refrigeration at 4°C and processed by the enrichment-PCR method within 24 hours. Water samples were filtered in one litre volumes for all sampling events. Filter papers (HA 0.45 μm filter membranes, Millipore) were put into 100 ml of m-Exeter broth (in Whirl Pak stomacher bags) for one litre volumes. Microaerophilic enrichment conditions and all further processing methods, including controls are as described in Chapter Two for the enrichment-PCR method.

In brief, the primary enrichment was incubated for an initial 4 hours at 37ºC and then incubated for a further 44 hours at 42ºC. All incubations were carried out under microaerophilic conditions to enrich for *Campylobacter*. Forty eight hours after the primary enrichment was incubated, a 100 μl volume of the primary enrichment was transferred to a secondary enrichment of 10 ml of m-Exeter broth. The secondary enrichment was incubated at 42ºC for 24 hours under microaerophilic conditions. After 24 hours a 1 ml volume was removed from the secondary enrichment and washed according to the method for cell harvesting and preparation for the PCR reaction (Chapter Two).

3.2.3.2 Collection and processing of water samples to determine the carriage of multiple subtypes of Campylobacter

The site from which samples were obtained for determination of multiple subtypes was the infiltration gallery that supplies the drinking water for the township of Ashburton. Water samples were collected at 10 a.m. and noon on three separate days within a period of six days. The samples were transported to the laboratory under refrigeration at 4°C and processed by the

enrichment-PCR method within 24 hours. Water samples were filtered in 100 ml volumes and one litre volumes for all sampling events. For two of the samples, 800 ml volumes were filtered instead of 1000 ml. This smaller volume was due to the difficulty of filtering turbid water. Filter papers (HA 0.45 μm filter membranes, Millipore) were put into 25 ml of m-Exeter broth for 100 ml filtered volumes and 100 ml of m-Exeter broth (in Whirl Pak stomacher bags) for one litre volumes. All further processing was as outlined in Section 3.2.3.1 above.

Enrichment broths that gave a positive result for *Campylobacter* by PCR were inoculated onto m-Exeter plates and individual colonies purified by multiple subcultures onto Columbia blood agar (CBA) and incubation at 42°C under microaerophilic conditions. Twelve to fifteen pure colonies were isolated from each water sample and subjected to *Campylobacter* multiplex PCR confirmation. Colonies testing positive for *C. jejuni* by PCR were confirmed by the hippurate hydrolysis method (Lior 1984). For long term storage of cultures, bacterial growth was removed from a 48 hour plate of CBA with a sterile, disposable inoculating loop and suspended in sterile Nunc cryotubes (Milian, Gahanna, Ohio, USA) containing sterilised glass beads with Brain Heart Infusion broth (BHI) supplemented with 20% glycerol. Cryotubes were left at room temperature for half an hour prior to freezing at -80°C.

3.2.4 Subtyping of *Campylobacter* isolates from water by pulsed-field gel electrophoresis (PFGE)

The method used for preparation of the agarose plugs, digestion of plugs and gel electrophoresis was based on the PFGE method for *C. jejuni* (Gibson *et al.* 1994). Modifications to this protocol are described below and included cell density preparation using the McFarland scale (bioMerieux: Cat No. 69280); incubation of plugs overnight to achieve cell lysis, and the use of the Lambda-concatamer PFGE marker (New England Biolabs, Ipswich, Massachusetts) as the electrophoretic molecular weight standard.

3.2.4.1 Preparation of agarose embedded chromosomal DNA

Growth from a 48 hour plate of CBA was collected using a sterile, cotton tipped applicator pre-moistened in Phosphate Buffered Saline (PBS) and resuspended in 2 ml of PBS in a Falcon 2054 tube (Becton Dickinson, Franklin Lakes, NJ, USA).

The turbidity of the cell suspension was adjusted to 1.0 on the McFarland Scale, this equated to resuspending 4-6 colonies of *Campylobacter*. The suspension was transferred into a sterile 1.5 ml Eppendorf tube and centrifuged for 10 minutes at 6,600 x g (4°C). The supernatant was discarded and the pellet resuspended in 1 ml of PBS. A 400 µl aliquot of the cell suspension was transferred to a 1.5 ml tube containing 20 µl of Proteinase K (20 mg ml^{-1}) solution and mixed gently to avoid shearing of the DNA. Molten Megabase agarose (1.4%) (Biorad, Alfred Noble Drive, Hercules, California), equilibrated to 55-60°C, was added to the cell suspension in an equal volume. Gentle mixing was achieved by pipetting two or three times using a 1 ml pipette tip immediately prior to dispensing the mixture into two wells of a plug mold (2 cm x 1 cm x 1.5 mm) (Biorad). Plugs were solidified at room temperature for 15 minutes or at 4°C for 5 minutes.

Solidified plugs were carefully removed from plug molds and placed in 50 ml polypropylene screw-capped Cell-Star tubes (Greiner Bio-One, Longwood, Florida) which contained 5 ml of EC Lysis Buffer and 25 µl of Proteinase K (20 mg ml^{-1}) solution. Tubes were incubated at 55°C overnight.

Following incubation the plugs were initially rinsed in 10-15 ml of sterile Milli-Q water (APS Water Services Corp., Van Nuys, California) preheated to 55°C. This was followed by two 15 minute washes in 10-15 ml of sterile Milli-Q water (55°C) while the tubes were held in a shaking 55°C waterbath.

The next three washes were performed in Tris-EDTA buffer (TE), preheated to 55°C. The tubes were shaken in a 55°C waterbath for 15 minutes. At the completion of washing the plugs were transferred to 2 ml graduated microcentrifuge tubes (Quality Scientific Plastics, Porex, Petaluma, California) containing 1.6 ml of TE buffer (room temperature) and stored at 4°C until required for enzyme digestion.

3.2.4.2 Restriction enzyme digestion of DNA in agarose plugs

Digests of 1 mm slices of *Campylobacter* DNA plugs were performed in 20 Units of *Sma*I enzyme (Roche Diagnostics GmbH, Sandhoferstrasse, Mannheim, Germany) in 100 µl of the appropriate (1x) restriction enzyme buffer (Roche Buffer A). Reactions were incubated at 25°C overnight.

3.2.4.3 PFGE electrophoresis of DNA embedded agarose plugs

Gels were prepared from 1% Megabase agarose (Biorad) in 100 ml of 0.5 x TBE buffer (prepared from 10 x TBE, USB Corp, Cleveland, USA) using 20-well combs. The plug slices of the samples were loaded into the pre-cast agarose wells. Three plugs of the molecular weight standard Lambda-concatamer PFGE marker (New England Biolabs, Ipswich, Massachusetts) were loaded into wells on the two outer most lanes and the centre lane. Plugs in the wells were covered with molten 1% Megabase agarose (Biorad) to prevent movement of the plugs. Electrophoresis was performed in 2.2 litres of 0.5 x TBE running buffer on a CHEF DRIII system (BioRad) with the cooling module set at 14°C. The conditions for each electrophoresis run were a gradient of 6.0 volts cm^{-1}; included angle of 120°, an initial switch time of 10 seconds and a final switch time of 35 seconds, and a running time of 22 hours.

Following electrophoresis, the gels were stained for 20 minutes in 400 ml of Milli-Q water containing 40 µl of 10 mg ml^{-1} ethidium bromide. Gels were destained in 400 ml of Milli-Q water for up to one hour. The electrophoretic image was captured by Polaroid photograph and band patterns were analysed manually.

3.3 Results

3.3.1 Identification of target campylobacters in chicken carcass samples

3.3.1.1 Prevalence of campylobacters in chicken carcasses

C. jejuni was the predominant species identified in chicken carcasses sampled from retail outlets, being detected in 56 of the 204 samples (27.5%), compared with two (1.0%) samples positive for *C. coli*.

3.3.1.2 Seasonality of campylobacters in chicken carcasses

The prevalence of *C. jejuni* in chicken carcasses was highest in the summer, autumn and spring quarters, with lower prevalence over winter (Figure 4). The low numbers of *C. coli* identified in the carcasses precludes representation of the data on a seasonal basis.

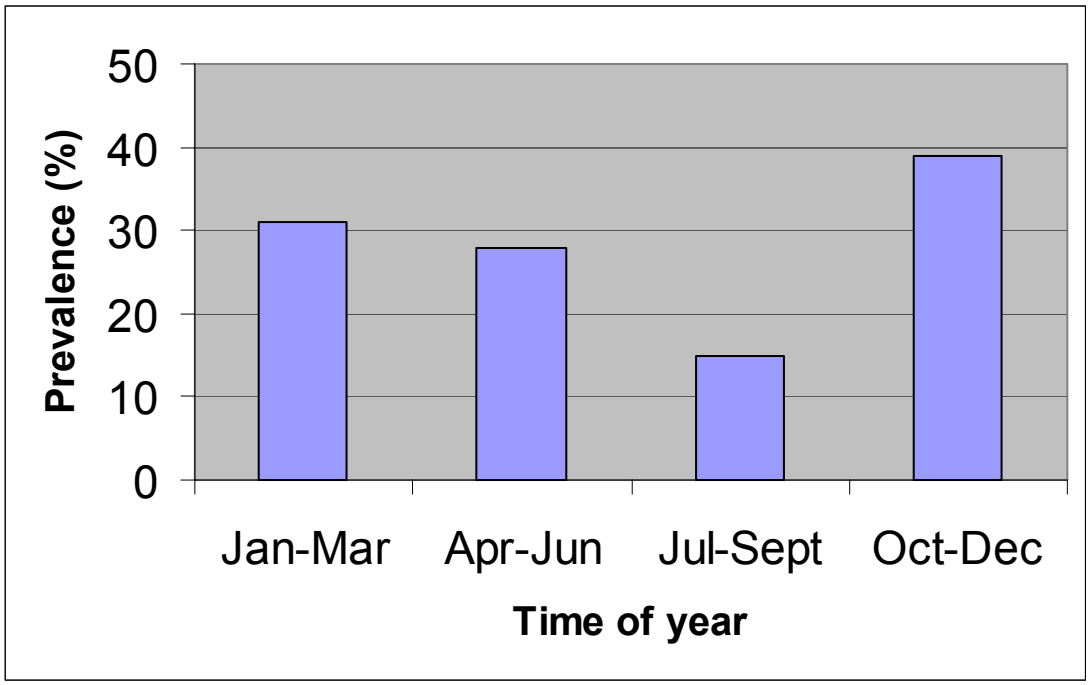

Figure 4: Seasonal prevalence of *Campylobacter* in chicken carcasses

3.3.2 Prevalence of *Campylobacter* in river water

The prevalence of *C. jejuni* and *C. coli* for each of the water sites is shown in Table 11. Region B is the area adjacent to both banks of the South Branch of the Ashburton River and Region C is the area surrounding two tributaries of the South Branch. Region A is below the confluence of the rivers flowing through Regions B and C and is the site of the infiltration gallery for the drinking water supply for the township of Ashburton. All sampling sites are adjacent to areas of sheep and dairy/cattle farming.

Samples of water from the river draining Region B demonstrate a lower prevalence of *C. jejuni* compared with the other two water sampling sites (χ^2, $p<0.0001$). *C. coli* was not isolated from Region B and C water sampling sites which are upstream of the Region A water site. Differences between water prevalence among regions are not significant for *C. coli* (Fisher's exact test, $p=0.46$). The isolation of *C. coli* from Region A water sampling site may be the result of a four fold higher sampling rate between this site and the other two water sites.

Table 11: Prevalence of *Campylobacter* in water from the Ashburton River

Site	% Isolation of *C. jejuni*	% Isolation of *C. coli*	Total sample numbers
Region A	59	6	193
Region B	16	0	50
Region C	60	0	50
Total	55.3	4.1	293

3.3.3 Seasonality of *Campylobacter* prevalence in water

As observed in previous studies (Obiri-Danso and Jones 1999, Brennhovd *et al.* 1992) the prevalence of *Campylobacter* in the river water was highest over the winter months and lower during the summer when water temperatures were highest (Figure 5). As can be seen in Figure 6 the coldest water temperatures in the Ashburton River occurred during the winter months of June-August.

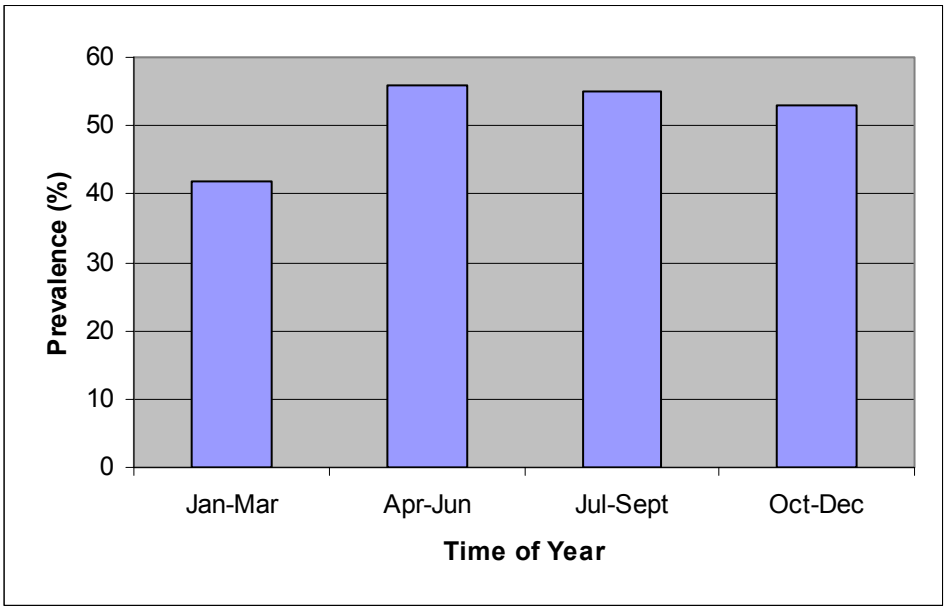

Figure 5: Seasonal trends for *Campylobacter* prevalence in river water

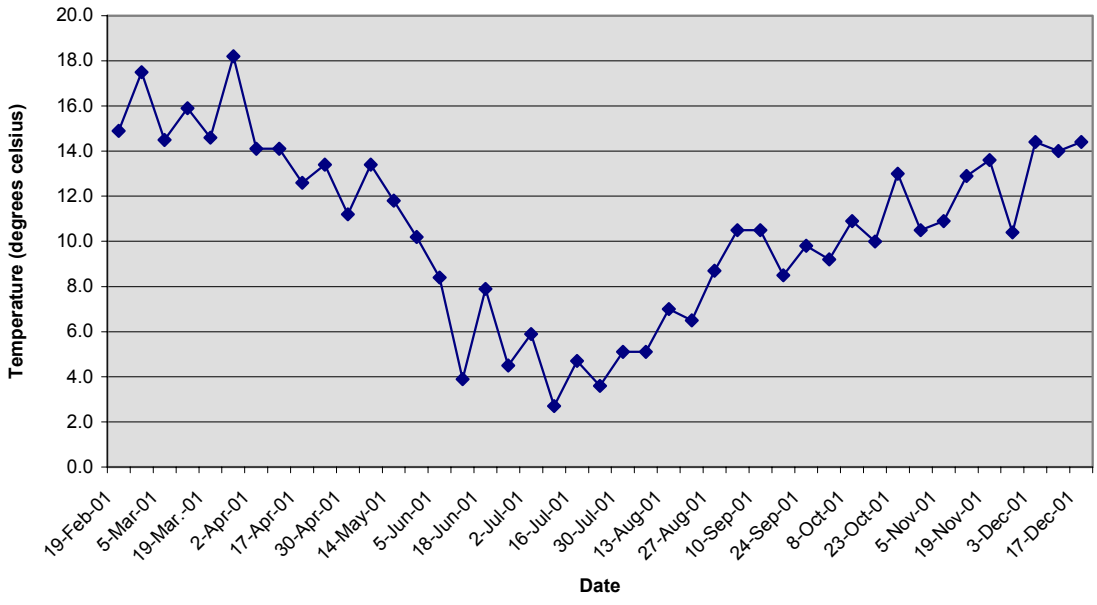

Figure 6: Seasonal variation of water temperatures in the Ashburton River

3.3.4 Detection of multiple *Campylobacter* subtypes in individual samples

Results for the identification of campylobacters in water samples from the Ashburton River are presented in Table 12. Three of the six 100 ml water samples were positive for *C. jejuni.* In contrast, five of the six 800 ml to one litre water samples were positive for *C. jejuni*. *C. coli* was not identified in any water sample. Two samples tested positive for a thermophilic *Campylobacter* species which was not identified as either *C. jejuni* or *C. coli*. Therefore, no further isolation of bacteria was performed on these samples.

Five of the six samples (83%) collected at 10 a.m. of each sampling day were positive for target campylobacters. This is in comparison to the samples collected at noon of each sampling day, where three of the six (50%) water samples were positive.

The number of colonies purified from the water samples varied between 12 and 15 colonies per sample. Seven PFGE *C. jejuni* subtypes were identified from the five *C. jejuni* positive water samples (Table 13). Three of the seven PFGE *C. jejuni* subtypes were identified on only one sampling occasion. No individual subtype was identified in more than two water samples. At least two subtypes were identified in every *Campylobacter*-positive sample and in one sample three subtypes were identified.

One PFGE subtype of *C. jejuni* was dominant in each sample (presented in boldface in Table 13) ranging from 87% to 93% of the total number of colonies. Of the four subtypes that were identified in two samples, three of the four were dominant in one sample and then detected as the minor subtype in the other sample. The fourth subtype (PFGE 7) was a minor subtype in both samples. Three subtypes were identified only once in a sample. Two of these subtypes (PFGE 3 and 6) were identified as the dominant subtype in their respective water samples.

Table 12: Identification of campylobacters isolated from the Ashburton River

Sample number	Time of sampling	Volume of water filtered	*Campylobacter* identified
299	10 am	100 ml	*C. jejuni*
		800 ml	*C. jejuni*
300	noon	100 ml	*C. jejuni*
		800 ml	*C. jejuni*
301	10 am	100 ml	Thermotolerant *Campylobacter*
		1000 ml	*C. jejuni*
302	noon	100 ml	Negative
		1000 ml	Thermotolerant *Campylobacter*
305	10 am	100 ml	*C. jejuni*
		1000 ml	*C. jejuni*
306	noon	100 ml	Negative
		1000 ml	*C. jejuni*

Table 13: *C. jejuni* PFGE subtypes identified in Ashburton river water

Water Sample	*Sma*I PFGE subtype	Number of isolates
299	**1**	13
	7	1
300	**2**	12
	4	1
301	**4**	12
	2	1
305	**6**	11
	7	1
306	**3**	13
	5	1
	1	1

3.4 Discussion

3.4.1 The prevalence of *Campylobacter* in chicken produce

It is important to validate the enrichment-PCR method by testing it on environmental samples. To this end, whole chicken carcasses were sampled at the point of purchase to the consumer. Sampling from retail outlets allowed a comparison of rates of *Campylobacter* isolation with previous New Zealand and international studies.

The prevalence of *C. jejuni* in fresh chicken was lower in this study than has been observed in previous New Zealand studies where the prevalence was found to range between 54 to 60% (Anonymous *et al.* 1999, Hudson *et al.* 1999, Campbell and Gilbert 1995). International studies, however, have shown a wide variation of prevalence of campylobacters in chicken products. A study of the prevalence of *Campylobacter* in raw retail meats in the USA identified 71% of chicken carcasses (n = 184) as being contaminated with *Campylobacter* (identified by PCR as either *C. jejuni* or *C. coli*) (Zhao *et al.* 2001). A United Kingdom study found that 77% of chicken portions (thigh and breast) (n= 198) were contaminated with *C. jejuni* and 7% were contaminated with *C. coli* (Kramer *et al*. 2000). In studies discussed by Stern (1992) *C. jejuni* was isolated from chicken carcasses at rates from 48 to 98%, although one study from the Netherlands yielded a low 16% of *C. jejuni* isolates.

Low prevalences were reported in a study from Finland where *C. jejuni* was isolated from chicken portions purchased from retail shops over a three-year period concentrating on the months, June to September, when cases of campylobacteriosis were at their peak (Hänninen *et al.* 2000). In the first year of sampling 13 % (n = 80) of chicken portions were positive, 14% (n = 206) positive in the second year of study and 21% (n = 243) in the third year. This study used enrichment in Lab M broth prior to culture on charcoal-cefoperazone-desoxycholate agar (CCDA) for the isolation of *C. jejuni* from chicken. A lower prevalence was also found in a Danish study when campylobacters were isolated from 35% of raw chicken products (n = 1096) (Anonymous, 2002).

Reasons for the lower prevalences reported in this study compared to previous New Zealand studies include differences in isolation methods. Other New Zealand studies (Hudson *et al.* 1999, Campbell and Gilbert 1995) used Preston as the enrichment broth and plated from the

broth onto selective agar plates to identify *Campylobacter* colonies. Another consideration is that whole chicken carcasses were sampled in this study and by Campbell and Gilbert (1995), in contrast to the chicken portions surveyed in the New Zealand study of Hudson *et al.* (1999). It is probable that the extra handling and manipulation required for the preparation of chicken portions may increase the likelihood of cross-contamination resulting in higher prevalence of campylobacters. This reasoning is supported by a recent New Zealand survey of *Campylobacter* contamination in retail chickens that had been minced, diced or supplied in strips (Wong *et.al*. 2005). The survey employed the same m-Exeter enrichment method as developed for the present study except that enrichment samples were plated out to determine positive campylobacters and then their identity was confirmed by the PCR assay developed in this enrichment-PCR method. These chicken samples (n = 230) in Wong *et.al*. (2005) were collected over a one year period from the nation's five main cities and reported a prevalence of 89%, with most of these isolates (>86.5%) being *C. jejuni*. In comparison, the whole chicken carcasses surveyed in this study were obtained from the two chicken brand names that had the highest volume of sales from retail outlets in the test area. It was subsequently discovered that both brands of chickens were from the same supplier, who is a nationwide provider of chicken products with one of the higher proportions of the market share. It may be that this large company has lower rates of *Campylobacter* contamination due to an improvement in controls during processing. Another possibility is that differences in sampling geography affect prevalence whereby localised survey results are compared with results from nationwide surveys.

3.4.2 The detection of *Campylobacter* in river water samples

Overall prevalence of *C. jejuni* isolated from the Ashburton River was 55.3% and 4.1% for *C. coli*. Water samples showed slightly lower prevalences in the summer period as compared with winter prevalence (Figure 5) and this can be seen to correlate with river temperatures. The annual temperature variations of the Ashburton River, as measured at Region A water sampling site, are presented in Figure 6. The temperatures varied from a summer high of 18°C in March to a wintertime low of 3°C in July. Seasonal variation of *Campylobacter* prevalence is usually observed in water samples due to differences in sunlight and water temperature, with isolations of *Campylobacter* spp. being more frequent in winter (Brennhovd *et al.* 1992).

A New Zealand study of the prevalences of *Campylobacter* in river water during the summer (n = 48) and winter periods (n = 36) showed lower prevalence in summer months (31%) in

comparison to the winter (75%) (Hudson *et al.* 1999). Another New Zealand survey of *Campylobacter* in river water identified prevalences of 60% for thermotolerant *Campylobacter* collected over a six month period (spring and summer) (Savill *et al.* 2001). Evaluation of the concentration of *Campylobacter* by the most Probable Number (MPN) technique identified a median range of 0.18 MPN 100 ml^{-1} and a range of <0.12 - >11 MPN 100 ml^{-1} in the river waters (Savill *et al.* 2001).

International studies have identified thermotolerant *Campylobacter* prevalences in surface waters at 74% (n = 69) (Sails *et al.* 2002); 53% (n = 60) (Rosef *et al.* 2001); 41% (n = 119) (Kemp *et al.* 2005); 44% (n = 96) (Brennhovd *et al.* 1992) and 17% (n =139) from a study of surface waters in Finland (Horman *et al.* 2004). Therefore the prevalences reported for *Campylobacter* in this study concur with previous studies.

In this study, *C. coli* was rarely identified in the water samples. Previous studies using culture and enrichment methods of campylobacters in surface waters have reported lower levels of detection of *C. coli* in contrast to *C. jejuni* (Horman *et al.* 2004, Brennhovd *et al.* 1992, Bolton *et al.* 1987). These data are supported by laboratory studies which report that *C. jejuni* survived longer in culturable form than *C. coli* in lake water incubated at 4°C and 20°C (Korhonen and Martikainen 1991). In contrast, higher prevalences of *C. coli* compared to *C. jejuni* were detected in studies where detection was also based on culture (Kemp *et al.* 2005, Sails *et al.* 2002, Rosef *et al.* 2001).

3.4.2.1 The detection of multiple subtypes of Campylobacter in river water samples

To test the ability of the method to enrich for multiple subtypes river water was chosen as a matrix that had a high probability of containing multiple *Campylobacter* subtypes in a single grab sample. This premise was based on the potential multiple inputs of *Campylobacter* e.g. from animal and bird faeces, that are received by a river system. The Ashburton River in Canterbury, New Zealand, was chosen as a suitable surface water for sampling as the river passes through densely farmed areas where sheep and cattle graze alongside the river and there are considerable populations of wild birds and feral animals in the surrounding environment.

The results suggest that sampling a larger volume (800 ml-one litre) of water is more likely to identify target campylobacters. The samples were collected on three different days over a six day period. On each sampling day, water was collected at 10 a.m. and noon to see if the time of

sampling affected the survival of *C. jejuni* and *C. coli*, as it is known that ultraviolet irradiation can be detrimental to *Campylobacter* survival in water (Obiri-Danso *et al.* 2001). Fifty percent of the samples collected at noon were positive for *C. jejuni*, this compares with 83% of the samples collected at 10 a.m. The small sample size, however, precludes statistical analysis of the findings. Analysis of results from a larger sample size for both time periods and water volumes would be required before confirming the suggestion that collectors should avoid sampling at times of the day when UV radiation is expected to be at its highest.

In all of the samples that tested positive for *C. jejuni* (5 of 6), more than one subtype was identified, with a maximum of three subtypes per sample. One subtype was dominant in each sample but importantly, each subtype that was identified as being >85% of the typed colonies was also identified as a minor subtype (6.7-8.3%) in another sample. This suggests that these subtypes are not selected preferentially over other strains and that the method allows for the detection of a wider range of subtypes when they are present. Whether a particular strain is dominant in an individual sample may be dependent on factors which include its ability to compete with other campylobacters based on its genomic repertoire of survival strategies (Moen *et al.* 2005, Murphy *et al.* 2003). Another factor is its concentration in the water at the time of sampling, including the numbers of injured cells present for that particular strain. The intermittent pattern of subtypes detected is not unexpected since many of the strains could be derived from direct/indirect input of faeces from the farm animals and wildlife that inhabit the fields surrounding the river system.

It is recognised that the problem of dominant subtypes may be overcome by employing more than one enrichment procedure for each sample to enable identification of individual subtypes that vary under different growth conditions (Kramer *et al.* 2000). One of the aims of this method development, however, was to enable the implementation of large surveys to better understand the transmission of *Campylobacter* through the environment to humans. The use of a single method for all matrices would allow cost effective identification of pathogenic campylobacters in a wide range of environmental matrices and facilitate a high sampling frequency within a routine laboratory set up.

The variation of PFGE subtypes identified in the study suggest that sampling at different times on one day will lead to the isolation of different *C. jejuni* subtypes. It also suggests, however,

that multiple sampling over an extended period has the potential to provide a better representation of *C. jejuni* strains present in a river system.

3.4.3 Conclusions

The prevalences of *C. jejuni* and *C. coli* in retail chickens were lower than that obtained from previous studies in New Zealand but are similar to international studies in Denmark, Finland and the Netherlands. Differences between the New Zealand studies may be due to this survey obtaining retail chickens from a major supplier for the country who is expected to have improved techniques which minimise the contamination of chicken carcasses during processing. Another point of difference is that this survey was conducted on whole fresh chickens rather than chicken portions, which due to the increased handling, might be expected to be exposed to more cross contamination events than the prepackaged whole chicken.

The prevalence of *C. jejuni* in river water was similar to international findings and previous studies of New Zealand rivers. The low prevalence of *C. coli* although supported by some international literature, is also in contrast to other overseas studies where *C. coli* has been identified at higher prevalences than *C. jejuni*. The identification of *Campylobacter* followed seasonal trends with lower prevalence in warmer summer water compared with winter conditions.

The results of this validation supported the proposal that the enrichment-PCR method is facilitating the identification of different *C. jejuni* PFGE subtypes and indicates that an overlap of *C. jejuni* subtypes will be identified when sampling at different time periods. It is a reasonable assumption, therefore, that the isolation regime of selecting one colony per sample over an extended temporal survey, as compared with typing multiple isolates from fewer samples, will reveal a variation of *Campylobacter* subtypes. This would result in an accurate assessment of the *Campylobacter* types present in an environmental matrix.

The greater the number of *Campylobacter* subtypes detected, the higher is the likelihood of establishing potential transmission routes of *C. jejuni* and *C. coli* from the environment to humans. This assessment of single colonies per sample may not hold true when investigating outbreak cases of campylobacteriosis, as will be examined in the following chapter.

In conclusion, this work supports the hypothesis that a robust enrichment-PCR assay has been developed to detect and identify pathogenic *Campylobacter* from chicken and water samples. Identification of multiple subtypes of *C. jejuni* in a river water sample validates the ability of this method to confirm or repudiate the third hypothesis for this current study that chicken carcasses carry multiple subtypes of the pathogenic species *C. jejuni*.

4 Multiple subtypes of *C. jejuni* in chicken carcasses

4.1 Introduction

As discussed in the previous chapter, the isolation of one colony from environmental samples is a valid strategy when testing large numbers of samples over an extended time period. However, when investigating outbreak cases of campylobacteriosis single isolates from a suspected source may not provide an accurate assessment of the presence of pathogenic subtypes. The distribution of *C. jejuni* subtypes in the environment may be more accurately represented if dominant and minor subtypes from potential reservoirs were isolated from the same sample. Recognition of the importance of multiple subtypes of campylobacters present in the same sample is beginning to emerge (Schouls *et al.* 2003, Dickins *et al.* 2002, Jørgensen *et al.* 2002, Newell *et al.* 2001). In general, these studies on *Campylobacter* have tested low numbers of colonies (3-5) per sample. They have, also, not presented statistical justification to establish the number of colonies required to determine the probable proportions of multiple subtypes present in a single sample.

4.1.1 The relevance of multiple subtypes of bacterial species in individual samples.

The relevance of multiple subtypes of a bacterial species in individual samples is illustrated by the following investigation into an outbreak of *E. coli* O157:H7 associated with ground beef. Analysis identified more than one subtype of *E. coli* O157:H7 in the single meat package recovered for testing (Proctor *et al.* 2002). From the nine patients tested for *E. coli* O157:H7 four genetically distinct subtypes of *E. coli* O157:H7 were identified, but epidemiological evidence strongly suggested that these cases were all linked. The authors highlight that based on the genotyping evidence, not all patients would have been linked to the same outbreak event as only six of the nine cases were identical to one of the two isolates from the meat package. The second isolate from the meat sample was unrelated to any of the four subtypes identified in the human faecal specimens. If this subtype was the only one isolated from the meat sample, then the importance of the ground beef as the source of the outbreak would have been unconfirmed. Proctor *et al.* (2002) concluded that based on the evidence from previous studies (Besser *et al.* 1997, Faith *et al.* 1996), which have recognised distinguishable but related PFGE patterns when

isolating *E. coli* O157:H7 from the faeces of the same cow, it is important to test multiple isolates from the same sample in order to detect all of the subtypes involved in an outbreak.

These findings have relevance for the investigation of campylobacteriosis cases. It is well documented that multiple subtypes of *C. jejuni* may be circulating on one farm (Hiett *et al.* 2002, Thomas *et al.* 1997) and some studies have observed multiple *C. jejuni* subtypes present in the same faecal sample from a human case (Steinbrueckner *et al.* 2001, Richardson *et al.* 2001). Therefore the identification of only one isolate per sample limits the information available for tracing the source of a campylobacteriosis incident. As most cases are recognised as being sporadic (Pebody *et al*. 1997), this information may suggest that at least some of these sporadic outbreaks are not recognised as being linked because not all of the available subtypes have been identified from either the clinical faecal specimens or the suspected source of the outbreak.

4.1.1.1 Identification of multiple subtypes in chicken carcasses

While previous studies have shown that flocks of chickens may carry multiple subtypes (Nadeau *et al.* 2002, Newell *et al.* 2001), there is mounting evidence that individual chickens also harbour multiple subtypes of *C. jejuni*. Schouls *et al.* (2003) performed a pilot experiment to assess the number of multiple subtypes that could be isolated from three chickens. Four colonies from a caecal sample of each chicken were isolated and typed by three different genotyping techniques. Three of the four colonies from each faecal sample were identified as *C. jejuni*, with the other colonies being identified as *C. coli.* Typing data revealed that all three *C. jejuni* isolates from an individual chicken were of different subtypes.

Newell *et al.* (2001) conducted research in a chicken abattoir which tracked the changes in composition of *C. jejuni* subtypes isolated from chicken carcasses as the individual carcasses travelled through the processing plant. Their research indicated that some of the *C. jejuni* subtypes identified in the bird prior to slaughter were not present in the carcass after processing and that other *C. jejuni* subtypes predominated after carcasses had travelled through the processing plant. Therefore, the dominant subtype detected in the final product from the abattoir may represent a *C. jejuni* subtype that was not identified in the initial flock prior to slaughter. This included subtypes identified in processed carcasses when the initial flock had tested negative for *Campylobacter* prior to slaughter or transport to the abattoir. The authors suggest

that changes in subtype composition in carcasses may be due to environmental selection pressures on the *C. jejuni* subtypes. Certain subtypes may be capable of better survival in the abattoir environment than the subtype identified in the original flock.

The study of Newell *et al.* (2001) would have benefited from a more rigorous statistical methodology in establishing the presence/absence of subtypes within individual birds. For example, only one or two colonies from the caeca of each individual bird (n = 10) were analysed for each flock prior to slaughter. This number of colonies would not be sufficient to fulfil the criteria for establishing the presence of minor subtypes in the caeca where high numbers of *Campylobacter* reside. A minor subtype is defined as a clone that represents <10% of typed isolates in a sample (Schlager *et al.* 2002). This survey of the subtypes colonizing a flock is highly significant to the rest of the study where subtypes isolated in the subsequent processing procedures are compared with the initial subtypes identified in the flock. The method used to determine the subtypes in the original flock must be robust to ensure the identification of the majority of subtypes present. If this is not the case then it calls into question the conclusion that subtypes identified in the original flock are replaced by more environmentally stable subtypes as the carcass progresses through the abattoir.

4.1.2 Statistical significance of multiple subtypes of bacterial species present in individual samples

In a study of *E. coli* subtypes in the human faeces, Schlager *et al.* (2002) have discussed the importance of multiple isolations from a single stool sample to identify both dominant and minor subtypes. They caution that basing a study on the isolation of dominant subtypes of a bacterial species may exclude significant information, especially where the study is examining the frequency of virulence factors in a bacterial population. Identification of minor subtypes from chicken carcasses may contribute to the knowledge about potential host specificity of *C. jejuni* subtypes and whether all subtypes identified in chickens contribute to the pathogenesis of humans. Schlager *et al.* (2002) describe a dominant subtype as a clone which is represented by >50% of typed isolates in a sample. A minor subtype is defined as a clone that represents <10% of typed isolates in a sample. They used a binomial formula to determine the number of randomly selected colonies required to achieve a 90% probability of identifying a minor clone: $1-(1-p)^n$, where p is the frequency of the minor clone (ie $p = 0.10$) and n is the number of colonies picked.

4.1.3 *Campylobacter* subtype prevalence in chicken farms

It is generally thought that although *Campylobacter* is known to be a genetically polymorphic organism (Parkhill *et al.* 2000), an individual chicken flock is colonised by only one or two subtypes within a *Campylobacter* species (Ayling *et al.* 1996). Examples of this include a high genetic diversity of *Campylobacter* subtypes reported from a survey of the caecal contents of 2,325 broiler chickens in Quebec, Canada (Nadeau *et al.* 2002). PFGE profiling identified 49 distinct genotypes in 56 of the positive lots. Despite this genetic diversity, the majority (76.8%) of these lots were colonised by a unique genotype, suggesting a single source of infection. A similar result was found using AFLP and RFLP typing of the flagellin gene (refer to Appendix IV) during a survey of 100 chicken farms in Switzerland. Despite an overall high genetic diversity of *C. jejuni* and *C. coli*, the study found that, in general, isolates from a single farm had an AFLP similarity level of >97% (Wittwer *et al.* 2005). Conversely, a study in the United States identified multiple clones of *Campylobacter* (up to six per farm) (Hiett *et al.* 2002). The study used *fla*A (flagellin gene) short variable region typing to distinguish subtypes. This finding was further supported by a Danish study which used genotyping (*fla*-typing and PFGE) and serotyping to identify up to 3 clones in broiler flocks and up to six clones in parent flocks supplying the broiler farms (Petersen *et al.* 2001b).

4.1.4 PFGE subtyping techniques

Researchers have suggested that indistinguishable patterns based on PFGE can only be ascertained with the employment of a minimum of two restriction enzymes (Singer *et al.* 2004). Comparisons by various researchers of the profile groups obtained with *Sma*I and other restriction enzymes, including *Kpn*I and *Sal*I have concluded that *Sma*I accurately determines *C. jejuni* subtype relationships (Fitzgerald *et al.* 2001b, On *et al.* 1998). However, they and other researchers have noted that some isolates giving the same profile for *Sma*I digestion could be further subdivided by the use of a second restriction enzyme (Lindmark *et al.* 2004, Damborg *et al.* 2004). It has been suggested that the use of two restriction enzymes is potentially useful in population studies where isolates are collected over a period of one year or longer (Tenover *et al.* 1995).

The two restriction enzymes most widely used to determine PFGE patterns of *C. jejuni* isolates are *Sma*I and *Kpn*I. A comparison of the two enzymes by researchers in Quebec, revealed that *C. jejuni* clusters that were epidemiologically related to one another correlated better with PFGE data and showed a higher diversity when based on *Kpn*I digestions rather than the restriction enzyme *Sma*I (Michaud *et al.* 2001). This observation was further supported by other studies (Hald *et al.* 2004a), including a study of human cases of campylobacteriosis in Christchurch, New Zealand, where the researchers identified 71 different *Kpn*I patterns for 183 *C. jejuni* isolates compared with 57 patterns when using *Sma*I as the restriction enzyme (Gilpin *et al.* 2006) When combined the results from these two restriction enzymes produced 77 *Sma*I/*Kpn*I profiles.

4.1.5 Standardisation of PFGE between laboratories

Pulsenet is a USA-based National Molecular Typing Network for Foodborne Disease Surveillance which was established by the National Centre for Infectious Diseases and the Centers for Disease Control and Prevention (CDC). A paper published by Pulsenet (Ribot *et al.* 2001) investigated the reproducibility of *Sma*I PFGE protocols for the subtyping of *C. jejuni*. The aim was to determine standardised protocols which would produce high quality interlaboratory comparisons of data. These protocols allow for rapid comparison of DNA fingerprints of *C. jejuni* isolates from geographically dispersed laboratories to enhance the national surveillance of foodborne diseases. In this survey, five independent laboratories typed the same seven isolates and gel image results were compared using computer-assisted analysis. In each case there was a perfect match between the PFGE patterns for each of the isolates, indicating the reproducibility and utility of this method. Pulsenet use the restriction enzyme *Sma*I as its primary enzyme for PFGE but acknowledge that a secondary enzyme can be useful for further discriminatory power where the results with *Sma*I are inconclusive.

It is expected that the importance of data exchange between international laboratories will increase as global epidemiological studies are undertaken to detect emerging infectious diseases and changes in disease aetiology (Woodward and Rodgers 2002, Wassenaar and Newell 2000, Olive and Bean 1999, Stephens and Farley 1996). It is recommended that this important aspect of data exchange be taken into consideration when adopting a particular methodology. The Pulsenet protocol for *Campylobacter* PFGE analysis was adopted in this section of the thesis, based on the reproducibility of results and the ability to exchange data between international

laboratories. In this section the PFGE molecular weight marker used as the standard on each of the agarose gels was *Salmonella* Braenderup H9812, which has recently been verified by Pulsenet as a universal standard for PFGE electrophoresis for all of their bacteria of interest (Hunter *et al.* 2005).

4.1.6 The stability of the *Campylobacter* genome in relation to genotypic subtyping methods

An important aspect of a subtyping system is its stability over an extended period of time. *Campylobacter* is a naturally competent bacterium (Duim *et al.* 1999) which means it is able to take up foreign DNA from its surrounding environment and incorporate the DNA into its own genome. This natural competence, as well as internal rearrangements of the genome may be important for increasing an organism's ability to survive within a changing environment (Manning *et al.* 2001). This is important for *Campylobacter* which can be isolated from a wide range of diverse habitats. Genetic instability could undermine the applicability of genetic subtyping. For example, RFLP subtyping of the *Campylobacter* flagellin gene locus has demonstrated hypervariable regions that are subject to recombination events (Harrington *et al.* 1997). Ideally, genetic subtyping methods need to target highly conserved genes with a low frequency of recombination. Methods, such as PFGE, which use the entire genome are inherently more stable than those which focus on one or two genes (Wassenaar and Newell 2000).

A Danish study compared serotypes, flagellin RFLP (*fla* type) and PFGE subtypes of *C. jejuni* isolates from broiler flocks, humans, wild animals and birds (Petersen *et al.* 2001a). The isolates were collected over a three-year period and a wide geographical area within Denmark. Comparison of the PFGE profiles produced by three different restriction endonucleases identified clonal lineages that had been genetically stable over long time periods (e.g. two and a half years) and wide geographical ranges (within Denmark). Studies by Manning *et al*. (2001) have demonstrated longer-term genetic stability of environmental isolates collected over a two month period in 1998, which clustered with human isolates from an outbreak in 1981. The related human and environmental isolates had the same PFGE subtype when cut with three different endonucleases. Furthermore, AFLP subtyping demonstrated 90% genetic homology between the same isolates. One of the isolates from the human waterborne outbreak was subsequently used as an international standard strain for *C. jejuni*. It showed the same genotypic

stability even though it had been subcultured frequently and showed reduced colonisation potential when used for infection studies of chickens. The presence of stable *Campylobacter* subtypes in a diverse range of habitats was supported by a United Kingdom study which conducted a three year survey of isolates from the farm environment and identified many genotypes continuously over the length of the study (Fitzgerald *et al.* 2001b). From these data Manning *et al.* (2001) have proposed that "genome shuffling may not be as essential for *Campylobacter* stress adaptation as previously thought" and that these mechanisms need further investigation but do not undermine the usefulness of genotyping, at least for short term epidemiological studies. Both of these studies confirmed the use of genotyping techniques such as PFGE for the investigation of complex epidemiologies.

4.1.6.1 The genetic diversity of C. jejuni

The genetic diversity attributed to the *Campylobacter* genome is due to a high frequency of both intra and inter-species recombination in *C. jejuni* (Schouls *et al*. 2003). This polymorphic nature of *Campylobacter* subtypes (Duim *et al*. 1999) adds to the complexity when trying to deduce the source of an infection.

The sequencing of the *C. jejuni* genome revealed that it contained hypervariable regions associated with genes known to encode the biosynthesis of modification of surface structures (Parkhill *et al.* 2000) which maybe important in mechanisms to evade the host immune system. This finding is supported by data from a meta-analysis study which used comparative genomic hybridisation to analyse the genetic diversity between *C. jejuni* isolates from dissimilar geographical and epidemiological backgrounds (Taboada *et al.* 2004). The study found that 36.6% of genes (n = 1,597) were variable in at least one of the 97 strains analysed. Analysis of the non-variable genes and those in which the sequence varied in only one of the 97 strains showed that 78.6% of the genes showed a high degree of intraspecies conservation and therefore probably form the core set of genes required by *C. jejuni*. This suggests that overall the *C. jejuni* chromosome has a low level of genome plasticity.

Most of the variable genes mapped to 16 defined hypervariable regions, which included functionally related groups of genes associated with lipopolysaccharide biosynthesis, capsular polysaccharide biosynthesis and flagellar biosynthetic loci. The heterogeneity in cell surface

structures conferred by these divergent loci is recognised as being important for adaptation of *C. jejuni* to different host environments.

From these studies it would appear that the PFGE subtype of *C. jejuni* isolates is relatively stable in the environment and although genomic rearrangements can occur in a population it is a rarer event than generally accepted (Manning *et al.* 2001) being confined to approximately one third of the genome that enables the pathogen to adapt to a variety of host habitats.

4.1.7 Objectives

- To determine if chicken carcasses carry multiple subtypes of *C. jejuni* by analysing isolates using PFGE typing with the restriction enzyme *Sma*I and based on the protocol implemented by Pulsenet, USA. To confirm that isolates of the same *Sma*I PFGE subtype are truly indistinguishable, *Kpn*I will be used as the secondary enzyme.
- To determine the prevalence of non-dominant clones of *C. jejuni* in chicken carcasses, by subtyping a maximum of 25 isolates from each of ten chicken carcasses. Based on the binomial formula used by Schlager *et al.* (2002), 25 isolates per sample would have a 93% probability of identifying a minor clone.
- To determine whether isolates from a single chicken carcass are related or distinct subtypes.

4.2 Materials and methods

4.2.1 Media and reagents

Media and Reagents used in this research were prepared as described in Appendices I and II (respectively). Unless otherwise stated, the chemicals used in this methods section were obtained from Sigma (Castle Hill, New South Wales, Australia).

4.2.2 Characterisation of *C. jejuni* from chicken carcasses

Twenty-five isolates from ten samples that had tested positive for *C. jejuni* only, in the chicken carcasses in Chapter Three were selected for further purification and subtyping by PFGE analysis. Purification of isolates was achieved by two subcultures on CBA agar and isolates were prepared for long term storage. Bacterial growth was removed from a 48 hour plate of CBA with a sterile, disposable inoculating loop and suspended in sterile Nunc cryotubes (In Vitro Technologies, Victoria, Australia) containing sterilised glass beads with Brain Heart Infusion broth (BHI) supplemented with 20% glycerol. Cryotubes were left at room temperature for half an hour prior to storage at -80°C.

4.2.2.1 Colony identification by PCR

Identification of bacterial colonies by PCR directly from plate cultures, was achieved by removal of a portion of a single isolated colony and resuspension in 27.0 µl of ddH_2O in a 0.5 ml thin-walled PCR tube. Within 15 minutes from the time of colony resuspension, the tube was heated for 3 min at 100°C and then cooled to 4°C. Prepared premix was added to the PCR tube to obtain a final volume of 50 µl. PCR analysis was performed as outlined in Chapter Two. PCR confirmation was performed on up to five isolates per PFGE subtype identified in each chicken carcass (Dickins *et al.* 2002).

4.2.2.2 Colony identification by biochemical analysis

Phenotypic characterisations of up to four isolates per PFGE subtype identified in each chicken carcass were subjected to confirmatory identification by the following biochemical tests.

Positive controls of *C. jejuni* (ERL96 3376) and *C. coli* (ERL 97/454) were included for all tests. Table 14 outlines the biochemical tests used to differentiate between thermotolerant *Campylobacter* and related organisms (Barros-Velázquez *et al.* 1999, Griffiths and Park 1990).

Table 14: Biochemical identification of the thermotolerant *Campylobacter*

Biochemical Test	*C. jejuni*		*C. coli*	*C. lari*	*C. upsaliensis*
	subsp. *jejuni*	subsp. *doylei*			
Gram Stain	-	-	-	-	-
Hippurate hydrolysis	+	+	-	-	-
Catalase production	+	+	+	+	-/W
Oxidase production	+	+	+	+	+
Nitrate reduction	+	-	+	+	+
Nalidixic acid resistance	S	S	S	R	S
Cephalothin resistance	R	V	R	R	S
Growth at 25°C	-	-	-	-	-
Aerobic growth	-	-	-	-	-

W: weak; V: variable; S: sensitive; R: resistant

4.2.2.2.1 Gram stain, colony and bacterium morphology

Gram stain, colony and bacterium morphology were determined on 48 hour cultures on CBA plates.

4.2.2.2.2 Oxidase test

The oxidase reaction was determined using the Dry Slide™ system (Becton Dickinson, Sparks, Maryland, USA).

4.2.2.2.3 Catalase Test

The catalase reaction was performed by addition of a colony portion to hydrogen peroxide droplets on a slide. Production of bubbles indicated a positive catalase reaction.

4.2.2.2.4 Hippurate hydrolysis

Hippurate hydrolysis was performed by the method of Lior (1984). Briefly, a large 10 µl loopful of the presumptive *Campylobacter* was incubated in 1.0% sodium hippurate at 37°C for 2 hours. Following incubation, 5 drops of ninhydrin reagent (stain dropper, Difco, BBL, Becton Dickinson) were added to the tube and incubation continued for a further 10 minutes. The appearance of a purple colour indicates hippurate hydrolysis (Lior 1984).

4.2.2.2.5 Antibiotic susceptibility

Sensitivity to nalidixic acid and resistance to cephalothin was confirmed by placing 30 µg antibiotic discs (BD, BBL, Sensi-Disc, Becton Dickinson) onto CBA plates inoculated by swabbing from a broth culture of the test isolate. Incubation of plates was performed microaerophilically at 42°C for 48 hours.

4.2.2.2.6 Aerobic growth

Aerobic growth was determined by inoculation of CBA plates and incubation at 25°C for one week. This test eliminated the misidentification of *Arcobacter* species, which are able to grow aerobically at 25°C, but are otherwise biochemically similar to the thermotolerant campylobacters.

4.2.3 Pulsed-field gel electrophoresis (PFGE)

The method used for preparation of the agarose plugs, digestion of plugs and gel electrophoresis employed a 24 hour methodology for the rapid analysis of gram negative microbes (Ribot *et al.* 2001) and is the method recommended by PulseNet, USA, except for the Megabase agarose, for which Pulsenet now recommends Seakem Gold agarose (FMC BioProducts, Rockland, Maine) (Swaminathan *et al.* 2001). The PulseNet protocol is presented below.

4.2.3.1 Preparation of agarose embedded chromosomal DNA

Campylobacter growth from a 48 hour plate of CBA was collected using a pre-moistened (in PBS) sterile, cotton tipped applicator and emulsified in 2 ml of PBS in a Falcon 2054 tube (Becton Dickinson).

The turbidity of the cell suspension was adjusted to 0.35-0.45 on the digital output of a Dade Microscan Turbidity Meter (Dade Behring, West Sacramento, California). A 400 µl volume of the cell suspension was transferred to a 1.5 ml Eppendorf tube containing 20 µl of Proteinase K

(20 mg ml^{-1}) solution and mixed gently. Molten Megabase agarose (1.4%) (Biorad, Alfred Noble Drive, Hercules, California), equilibrated at 55-60°C was added to the cell suspension in an equal volume. Gentle mixing was achieved by pipetting two or three times using the 1000 µl pipette tip, immediately prior to dispensing the mixture into two wells of a plug mould (2 cm x 1 cm x 1.5 mm, Biorad). Plugs were solidified at room temperature for 15 minutes or at 4°C for 5 minutes.

Solidified plugs were carefully removed from plug moulds and placed in 50 ml polypropylene screw-capped tubes Cell-Star tubes (Greiner Bio-One) containing 5 ml of EC Lysis Buffer and 25 µl of Proteinase K (20 mg ml^{-1}) solution. Tubes were incubated in a shaking waterbath (175-200 rpm) at 55°C for one hour.

Following incubation the plugs were rinsed in 10-15 ml of sterile Milli-Q water preheated to 55°C. This was followed by two 15 minute washes in 10-15 ml of sterile Milli-Q water (55°C) where the tubes were held in a shaking 55°C waterbath.

The next three washes were performed in TE buffer, preheated to 55°C. The tubes were shaken in a 55°C waterbath for 15 minutes. At the completion of washing, the plugs were transferred to 2 ml graduated microcentrifuge tubes (Quality Scientific Plastics) containing 1.6 ml of TE buffer (room temperature) and stored at 4°C until required for enzyme digestion.

4.2.3.2 Restriction enzyme digestion of DNA in agarose plugs

4.2.3.2.1 SmaI restriction enzyme digestion

Digests of 1 mm slices of *Campylobacter* DNA plugs were performed in 40 Units of *Sma*I enzyme (Roche) in 100 µl of the appropriate (1x) restriction enzyme buffer (Roche Buffer A). Reactions were incubated at 25°C for 2-4 hours. Digests of the PFGE molecular size standard: *Salmonella* Braenderup H9812, were performed in 30 Units of *Xba*I enzyme (Roche) in 100 µl of the appropriate (1x) restriction enzyme buffer (Roche Buffer H) (Hunter *et al.* 2005). Reactions were incubated at 37°C for 2-4 hours.

4.2.3.2.2 KpnI restriction enzyme digestion

A subset of the 25 isolates from each chicken sample (refer Section 4.2.3.2.3) were restricted with a second enzyme *Kpn*I (New England Biolabs (NEB), Hitchin, United Kingdom) to establish if the isolates, which were indistinguishable from each other when digested with *Sma*I, showed differing DNA fingerprints with digestion in *Kpn*I.

Digests of 1 mm slices of *Campylobacter* DNA plugs were performed in 40 Units of *Kpn*I enzyme (NEB) in 100 µl of the appropriate (1x) restriction enzyme buffer (NEB Buffer A). Reactions were incubated at 37°C for 1 hour. The preparation of the PFGE molecular size standard, *Salmonella* Braenderup H9812, was performed as outlined in Section 4.2.3.2.1 above.

4.2.3.2.3 Statistical calculation of sample size for KpnI digestion

Due to budget and time constraints not all of the 25 isolates from each chicken sample were analysed by *Kpn*I digestion and electrophoresis. A statistical method termed Sampling Attribute Plan was employed to calculate the sample size required to determine 95% confidence levels in the results (Speck 1984).

This analysis determined that if a sample of ten isolates from a batch of 25 indistinguishable *Sma*I subtypes was tested and identified, and none of the ten isolates produced a different *Kpn*I profile then there would be 95% confidence that the batch of 25 will have no more than eight isolates (0-30.8%) that have a different *Kpn*I profile (Table 18, Appendix III). If the sample number is increased to 15 from the batch of 25 indistinguishable *Sma*I subtypes, and none of the 15 isolates produced a different *Kpn*I profile then there would be 95% confidence that the batch of 25 will have no more than five isolates (0-21.8%) with a different *Kpn*I profile. Based on these confidence levels, 15 from each batch of 25 isolates were digested with *Kpn*I enzyme to determine the presence of non-dominant PFGE subtypes.

4.2.3.3 PFGE electrophoresis

Gels were prepared from 1% Megabase agarose (Biorad) in 100 ml of 0.5 x TBE buffer (prepared from 10 x TBE, USB Corp, Cleveland, USA) using 20-well combs (BioRad). The

plug slices of the samples and standards were loaded into the pre-cast agarose wells and electrophoresed in 2.2 litres of 0.5 x TBE running buffer on a CHEF DRIII system (BioRad) with the cooling module set at 14°C.

4.2.3.3.1 Running conditions for electrophoresis of SmaI restriction enzyme digested plugs

The conditions for each electrophoresis run were a gradient of 6.0 volts cm^{-1}; included angle of 120°, an initial switch time of 6.8 seconds, a final switch time of 38.4 seconds and a running time of 18 hours. Please note that these conditions are different from the running conditions used in Chapter Three for the water isolates of *C. jejuni*.

4.2.3.3.2 Running conditions for electrophoresis of KpnI restriction enzyme digested plugs

The conditions for each electrophoresis run were a gradient of 6.0 volts cm^{-1}; included angle of 120°, an initial switch time of 5.2 seconds, a final switch time of 42.3 seconds and a running time of 19 hours.

4.2.3.3.3 Staining of gels

Following electrophoresis, the gels were stained for 20 minutes in 400 ml of Milli-Q water containing 40 µl of 10 mg ml^{-1} of ethidium bromide. Gels were destained in 400 ml of Milli-Q water for up to 1 hour.

4.2.3.3.4 Bionumerics software analysis

The electrophoretic image was captured with a Gel Doc 2000 gel documentation system (BioRad), and band patterns analysed and compared using BioNumerics software (Applied Maths, Kortrijk, Belgium). Only fragments in the range 700 to 50 kb were analysed for *Sma*I and 700 to 80 kb fragments were analysed for *Kpn*I (Michaud *et al.* 2005). Smaller fragments were not consistently resolved. The analytic parameters used were in accordance with the PulseNet, USA standard procedure and used the band-based Dice similarity coefficient and the unweighted pairs geometric matched analysis (UPGMA) dendrogram type with a position tolerance setting of 1.5% for optimization and position tolerance of 1.5% band comparison. All test isolates were normalized to the known molecular size bands of the *Salmonella* Braenderup

H9812 standard subtype (Hunter *et al.* 2005). In addition, all gels were run with a well-characterised subtype of *C. jejuni*: CPH011453 which acted as a control for the digestion reaction for both restriction enzymes.

4.2.3.3.5 Determination of related PFGE subtypes

The following section describes the criteria employed in this study to determine if two isolates, with similar but distinguishable PFGE subtypes, were clonally related.

Definition of "clonally related"

Tenover *et al.* (1995) described clones as genetically related isolates which are:

> isolates that are indistinguishable from each other by a variety of genetic tests (e.g., PFGE and ribotyping) or that are so similar that they are presumed to be derived from a common parent 'Given the potential for cryptic genetic changes detectable only by DNA sequencing or other specific analyses, evidence for clonality is best considered relative rather than absolute' (Eisenstein 1989).

Interpretative criteria for determining relatedness between isolates have been proposed by Tenover *et al.* (1995) for outbreaks of pathogens. It is more difficult, however, to apply these criteria over the time period of longer-term studies. Ribot (2002) suggests that studies which collect samples over a period of more than one year require careful interpretation of results. It is recognised that there are differences in genome stability between pathogenic species. For example, *Escherichia coli* O157:H7, is considered a highly clonal organism and has a stable genome and therefore single band differences may signal unrelatedness (Ribot 2002). This is in contrast to *C. jejuni* which is now regarded as genetically diverse with a high frequency of DNA recombination events within and between organisms (de Boer *et al.* 2002). Therefore one to three PFGE band differences may be interpreted as signaling a degree of relatedness. Ribot (2002), however, cautions against the over interpretation of results and stresses the importance of epidemiological information to confirm linkages.

The isolates for this study were collected over a seven month sampling period and therefore analysis of related PFGE subtypes was based on a conservative interpretation of the criteria developed by Tenover *et al*. (1995). All subtypes defined as clonally related had to have information on both restriction enzyme profiles, *Sma*I and *Kpn*I, which suggested that both

profiles were either indistinguishable or clonally related within their respective RE digestion profile. PFGE subtypes were considered to be "clonally related" when:

i) The subtypes digested with the same enzyme differed by one band shift which indicated a single genetic event had occurred resulting in a DNA fragment running as a larger or smaller band due to either an insertion or deletion of DNA (respectively).

ii) A large molecular weight band was replaced by two smaller molecular weight bands, the sum of whose DNA approximated the original larger molecular weight band. This change in PFGE pattern using the same RE, represents a single genetic event, indicative of the gain of a new restriction site resulting in the formation of two new bands, and the loss of the larger molecular weight band.

4.3 Results

4.3.1 Results of PFGE analysis of *C. jejuni* isolates from chicken carcasses

Ten of the *C. jejuni*-positive chicken carcasses (Chapter Three) were further analysed for multiple subtypes of *C. jejuni*. This was achieved by selecting 25 isolates derived from a single chicken sample enrichment cultured on m-Exeter agar. Initially, a single isolate from a batch was confirmed as *C. jejuni* by PCR analysis (Chapter Two). Following identification of indistinguishable PFGE profiles from the same chicken sample up to five isolates per PFGE profile (Dickins *et al.* 2002) were subjected to multiplex PCR (Chapter Two) and biochemical confirmation of *C. jejuni* (Table 14). These isolates were identified as *C. jejuni* by the multiplex PCR and biochemical tests. All tested isolates were gram negative, oxidase, catalase and hippurate positive, grew at 42°C microaerophilically but not at 25°C aerobically, and were nalidixic acid susceptible and resistant to cephalothin.

Attrition due to isolate death during storage (maximum of a three year period) reduced the real number of isolates per sample to a range of 21 to 25, with a mean of 22.7 analysed by *Sma*I PFGE per sample. Based on the binomial formula of Schlager *et al.* (2002), this would achieve a 91% probability of identifying a minor clone for an average of 23 isolates per sample and a 89% probability of detection of minor clones if 21 isolates per sample were analysed (Section 4.1.2 above).

To differentiate isolates further, and confirm whether isolates with indistinguishable *Sma*I profiles were of the same subtype, *Kpn*I digest was performed on a subset of these isolates. A minimum of 15 isolates per chicken carcass sample was subjected to *Kpn*I digestion and electrophoresis.

4.3.1.1 Characterisation of PFGE profiles

Employing *Sma*I as the initial restriction enzyme revealed that four of the ten chickens carried two subtypes of *C. jejuni*, while only one *C. jejuni* subtype per carcass was identified in the remaining six (Table 15) (Figure 7). Further differentiation by *Kpn*I digestion showed that one more chicken carcass was carrying multiple subtypes. Isolates from chicken sample CPH012693 did not cut with *Sma*I, but digestion with *Kpn*I revealed three distinguishable

subtypes (Figure 8), two of which were clonally related (refer to Section 4.2.3.3.5 above). CPH012077 carried two subtypes of comparable prevalences, which looked visually similar by *Sma*I digestion but were revealed as clonally unrelated by the many band differences in the *Kpn*I profile (Figure 9). Chicken sample CPH0111167 carried two clonally related *Sma*I *C. jejuni* subtypes (Figure 10) and the *Sma*I subtype Sm0106 was further differentiated into two clonally related *Kpn*I subtypes (Figure 11) suggesting three different, but closely related, subtypes were carried by the carcass (Table 15).

Overall five of the ten chicken carcasses carried more than one subtype of *C. jejuni* (Figure 7). In total, 15 distinguishable subtypes were identified from the ten carcasses, and this included the identification of subtype Sm0030/Kp0056 in three different chicken samples. Sample CPH014912 as seen in Figure 7 carried a band at approximately 25 kb which was present intermittently, suggesting it may have been a plasmid. As *Sma*I profiles were analysed between 700 and 50 kb only, the intermittent nature of this DNA band did not affect interpretation of the subtype. In the five chicken carcasses that were identified with multiple subtypes, two subtypes were present in three carcasses and three subtypes present in two carcasses.

C. jejuni CPH011453 was included on gels as a standard for the digestion reaction and replicate profiles of this strain were related at the 99.99% level for *Sma*I digests and 94.7% level for *Kpn*I digests. The cutoff for determining an indistinguishable strain was set at 90% (Nadeau *et al.* 2003, de Boer *et al.* 2000). From Table 15 it can be seen that the cutoff value for each subtype in a sample ranged between 95.00 - 99.99% for *Sma*I digests with an average cutoff of 99.57%. For *Kpn*I digests the range was 95.24 - 99.99% with an average cutoff of 98.08%. Thus technical reproducibility was high.

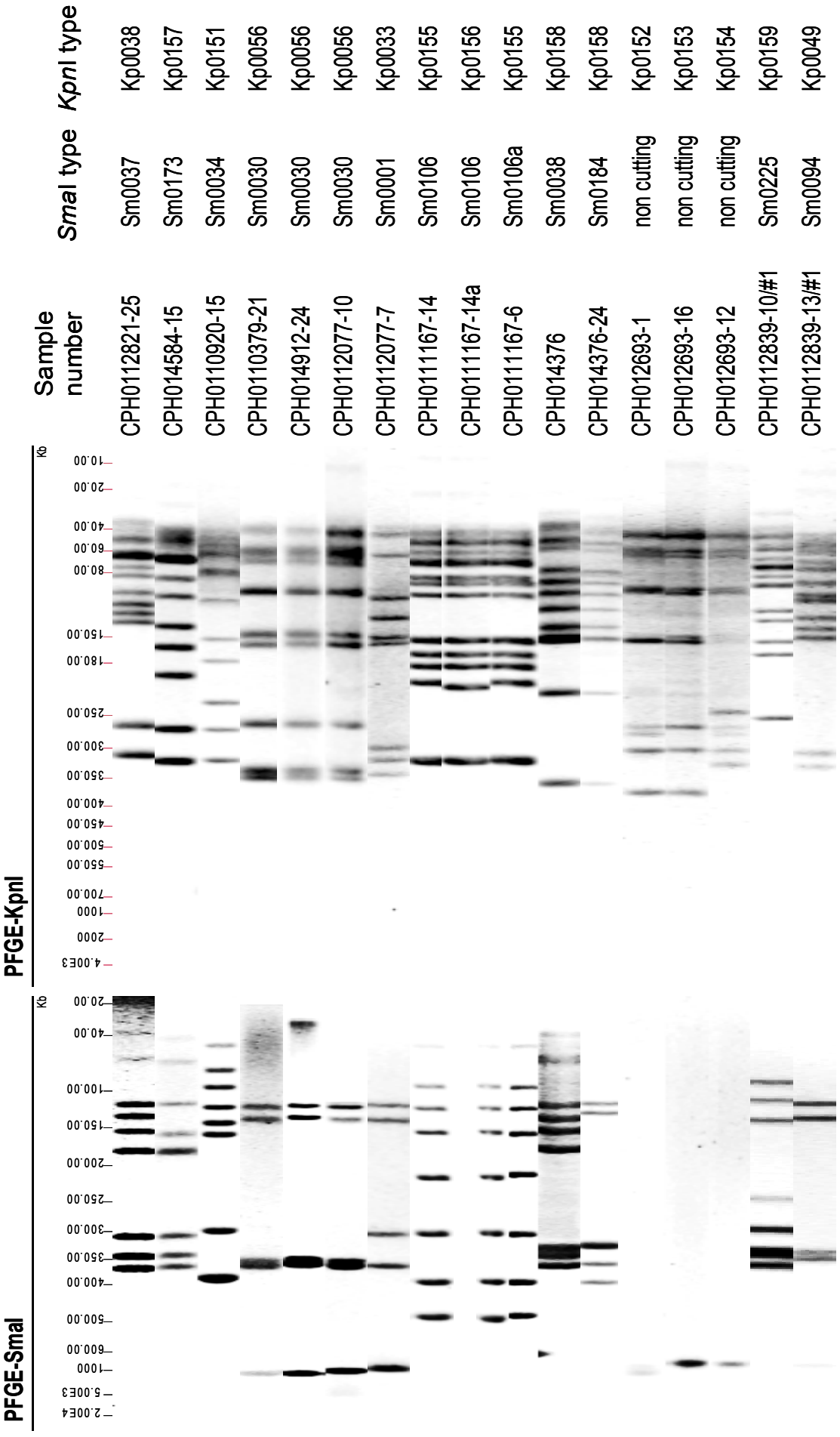

Figure 7: Subtypes of *C. jejuni* isolated from ten chicken carcass samples

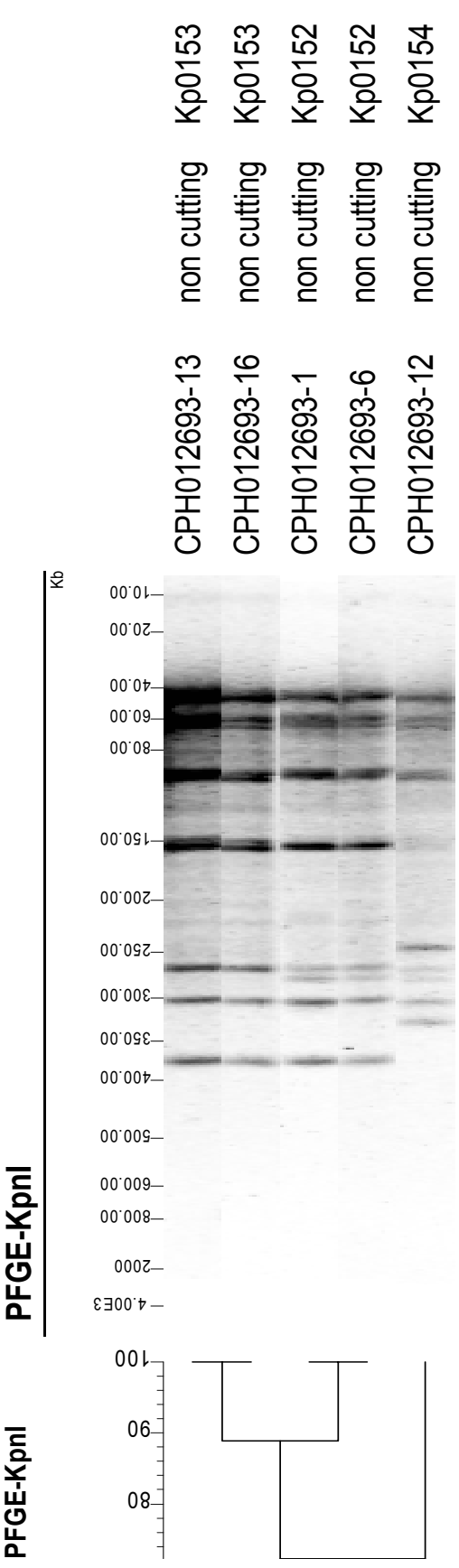

Figure 8: *Kpn*I profiles of CPH012693 isolates

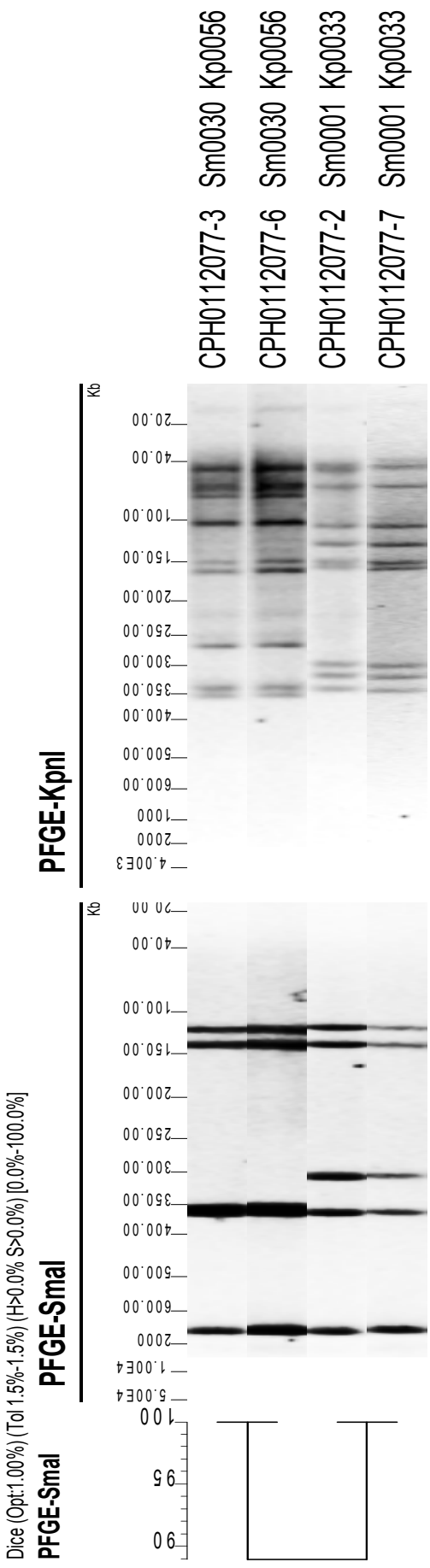

Figure 9: *Kpn*I digestion of visually similar *Sma*I subtypes

Table 15: Subtypes of *C. jejuni* identified in each chicken sample

Chicken isolate	*Sma*I type	Cutoff for *Sma* I pattern (%)	*Kpn*I type	Cutoff for *Kpn* I pattern (%)
Chicken samples carrying single subtypes				
CPH0112821	Sm0037 n = 21	99.99	Kp0038 n = 15	95.70
CPH014584	Sm0173 n = 22	99.99	Kp0157 n = 16	95.24
CPH0110920	Sm0034 n = 25	99.99	Kp0151 n = 15	99.99
CPH0110379	Sm0030 n = 21	99.99	Kp0056 n = 15	99.99
CPH014912	Sm0030 n = 23	99.99	Kp0056 n =15	99.99
Chicken samples carrying multiple subtypes				
CPH0112077	Sm0030 n = 12	99.99	Kp0056 n = 8	99.99
	Sm0001 n = 11	99.99	Kp0033 n = 8	99.99
CPH0111167	Sm0106 n = 18	99.99	Kp0155 n = 13	96.00
	Sm0106 n = 2	99.99	Kp0156 n = 2	99.99
	Sm0106a n = 1	99.99‡	Kp0155 n = 1	96.00†
CPH014376	Sm0038 n = 1	99.99*	Kp0158 n = 1	96.00
	Sm0184 n = 23	99.99	Kp0158 n = 16	96.00
CPH012693	Non cutting n = 25	NA	Kp0152 n = 14	99.99
			Kp0153 n = 2	99.99**
			Kp0154 n = 1	NA* unique Kpn
CPH0112839	Sm0225 n = 5	99.99	Kp0159 n = 5	96.30
	Sm0094 n = 17	95.00	Kp0049 n = 10	96.01

NA = not applicable to calculate cutoff point

NA* = not applicable to calculate cutoff point as a single isolate of this subtype

‡ 99.99% similarity to Sm0106 (refer to text for explanation)

†96.00% cutoff point determined by comparison with other Kp0155 profiles ie subtype Sm0106/Kp0155

* cutoff point determined by comparison with other *C. jejuni* from different samples with same PFGE profile

**94.1% similarity to Kp0152

4.3.1.2 Dominant versus minor subtypes in the same chicken sample

Table 16 outlines the percentage of minor, intermediate and dominant subtypes identified in each of the five carcasses carrying multiple subtypes. The percentages calculated in the first three samples were based on the total number of isolates per sample because the *Kpn*I digestion did not further differentiate the *Sma*I subtype. In contrast, the last two samples contained subtypes that were further resolved by *Kpn*I digestion and therefore the percentage calculations were based on the number of isolates subjected to *Kpn*I digestion only (n = 17 and 16 respectively). The exception was minor subtype Sm0106a/Kp0155 where the calculation was based on the total number of Sm0106 isolates (n = 21).

Based on the definition of dominant and minor subtypes (>50% and <10% of typed isolates in a sample, respectively) proposed by Schlager *et al.* (2002), each chicken carcass carried one dominant subtype. Two samples (CPH0112839 and CPH0112077) carried another subtype that would be described as intermediate in prevalence. The dominant subtype in CPH0112077 (Sm0030/Kp0056) was also identified as the only subtype harboured by two other chicken carcasses. Sample CPH014376 carried a minor and a dominant subtype, while CPH012693 and CPH0111167 each carried a minor and intermediate subtype as well as the dominant subtype.

Table 16: Dominant versus minority subtypes in the same chicken sample

Sample number	Subtype designation		Total isolate numbers per sample	Number of each subtype	% subtype
	*Sma*I type	*Kpn*I type			
CPH0112839	Sm0225	Kp0159	22	5	23
	Sm0094	Kp0049		17	77
CPH0112077	Sm0001	Kp0033	23	11	48
	Sm0030	Kp0056		12	52
CPH014376	Sm0038	Kp0158	24	1	4
	Sm0184	Kp0158		23	96
CPH012693	Non-cutting	Kp0154	17	1	6
		Kp0153		2	12
		Kp0152		14	82
CPH0111167	Sm0106	Kp0156	16	2	13
	Sm0106	Kp0155		14	87
	Sm0106a	Kp0155	21	1	5

4.3.1.2.1 Genetic relatedness of isolates from the same chicken carcass

Comparison of the *Kpn*I digests of isolates from sample CPH012693 identified two genotypes (Kp0152 and Kp0153) which were likely to be clonally related due to a one band difference between the isolates (Figure 8). Profile Kp0154, although looking superficially similar to Kp0152 and Kp0153, had too many different bands from either subtype (5 and 4 band differences, respectively) to be considered clonally related by the criteria of Tenover *et al.* (1995) outlined in Section 4.2.3.3.5.

The benefits of a double RE digestion were revealed in Figure 9, where the *Sma*I fingerprint for the two subtypes isolated from sample CPH0112077 suggested that the two subtypes were visually similar and could be clonally related. The large 370 kb band in subtype Sm0030 could have been due to the co-migration of two DNA fragments of a similar molecular weight. It was possible that the appearance of the approximately 300 kb band in subtype Sm0001 was due to a single deletion event in one of the approximately 370 kb bands in Sm0030. Further discrimination was revealed by the *Kpn*I digest, however, and the patterns of the two isolates no longer suggested that they were clonally related.

Chicken sample CPH0112839 contained two distinct subtypes as distinguished by *Sma*I and *Kpn*I PFGE profiles (Figure 7). One isolate from sample CPH014376 had the same unique *Kpn*I profile as the other isolates in the same sample, but a different *Sma*I pattern (Sm0038). Therefore sample CPH014376 carried two distinct subtypes, which based on the dissimilarity of the *Sma*I profiles could not be described as clonally related (Figure 7).

Sample CPH0111167 contained one isolate out of 21 that was a minor subtype by *Sma*I digestion (Figure 10) and two isolates out of 16 that were an intermediate subtype by *Kpn*I digestion (Figure 11). In both cases, these subtypes were likely to be clonally related to the dominant subtype as they had a single band difference. These band shifts were not seen as differences by the parameters used for computer analysis as all minor subtypes were 99.99% similar to the dominant subtype. However when isolates were run together on the same gel these band shifts were clearly differentiated (Figure 10 and Figure 11). Based on the data from this study the direction of genetic change between subtypes could not be confirmed, however it was reasonable to assume that the band differences were due to single deletion or insertion events in the slightly higher molecular weight (MW) band seen in the dominant subtypes.

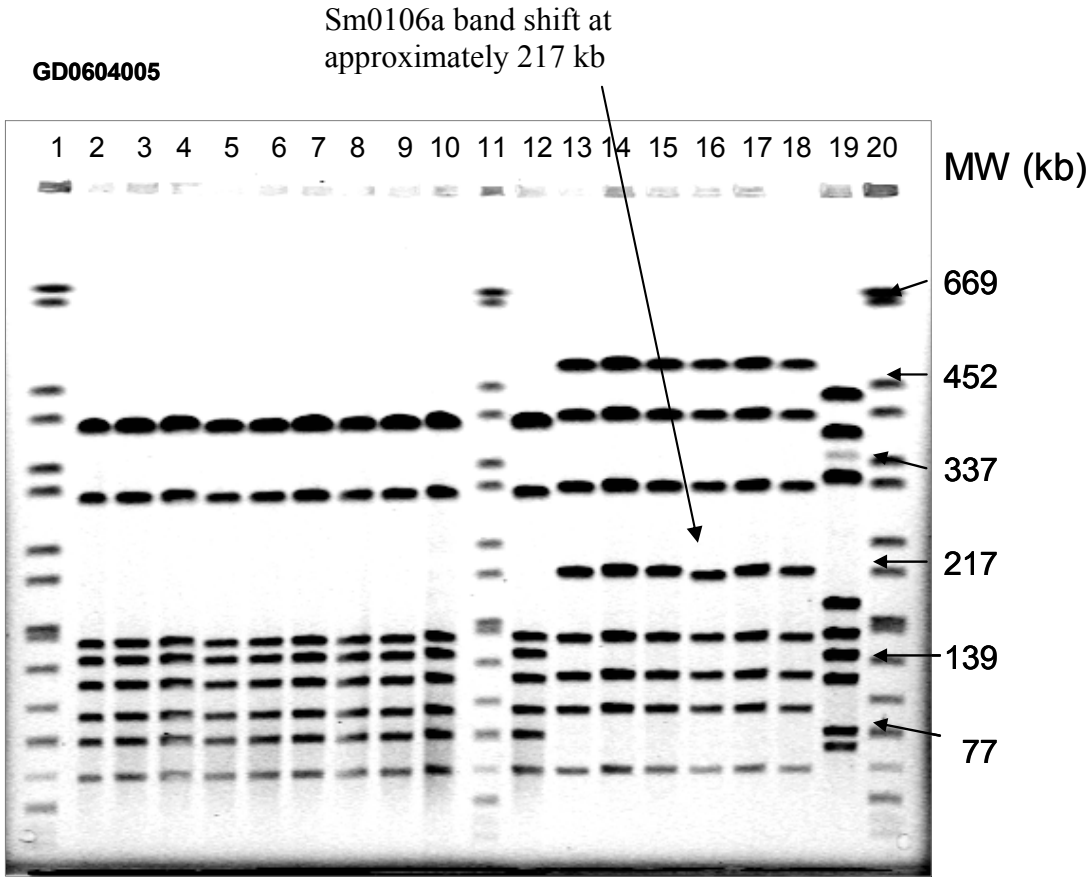

Figure 10: Clonal isolates from chicken carcass CPH0111167 with different *Sma*I profiles

Lanes 1, 11, 20, *Salmonella* Braenderup H9812 molecular weight marker;

Lanes 2-10, 12 isolates from sample CPH0110920

Lanes 13-18 isolates from sample CPH0111167; Lane 16, Sm0106a, band shift at approximately 217 kb band; Lane 19 digestion standard CPH011453.

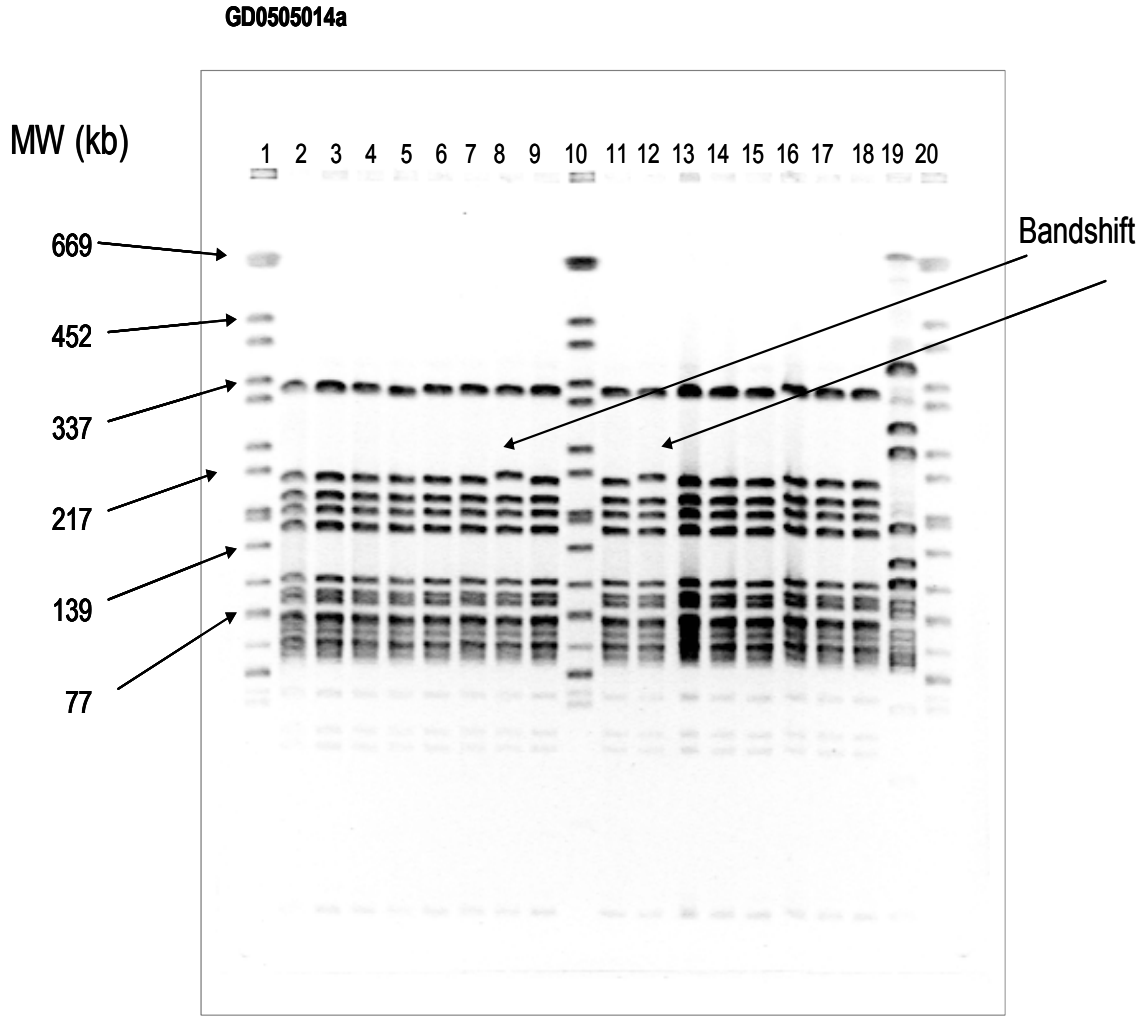

Figure 11: Clonal isolates from chicken carcass CPH0111167 with different *Kpn*I profiles

Lanes 1, 10 and 20, *Salmonella* Braenderup H9812 molecular weight marker;

Lanes 2-18 isolates from sample CPH0111167;

Lanes 8 and 12 band shift; Lane 19 Digestion Standard CPH011453.

4.4 Discussion

4.4.1 Multiple *Campylobacter* subtypes identified in studies of chicken matrices

The study by Wassenaar *et al.* (1998) highlighted the unusual occurrence where 14 clonally related subtypes of *C. jejuni* were identified from 30 packs of chicken (n = 21 isolates in total) collected sequentially after packaging. Although not isolated from an individual chicken, these subtypes were identified from chicken products derived from the same source farm and highlighted the extensive recombination events that can occur within a flock, leading to minor variations in PFGE patterns even though isolates were identified as having the same serotype and *fla* type. Due to the high level of clonality observed between isolates it is unlikely that their presence on the chicken products was caused by cross-contamination during processing. Therefore if such variation can be identified within a batch of chicken products then the variations in related PFGE patterns of subtypes isolated from an individual chicken is not unexpected.

In this study, multiple subtypes were isolated from five of ten chicken carcasses with a maximum of three subtypes isolated from an individual carcass on two occasions (CPH0112693 and CPH0111167). All three subtypes carried by sample CPH0111167 and at least two subtypes identified in CPH012693 had similar PFGE patterns suggestive of a clonal relationship between subtypes from the same carcass. The other three chicken samples that harboured multiple subtypes all contained two subtypes that were genetically distinct from each other.

An investigation of *Campylobacter* contamination of raw meat and poultry utilised both direct plating and enrichment methods for the isolation of campylobacters (Kramer *et al.* 2000). Thirty-five samples (17.7%) of *Campylobacter*-positive chicken pieces contained multiple subtypes of *C. jejuni* as determined by a combination of serotyping, phage typing and antibiotic resistance typing. Multiple subtypes of *C. coli* were not identified in the chicken portions. Selection of multiple isolates was based on a single colony from both the direct and the enrichment plates and further colonies were isolated from either method based on differing colony morphology.

In another study, 70 different PFGE patterns were identified from 59 *Campylobacter*-positive chicken carcasses by a direct plating method (Dickins *et al.* 2002). An average of 6.2 isolates was obtained from each carcass with a range of 1 to 10 isolates per carcass. In the carcasses (n = 39) where only *C. jejuni* was identified, 33% harboured two subtypes, 10% each harboured three and four subtypes, and one carcass (2.6%) harboured five subtypes. For the carcass where five subtypes were identified the subtypes could be split into three groups with two groups containing clonally related PFGE types. Another study analysed the caecal swabs of three chickens and identified four *Campylobacter* isolates from each chicken by direct plating onto an unspecified agar (Schouls *et al.* 2003). Three *C. jejuni* isolates were identified from each chicken and the typing data revealed that each chicken carried three different subtypes of *C. jejuni* as determined by genotyping techniques that included MLST and AFLP.

Lindmark *et al.* (2004) carried out a pilot study using enrichment of chicken carcass rinses in Preston broth at 42°C under microaerophilic conditions to determine the number of genotypes of *C. jejuni* carried by a chicken carcass. Two to five *C. jejuni* colonies from each chicken carcass (n = 10) were subtyped by PFGE revealing that all isolates from an individual carcass belonged to the same PFGE subtype.

None of the research papers discussed above selected enough colonies for subtyping to meet the criteria of Schlager *et al.* (2002) who used a binomial formula to determine the number of randomly selected colonies required to achieve a 90% probability of identifying a minor clone (refer 4.1.2 above). The higher numbers of subtypes of *C. jejuni* identified from individual chicken carcasses by some researchers may be dependent on the method of isolation e.g. direct plating (Schouls *et al.* 2003, Dickins *et al.* 2002) versus the enrichment-PCR method developed here. Although the Schouls *et al.* (2003) paper only tested four isolates from three chicken faecal samples and Dickins *et al.* (2002) tested 72 carcasses with an average of 6.2 colonies from each carcass, both papers report a higher number of multiple subtypes from an individual sample compared to the enrichment-PCR method, where an average of 23 colonies were tested per carcass. Comparisons between carcasses and faeces, however, are invalid due to the higher numbers of *Campylobacter* routinely encountered in faeces compared to carcasses.

The enrichment step employed in this assay was designed to detect only viable *C. jejuni* and *C. coli* cells as well as aiding the recovery of sublethally injured bacteria from stressful environments such as food surfaces. The enrichment procedure, however, may allow selection

of rapidly growing *Campylobacter* strains which outcompete slower growing strains that otherwise may be detected by direct plating (Dickins *et al.* 2002). To obtain a better representation of the true population of strains harboured on chicken carcasses and other food matrices, it maybe necessary to utilise both an enrichment and a direct plating method (Kramer *et al.* 2000). Furthermore, improvement of recovery of injured strains in the broth enrichment may be enhanced by delayed addition of antibiotics (Humphrey 1986).

4.4.1.1 Minor and dominant subtypes in a sample

The results of subtyping multiple isolates of *C. jejuni* in chicken carcasses are presented in Table 16. Samples CPH014376, CPH0111167 and CPH012693 contained minor subtypes which have a lower probability of being isolated compared with dominant subtypes when less than five colonies per sample are isolated. This could also hold true for those "intermediate" subtypes (Sm0225/Kp0159, NC/Kp0153, Sm0106/ Kp0156) that constitute less than 25% of the isolates in their respective samples. Sample CPH0111167 carried three subtypes that had single small insertions/deletions of DNA. Although visually similar, these differences involve DNA of several thousand base pairs and could relate to different phenotypic characteristics between the subtypes which may be important in determining virulence and thus pathogenicity potential. The relevance of minor and intermediate subtypes to human cases of campylobacteriosis will be further investigated in Chapter Five.

4.4.1.1.1 Clonal relationships between C. jejuni subtypes from the same chicken carcass

In this study, recognition of the clonal relationship between different isolates from the same chicken carcass led to the question of stability of *C. jejuni* subtypes during colonisation of the chicken intestine. In three of the five chicken carcasses that contained multiple subtypes, the subtypes were not clonally related. In comparison, the sample CPH0112693 contained a mixture of related (NC/Kp0152 and NC/Kp0153) and unrelated subtypes (NC/Kp0154), whereas sample CPH0111167 carried three subtypes with minor PFGE variations (Figures 8, 10 and 11).

Previous studies have reported varying degrees of genetic instability during both *in vivo* and *in vitro* experiments as outlined below. The genomic instability observed by Wassenaar *et al.* (1998) in 21 clonally related *C. jejuni* subtypes derived from a single batch of processed poultry was attributed to genomic variation due to *in vivo* recombination events. The same study

investigated the stability of the 14 PFGE subtypes *in vitro* and confirmed that, after repeated subculturing (ten passages) of each isolate, the PFGE subtypes remained stable. Another study examined the stability of four isolates over 50 *in vitro* passages in the laboratory (Dickins *et al.* 2002). Only one of the four isolates revealed minor changes (one and two band differences) to the original isolate after 30 passages. These isolates were labelled variant A and B and from passage 30 onwards the original PFGE pattern was not isolated. One of the isolates was also passaged *in vivo* in two 1-day old chicks. All 20 *C. jejuni* re-isolated after five days from each chick's caecal contents had the same PFGE genotype as the original infective strain. This suggests no recombination events had occurred during *in vivo* passage of *C. jejuni*.

A similar study by Hänninen *et al*. (1999), however, did find phenotypic and genomic changes detected by PFGE and serotyping in two of twelve *C. jejuni* isolates inoculated into chicks. The occurrence of natural transformation within the chicken gut was also investigated by the *in vivo* passage of isogenic mutants containing two different antibiotic resistance markers (Wassenaar *et al.* 1998). There was no DNA exchange observed between these two mutants.

A study by de Boer *et al.* (2002) provides substantial experimental evidence for horizontal DNA transfer among heterologous *C. jejuni* strains during their colonisation of chickens. Intragenomic alterations were also observed which added to the genetic diversity detected by changes in PFGE subtypes. Subsequently, these same strains were passaged more than 300 times in the laboratory and showed no genomic recombinations when typed by PFGE. These findings concur with those mentioned above, indicating the stability of strains cultured in the laboratory and that genetic differences may be generated by *in vivo* environmental selection pressures. It may be worthwhile to characterise further the subtypes identified on chicken carcass CPH0112693 to determine their level of relatedness by MLST, which can provide information on the evolutionary divergence of clonal complexes (Feil *et al.* 2004).

4.4.2 Conclusions

Multiple subtypes of *C. jejuni* were identified on individual chicken carcass samples using the enrichment-PCR isolation method developed in Chapter Two. Subtyping was determined by PFGE typing using *Sma*I as the initial enzyme and employing *Kpn*I as the secondary enzyme for further discrimination of subtypes with an indistinguishable *Sma*I profile. An average of 23 colonies were analysed by *Sma*I PFGE per sample and based on the binomial formula of

Schlager *et al.* (2002), this would achieve a 91% probability of identifying a minor clone. Although the cutoff for determining an indistinguishable strain was set at 90% (de Boer *et al.* 2000), the average cutoff for *Sma*I digests was 99.6% and 98.1% for *Kpn*I digests demonstrating that technical reproducibility was high.

Five of the ten carcasses revealed multiple subtypes. Fifteen distinguishable subtypes were identified from the ten carcasses, and this included the identification of one subtype Sm0030/Kp0056 in three different chicken samples. In the five chicken carcasses that were identified with multiple subtypes, two subtypes were identified in three carcasses and three subtypes in two carcasses.

Two carcasses carried subtypes that were clonally related, and four carcasses carried subtypes that were not clonally related. In the example where the same subtype was identified on three carcasses, on one of those carcasses, it was identified as the co-dominant strain and the other strain was clonally unrelated.

5 Comparison of *C. jejuni* subtypes isolated from chicken meat and human clinical specimens

5.1 Introduction

5.1.1 The association between chicken meat and campylobacteriosis cases in humans

The high prevalence of *Campylobacter* in chicken and its derived meat products is now well established (Kramer *et al.* 2000, Nielsen and Nielsen 1999). In addition, numerous studies comparing the subtypes of *C. jejuni* from various matrices including humans, broilers and chicken products have concluded that subtypes of human isolates are highly represented in the subtypes isolated from chickens and chicken meat (Lindmark *et al.* 2004, Karenlampi *et al.* 2003, Broman *et al.* 2002, Petersen *et al.* 2001a, Hänninen *et al.* 2000, Duim *et al.* 1999, Ziprin *et al.* 1999, Hänninen *et al.* 1999). A New Zealand study by Kakoyiannis *et al.* (1988) used genotypic typing to show that nearly half (49.7%) of the human isolates typed were indistinguishable from poultry isolates. This was supported by another New Zealand study (Hudson *et al.* 1999) which used Penner serotyping and PFGE to show common subtypes of *C. jejuni* present in chicken portions and human cases of campylobacteriosis.

Most of these studies also noted, however, a high diversity of genotypes in *C. jejuni* isolates from human specimens, surface waters and various meat products, including chicken. In one study, Lindmark *et al.* (2004) identified five clusters of PFGE subtypes which comprised 88 of the sample set of 162 *Sma*I profiles. Following *Kpn*I digestion, most of these isolates were still indistinguishable within each cluster apart from the fifth cluster which was separated into many smaller clusters. Although PFGE revealed a high level of diversity, the study also concluded that there are some PFGE subtypes which are isolated frequently. The largest cluster comprised of human, chicken meat and one water isolate, although in general, the water isolates were not present in the clusters. Other clusters were comprised of human and meat isolates. Lindmark *et al*. (2004), along with other researchers (Gilpin *et al.* 2006, Michaud *et al.* 2001) have suggested that the clusters of cases may be caused by subtypes responsible for smaller outbreaks which are not detected by routine surveillance strategies. The non-recognition of outbreaks may occur because of time delays between the identification of indistinguishable subtypes of

Campylobacter and the epidemiological follow-up of cases, which could connect seemingly unrelated episodes of campylobacteriosis.

Outbreaks of campylobacteriosis directly attributed to the consumption of chicken have been reported (Pearson *et al.* 2000, Rosenfield *et al.* 1985), but outbreaks, in general, are infrequent relative to the large number of sporadic cases of campylobacteriosis (Hedberg *et al*. 2001, Blaser 1997). For example, in New Zealand in 2004 there were 31 recorded outbreaks which involved 130 of the 12,213 notified cases for the entire year (Anonymous 2005). More research involving rapid epidemiological follow-up of human cases is required to better define the sporadic nature of campylobacteriosis in comparison to outbreak cases (Gilpin *et al*. 2006, Michaud *et al.* 2001).

5.1.2 Host specificity

With the advent of new genotypic techniques for the typing of bacterial species there has been renewed interest in the typing of *Campylobacter* strains to assess the host specificity of a particular *C. jejuni* strain. A study using Multilocus Sequence Typing (MLST) to study *C. jejuni* isolates from farm animals and their environment concluded that there was some evidence for an association between some strains and a farm animal host (Colles *et al.* 2003). As an example, it was noted that certain strains were dominant among the poultry isolates but absent from sheep isolates and the converse also occurred. This work is supported by MLST studies of *C. jejuni* isolates from human and various animal and food matrices (Siemer *et al.* 2004, Manning *et al.* 2003). Although both studies identified a high diversity of genotypes among individual matrices, including humans, several clonal complexes were recognised that indicated some host specificity may exist.

A study, which conducted a structured spatiotemporal sampling of farm environments in a 100 km^2 area over a ten week time interval (French *et al.* 2005), concluded that there was evidence for a relationship between genotypes of *C. jejuni* (as determined by MLST) and host. The samples collected from wildlife (birds, rabbits and badgers) and water formed a cluster with many of the genotypes being unique to that study. In addition, many of these water/wildlife isolates of *C. jejuni* shared common alleles which were found infrequently in isolates from other matrices. A comparison with human cases of campylobacteriosis, however, did identify some important human–associated sequence types in the isolates from water and wildlife, suggesting

that these matrices could still be significant as reservoirs of pathogenic campylobacters. Subtypes isolated from cattle clustered into several large clonal clusters, one of which was frequently associated with human infection. The French *et al.* (2005) study supports the conclusions from a year long New Zealand survey in a rural environment which compared subtypes isolated from a variety of environmental matrices with human clinical isolates. The study concluded that the greatest similarity of indistinguishable serotype:PFGE subtypes of *C. jejuni* to isolates from humans was with isolates from ruminants (Devane *et al.* 2005, Baker *et al.* 2002).

In contrast, other studies comparing subtypes between matrices did not reveal any association with a particular host (Hopkins *et al.* 2004, Schouls *et al.* 2003). Matrices investigated in these studies included humans, pigs, cattle, poultry and retail meats. They concluded that *C. jejuni* strains of the same subtype colonise a wide range of hosts, which means that it is difficult to identify the source of an infection when investigating an outbreak of campylobacteriosis. Interestingly, in the study of Schouls *et al.* (2003) the genotypes isolated from the cattle specimens were more closely associated with human subtypes than with poultry subtypes. Based on their observations of the numbers of multiple *Campylobacter* subtypes colonising individual chickens Schouls *et al.* (2003) have suggested that perhaps the *C. jejuni* that cause disease in humans are minor subtypes in chicken microflora and therefore are not being identified by traditional bacteriological techniques where only a single colony is selected for typing.

Champion *et al*. (2005) used comparative phylogenomic analyses via DNA microarrays to identify potential genes associated with a specific host. Analysis revealed two separate clades comprising livestock-derived isolates and non-livestock isolates. Only 44% of the 111 human isolates were found in the livestock clade which was comprised of chickens, bovines and ovines. This again highlighted that there are other reservoirs/transmission routes of *Campylobacter* in the environment besides farmed animals and their meat products.

As indicated above, previous international comparisons of the subtypes isolated from chicken carcasses and human faecal specimens have identified unique subtypes in both matrices, as well as common subtypes between the two matrices. When trying to establish host specificity, it is important that the samples are collected with regard to a relationship in time and place. The identification of unique subtypes, which maybe host specific, suggests that not all subtypes are

pathogenic to humans or survive to cause human exposure. Characterisation of those subtypes that occur in multiple animal matrices allows for a comparison with subtypes unique to one matrix. This could lead to the investigation of potential virulence factors which confer an advantage to those subtypes capable of infecting multiple animal types. Recognition of genes encoding virulence factors could provide an efficient screening test for potentially pathogenic subtypes. Identification of multiple matrix subtypes may also enhance the management of contamination controls, allowing producers to focus on eradication of virulent subtypes which are of public health significance.

Inoculation of chickens with strains of *C. jejuni* and *C. coli* isolated from dairy cows demonstrated the ability of these isolates to colonise the ceca of chicks with 100% efficiency (Ziprin *et al.* 2003). The challenge isolate of *Campylobacter* was recovered from the caeca of the chickens 1-2 weeks after inoculation and determined to be the same colonizing strain by comparison of their ribotypes. These results suggest that these particular *Campylobacter* strains are not host specific.

It is possible to determine that a particular subtype is highly prevalent in one matrix compared to another matrix, but it is very difficult to demonstrate that a subtype is restricted to a particular host. Subtypes have been identified which are associated only with cases of human campylobacteriosis and, to date, recorded cases of human-to-human transmission of *Campylobacter* are infrequent, and generally occurred where there was direct contact with infectious material such as the changing of dirty nappies, bathing of children together and nosocomial infection between neonates (Graham *et al.* 2005, Llovo *et al.* 2003, Friedman *et al.* 2000); and poor hygiene associated with foodhandlers (Fitzgerald *et al.* 2001a). It is generally assumed, therefore, that not all of the reservoirs of *Campylobacter* have been identified and/or the particular subtypes have not, thus far, been isolated from known environmental reservoirs. It is yet to be conclusively proven that a particular *Campylobacter* subtype has adapted to a specific host.

5.1.3 Identification of multiple subtypes of *Campylobacter* in human clinical samples

A study to determine the presence of multiple *Campylobacter* strains in individual human clinical samples concluded that human infection with more than one *Campylobacter* strain was a rare event (Steinbrueckner *et al.* 2001). Four individual colonies from each patient (n=50)

were analysed by the two genotypic methods of PFGE and ERIC-PCR (refer Appendix 4). A dual infection with *C. jejuni* and *C. coli* was not detected in any of the samples and two co-infecting strains of *C. jejuni* were identified in only two of the clinical samples. For one of these samples the band patterns for the two different isolates were very similar indicating a strong genetic relationship, perhaps supporting the hypothesis of *in vivo* recombination events. Therefore out of 50 clinical samples only one sample was confirmed as having a multiple infection with more than one strain of *C. jejuni*.

The results of Steinbrueckner *et al.* (2001) were supported by a similar study which used three phenotypic methods and two genotypic methods to type isolates from individual human faecal samples (Richardson *et al.* 2001). *Campylobacter* was isolated from 53 human faecal samples. Two methods of isolating *Campylobacter* were employed and five colonies were taken from each isolation method resulting in 10 colonies being typed for each sample. Only one patient was infected with *C. coli*, the remainder of the strains were identified as *C. jejuni*. The results of typing the isolates identified four of the samples (7.5%) as having two strains of *C. jejuni* present.

A study of campylobacteriosis in the population of Central Australia identified 86% of cases (n=218) as harbouring *C. jejuni* and 14% harbouring *C. coli* and 4% of cases who had a mixed infection of *C. jejuni* and *C. coli* (Albert *et al.* 1992). At least 13 cases were identified as having multiple infections of *C. jejuni* as determined by the presence of up to three different serotypes in single stool samples. Some cases were also found to have repeated reinfection with different *Campylobacter* serotypes and long term excretion (up to 73 days in one case) of the same serotype. It could not be ruled out, however, that the long term excretion was not due to another reinfection event by the same serotype. Kramer *et al.* (2000) who recognised multiple phenotypic subtypes of *C. jejuni* in red meat and chicken also reported that the Public Health Laboratory Service had identified co-infection with multiple strains of *Campylobacter* species in 5-10% of human cases. Their findings led them to highlight the importance of selecting more than one isolate per sample.

5.1.4 Objectives

- To compare subtypes identified in chicken carcasses (Chapter Four) with human clinical subtypes of *C. jejuni* collected nationwide, including a subset of human isolates collected from the same geographical area and over the same timeframe as the chicken samples.
- To identify if isolates from chicken meat are pathogenic to humans.
- To address the issue of whether the subtyping information generated is limited by the selection of single isolates from samples when compared with selecting multiple isolates which have a higher probability of identifying non-dominant clones in a sample.
- Using the findings of this study, propose appropriate strategies for the use of subtyping in the tracking of the environmental sources of *Campylobacter* with respect to both sporadic and outbreak cases.

5.2 Materials and methods

The 15 PFGE subtypes identified from chicken carcasses were compared with the 1518 isolates held in the PulseNet Aoteoroa New Zealand Database, Environmental Science and Research Ltd (ESR) Christchurch, New Zealand. This database contained data for 376 human isolates collected nationwide, including 61 human clinical isolates collected from the same geographical area and within the same time frame as the chicken carcass isolates analysed in this study. Human clinical isolates of *C. jejuni* in the database which had not been analysed with RE *Kpn*I and were of interest to this study were subjected to PFGE *Kpn*I digestion as described in Chapter Four.

5.2.1 Analysis of PFGE subtypes

PFGE band patterns were analysed and compared using BioNumerics software (Applied Maths, Kortrijk, Belgium). Only fragments in the range 700 to 50 kb were analysed for *Sma*I and 700 to 80 kb fragments were analysed for *Kpn*I (Michaud *et al.* 2005). Smaller fragments were not consistently resolved. The analysis parameters used were in accordance with the PulseNet, USA standard procedure and utilized the band-based Dice similarity coefficient and the unweighted pairs geometric matched analysis (UPGMA) dendrogram type with a position tolerance setting of 1.5% for optimization and position tolerance of 1.5% band comparison. All test isolates were normalized to the known molecular size bands of the *Salmonella* Braenderup H9812 standard subtype (Hunter *et al.* 2005). In addition, all gels were run with a well-characterised subtype of *C. jejuni*: CPH011453 which acted as a control for the digestion reaction for both restriction enzymes.

5.3 Results

5.3.1 Comparison of *C. jejuni* isolates from chicken carcasses and humans

Results of the comparison between *C. jejuni* isolates from humans and chicken carcasses are presented in Table 17. Additional information on their identification in other matrices and other chicken meat samples from previous studies is also shown. Not all of the 1518 isolates in the database have, as yet, information on *KpnI* digestion and therefore it has been noted in Table 17 where the correlation with environmental matrices is by *Sma*I typing data only. Human isolates that had the same PFGE *Sma*I subtype as a chicken isolate were digested with *Kpn*I to establish whether they were an indistinguishable or a related subtype to the chicken isolate.

Twelve of the 15 subtypes isolated in chicken carcasses were not identified in any other matrix (Table 17). Furthermore, the same twelve subtypes were not implicated in any of the human cases of campylobacteriosis. Three subtypes were identified in human clinical samples and two of those subtypes were identified in three and four human clinical samples each. Although only three subtypes were identified as indistinguishable to isolates from human cases, five of the ten chicken carcasses carried those pathogenic *Campylobacter* subtypes. Eight clinical isolates were identified as indistinguishable to chicken subtypes but seven of those human isolates were collected at a different time and location to the chicken carcasses.

For four of the chicken samples that carried multiple subtypes, none of the non-dominant subtypes correlated with any isolates from human cases. In the fifth sample (CPH0112077) that carried two subtypes which were both close to 50% prevalence, one of those subtypes was identified in three isolates from human cases. This subtype (Sm0030/Kp0056) was also identified in two other chicken carcasses tested in this study and collected at different time periods.

5.3.2 Clonal relationships between human and chicken isolates of *C. jejuni*

Both of the subtypes from sample CPH0112077 had human isolates that were clonally related, as assessed by examination of their subtype profiles. As noted in Chapter Four, these two isolates have *Sma*I types Sm0001 and Sm0030 which are visually similar due to a single band

shift (Figure 12). The *Kpn*I profile of chicken isolate Sm0030/Kp0056 is presented in Figure 13 along with the three human isolates that had an indistinguishable profile by *Sma*I and *Kpn*I digestion. Also shown are two human isolates that are potentially clonally related to Sm0030/Kp0056. Human *C. jejuni* isolate CPH0210257 (Sm0001/Kp0200) is 89% similar to Kp0056 using position tolerance 1.5% and optimization of 1.0% and human isolate CSC-_CPH0311539-01 (sm0030/Kp0116) is 84% similar to Kp0056 using the same parameters (Figure 13).The critieria of Tenover *et al.* (1995) (refer to Chapter Four) is more relevant for determining clonal relationships than basing relatedness on a dendrogram. By employing these criteria the *Kpn*I profile of CPH021057 is assessed as closely related to the chicken isolate (Sm0030/Kp0056) as it has two band shifts in comparison to the Kp0056 profile. This suggests a single genetic event whereby a deletion of DNA from the band at approximately 350 kb in Kp0056 has resulted in that band disappearing in subtype Kp0200 and a smaller DNA band appearing at approximately 300 kb (Figure 13).

The human isolate CSC_CPH0311539-01 has the same *Sma*I profile as chicken subtype Sm0030/Kp0056 but a different *Kpn*I profile (Figure 13). This *Kpn*I profile (Kp0116) is, by the criteria of Tenover *et al*. (1995) "possibly related" because it has four band differences to Kp0056. These band differences are consistent with the occurrence of two genetic events, being deletions in the bands occurring at approximately 350 and 175 kb in Kp0056 and appearing as bands at approximately 250 and 130 kb (respectively) in Kp0116. It should be noted that directionality of the band shifts between subtypes cannot be inferred from these experiments and therefore the differences in band patterns for all of the above genetic events could be either deletions or insertions of DNA.

Isolate Sm0001/Kp0033 which was identified in 48% of the isolates from chicken carcass CPH0112077 was not identified in any human cases of campylobacteriosis. Subtype Sm0001/Kp0032, however, is clonally related to Sm0001/Kp0033 as the band at approximately 160 kb in Kp0033 does not appear in Kp0032, but two bands appear at approximately 70 and 90 kb in Kp0032 (Figure 14). This is suggestive of a single genetic event resulting in a new restriction enzyme site in the 160 kb fragment (Kp0033) which is cleaved by *Kpn*I into two fragments of 70 and 90 kb (Kp0032). The subtype Sm0001/Kp0032 was isolated from a human case of campylobacteriosis (CSC_CPH0214589-01) and from a sample of roof water (CSC_CPH0315218) (Figure 14).

Table 17: Comparison of *C. jejuni* isolates from chicken carcasses with isolates from human cases of campylobacteriosis and other matrices

Chicken sample	*PFGE subtype	Subtype prevalence	Indistinguishable *Sma*I/*Kpn*I PFGE isolates from human cases †	Indistinguishable *Sma*I/*Kpn*I PFGE isolates from other matrices and previous studies ‡	Indistinguishable *Sma*I PFGE isolates from other matrices and previous studies ‡
CPH0111167	Sm0106/Kp0155	Dominant (87%)	none	none	Chicken meat
	Sm0106/Kp0156	Intermediate (13%)	none	none	-
	Sm0106a/Kp0155	Minor (5%)	none	none	-
CPH014376	Sm0184/Kp0158	Dominant (96%)	none	none	Chicken meat sheep liver, river water
	Sm0038/Kp0158	Minor (4%)	none	none	-
CPH012693	NC/Kp0152	Dominant (81%)	none	none	-
	NC/Kp0153	Intermediate (12%)	none	none	-
	NC/Kp0154	Minor (6%)	none	none	-

CPH0112839	Dominant (77%)	Sm0094/Kp0049	1	Chicken meat	Chicken meat
	Intermediate (23%)	Sm0225/Kp0159	none	Chicken meat	Chicken meat
CPH0112077	Dominant (52%)	Sm0030/Kp0056	3	none	Chicken meat, mutton, (sheep), veal, pork
	Intermediate (48%)	Sm0001/Kp0033	none	none	Chicken meat, lamb, veal, pork, river sediment, oxidation pond, duck and sheep faeces
CPH0110920	Single isolate	Sm0034/Kp0151	none	none	Chicken meat, river water, lamb
CPH0112821	Single isolate	Sm0037/Kp0038	4††	Dairy cow faeces, chicken meat	Chicken meat
CPH014584	Single isolate	Sm0173/Kp0157	none	none	-
CPH0110379 and CPH014912	Single isolates	Sm0030/Kp0056	Same as CPH0112077	none	Chicken meat, mutton, (sheep), veal, pork

* Sm0000/Kp0000 = *Sma*I digestion profile/*Kpn*I digestion profile

** NC, non-cutting

† compared with 376 human isolates, including 61 isolates from the same test period and area as the chicken isolates

‡ compared with 1142 non-human isolates.

†† There was a temporal and spatial relationship between one of these four isolates from humans and the chicken subtype.

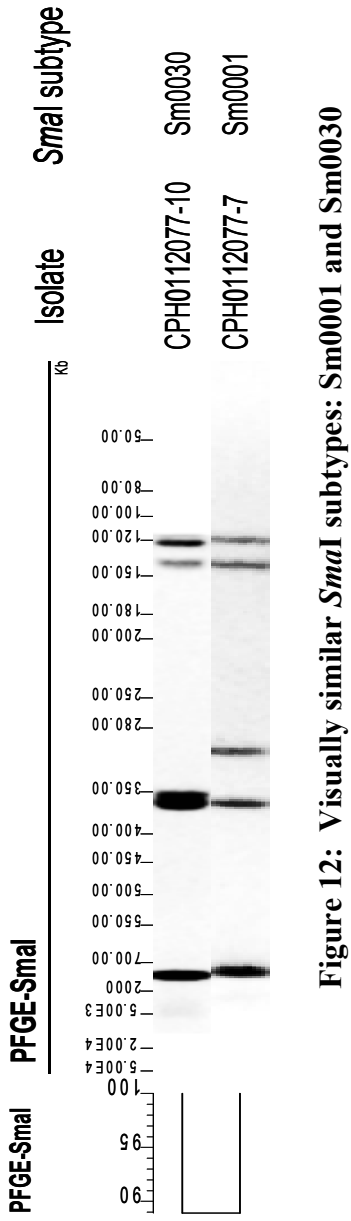

Figure 12: Visually similar *Sma*I subtypes: Sm0001 and Sm0030

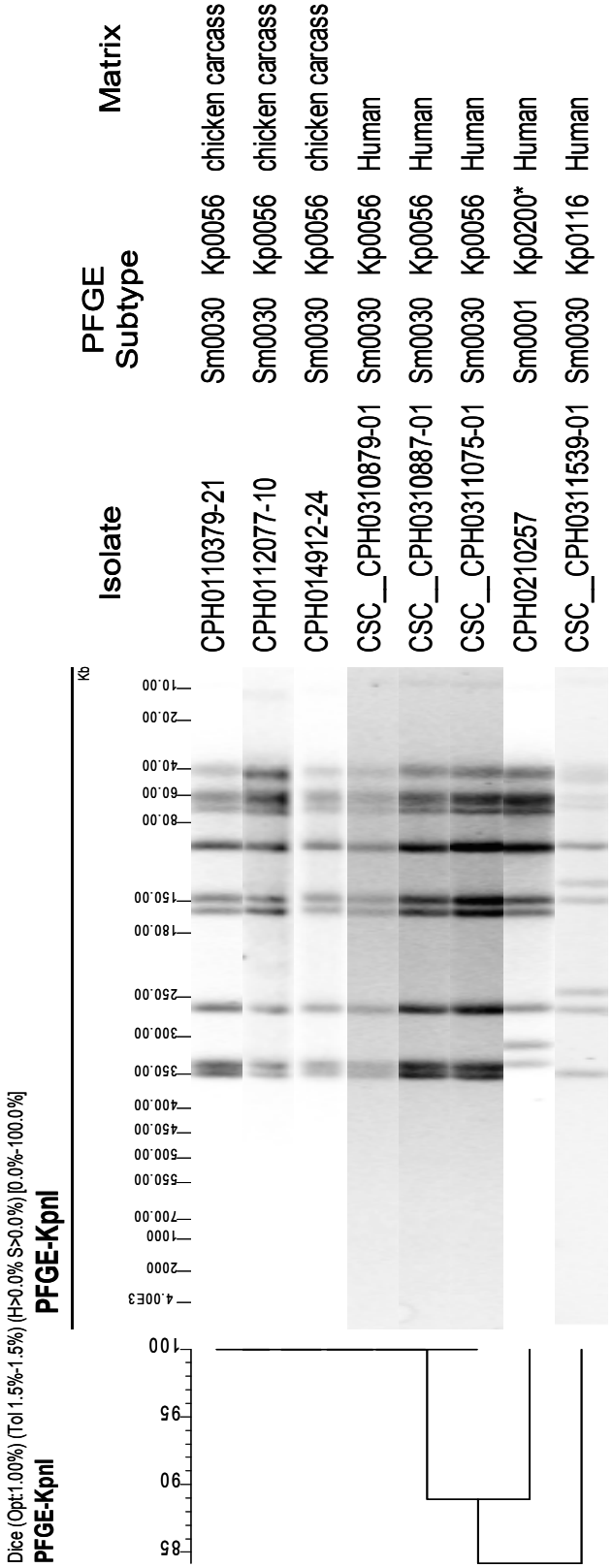

Figure 13: Clonal relationships between human isolates and subtype Sm0030/Kp0056 from chicken

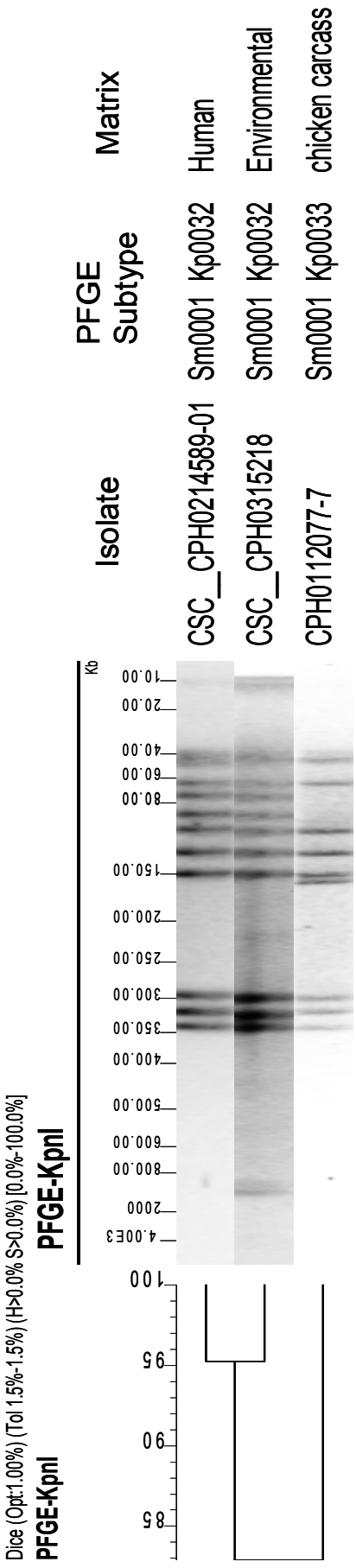

Figure 14: Clonal relationships between human isolates and subtype Sm0001/Kp0033 from chicken

5.4 Discussion

5.4.1 Comparison of *C. jejuni* isolates from chicken carcasses and humans

It has been established in previous discussions (Chapters Two to Four) that broiler chickens and their chicken products carry pathogenic campylobacters which represent a wide diversity of *C. jejuni* genotypes. There still remains a lack of understanding, however, as to the impact of these findings on the incidence of campylobacteriosis in the human population.

Chapter Five was designed to evaluate the third hypothesis of this thesis that all subtypes of *C. jejuni* identified on chicken carcasses are also identified in human faecal specimens. This is important to determine so as to increase the understanding of the impact of chicken consumption on the number of campylobacteriosis cases, as there are varying reports ranging from 40 up to 70% of human cases (Michaud *et al.* 2004, Vellinga and Van Loock 2002, Stern 1992) being attributed directly to chicken meat. It is widely accepted, however, that the role of chickens and chicken meat is very important in the transfer of *Campylobacter* to humans (Friedman *et al.* 2004, Rosenquist *et al.* 2003).

The 15 subtypes isolated from the ten chicken carcasses tested in this study were compared with the 1518 *Campylobacter* isolates in the ESR database. The database contains 376 human isolates, including 61 human isolates collected from the same location and timeframe as the chicken isolates during a year long survey in a rural district. Three subtypes were identified in up to four human clinical samples each (Table 17). Therefore, twelve of the subtypes were not implicated in any of the human cases of campylobacteriosis, leading to a repudiation of the third hypothesis.

Subtype Sm0094/Kp0049 was isolated as the dominant subtype (77% prevalence) from sample CPH0112839 and was also identified in one human clinical isolate and in chicken meat from previous studies, but no other matrices. The *Sma*I subtype was identified in three other chicken carcasses, but in comparison, these isolates all had a different and unrelated *Kpn*I profile, namely Kp0056. The intermediate subtype identified in the same sample CPH0112839 was not clonally related to the dominant subtype nor was it identified in any other matrices.

Subtype Sm0037/Kp0038 was the sole subtype identified in sample CPH0112821 and it had been isolated from four human clinical samples as well as dairy cow faeces and chicken meat in other studies.

Subtype Sm0030/Kp0056 was identified in three chicken carcasses, where it was the only subtype detected in two of those samples and found at close to 50% prevalence in the third sample (CPH0112077). This subtype had been isolated from three clinical cases and therefore is pathogenic to humans. Sm0030 had also been previously identified in other chicken meat samples, sheep faeces, veal and pork, suggesting that this *Sma*I type is ubiquitous in the environment. The two subtypes identified in almost equal proportions in sample CPH0112077 had *Sma*I profiles (Sm0001 and Sm0030) that differed by the presence of one band and thus the profiles were visually similar (Figure 12). Their *Kpn*I profiles were very different, however (seven band difference) and therefore they were not defined as clonally related.

Interestingly, one human isolate CPH0210257 (Sm0001/Kp0200) was identified as clonally related to Sm0030/Kp0056, the subtype identified in three chicken carcasses. The PFGE profile of CPH0210257 had one band difference to Sm0030 and two band differences to Kp0056 (Figure 13). This tentatively increased the number of human isolates to four for the subtype Sm0030/Kp0056 (not shown in Table 17). Furthermore, another human isolate CSC_CPH0311539-01 was determined to be "possibly clonally" related to chicken isolate Sm0030/Kp0056 because it had an indistinguishable *Sma*I type and the difference in its *Kpn*I profile could be attributed to two genetic events (Figure 13).

The issue of clonality is further exemplified by the case where an isolate (Sm0001/Kp0033) that was co-dominant in a chicken carcass did not correlate with indistinguishable subtypes isolated from human cases. A clonally related isolate, however, was identified in a human case of *Campylobacter* and in a sample of roof water, which could have implications for waterborne transmission of this subtype (Figure 14). In addition, the *Sma*I subtype that this isolate belonged to is one of the most common subtypes encountered in the environment as can be observed from its isolation from all four meat matrices, duck and sheep faeces, and river sediment (Table 17). However, when *Kpn*I digestion is applied to isolates of this *Sma*I type, the number of different subtypes generated is large (personal communication, Brent Gilpin, ESR), which emphasizes the importance of basing subtyping information on two restriction enzymes.

Evidence for putative genetic divergence during *Campylobacter* colonisation of the human intestine has also been suggested. This divergence gives rise to clonally related subtypes during the bacterium's passage through the human host, which further complicates the tracking of an outbreak source. The same paper that investigated the occurrence of multiple subtypes of *Campylobacter* in individual human faecal samples (Section 5.1.3 above) also studied the subtypes isolated from the same patient over the course of a *Campylobacter* infection (Steinbrueckner *et al.* 2001). The time interval between the collection of isolates from each patient was on average 23 days with a median of 15 days. Fifty-two patients were involved in the study and only four patients were identified as harbouring more than one subtype of the same *Campylobacter* species over the course of their infection. Because of the time intervals between collections of samples, it could not be determined, definitively, if the different isolates were due to re-infection events or co-infection by the two subtypes. Two of the patients, however, exhibited subtypes with between two and six band differences (as determined by PFGE) suggesting that they may have been clonally related (Tenover *et al.* 1995). The authors concluded that based on the low level of simultaneous infection (4%) observed in the first part of the study (Section 5.1.3 above), that it was likely that the isolation of clonally related subtypes of the same species was due to genetic instability of the primary infecting strain. These isolates were passaged *in vitro* 45 times on non-selective media, and as for previous studies (reviewed in Chapter Four) the PFGE patterns of the isolates did not change during laboratory passage suggesting that the genetic instability of the strains was not high. The authors suggest that the observation whereby *Campylobacter* strains can undergo genetic changes during an infection episode, must be taken into account when conducting epidemiological investigations.

Overall, the number of subtypes isolated from chicken samples and correlated with subtypes from human clinical specimens was low at 20%. However, the three subtypes identified in human cases were isolated from five (50%) of the ten chicken carcasses, although the small sample number precludes meaningful conclusions. Furthermore, there was no bias in the subtype correlations towards the 61 human isolates collected from the same region and the same time period. Only one of those 61 human isolates had an indistinguishable subtype to a chicken isolate (Subtype Sm0037/Kp0038). In addition, subtyping analysis of all the *Campylobacter* isolates (n = 58) identified in chickens during the same one year survey (n = 204 chickens) showed a low overall similarity to the 61 human isolates (Devane *et al.* 2005, Baker *et al.* 2002). The other seven human isolates that were indistinguishable to the chicken subtypes were not linked to the chicken isolates by a temporal/spatial relationship. However, all eight human

isolates were from people who lived in the same province where the chicken and 61 human isolates were collected but the campylobacteriosis cases occurred nine to 15 months after the conclusion of the year long survey. In addition, five of the eight cases lived in the largest urban centre (population 400,000) of that province.

Twelve of the 15 subtypes were not identified in any other environmental matrix represented in the 1518 isolates in the ESR database. When based on *Sma*I profiles alone, three of those twelve subtypes were identified in other matrices besides chicken meat (Table 17). The matrices in the database include sheep, cattle, dairy cow, duck, possum and rabbit faeces, river water, oxidation pond water, river sediments, beef, sheep and pig liver, veal, lamb, beef and pork. These results are not definitive of host specificity of the subtypes to chicken meat as there are many reasons why a subtype has not been identified in another matrix. These reasons include that the number of isolates present in the database is likely to be miniscule in comparison to those present in the environment. Also, the lack of consistency in PFGE typing designations and standardized methods between laboratories prevents comparison of PFGE subtypes between international laboratories and even between laboratories within New Zealand. This leads to the non-recognition of indistinguishable subtypes in different research projects. This issue is being addressed by the establishment of the PulseNet Aoteoroa NZ Database and international linkages with databases using the same standardised methods for typing.

5.4.2 Non-dominant subtypes

One of the aims of this study was to ascertain the importance of minor and intermediate subtypes and whether isolating one or two colonies was limiting the subtype information pertinent to tracking the source of an outbreak in a community. Furthermore, did the low probability of isolation of these non-dominant subtypes prevent the identification of an outbreak which was associated with a minority subtype? This finding would have suggested that the sporadic nature of campylobacteriosis cases was over reported.

From the information derived from this study, non-dominant subtypes identified in chicken meat did not correlate with any isolates from human cases. This result suggests that important information linking human cases to a common source is not being missed by employing the enrichment-PCR method and other methods that isolate only a few colonies from an individual sample. The caveat to this is the low number of carcasses tested for multiple isolates and that

the results reflect the situation in chicken meat only. As noted by various researchers, however, using two or more methods such as direct plating and enrichment has increased the diversity of genotypes identified in a sample (Dickins *et al.* 2002, Newell *et al.* 2001). It may be useful to employ the same comparative subtyping between human and chicken isolates, as outlined in this chapter, to evaluate whether the employment of two isolation methods increases the probability of tracing the source of an infection. It could also establish whether it is more efficacious to subtype one/two isolates from each method, rather than analyzing a large number of colonies derived from a single method.

5.4.3 Tracking the source of *Campylobacter* infection in human cases

A survey conducted in Quebec, Canada endeavoured to combine the power of *C. jejuni* subtyping by *Kpn*I with epidemiological information from sufferers of campylobacteriosis (Michaud *et al.* 2005). They were specifically questioning the current idea that most cases of campylobacteriosis are not associated with an outbreak incident (Blaser 1997, Pebody *et al*. 1997). Some researchers, however, have identified a high proportion of cases that report other illness in the home or community concurrent with their illness, which could be suggestive of an outbreak incident (Gillespie *et al.* 2003). The aim of the Michaud *et al*. (2005) study was to determine if rapid typing of clinical *Campylobacter* isolates could be combined with a well-timed epidemiological follow-up of clinical cases to identify a link between cases that had the same *Campylobacter* PFGE subtype. This information was used to establish if there was an identical source, indicative of an outbreak incident, between apparently unrelated cases.

Their study, however, led them to conclude that the epidemiological information was insensitive and unreliable as analysis rarely led to the establishment of a common infection source for indistinguishable *C. jejuni* subtypes. Reasons for this interpretation about the clinical descriptive data included the time delay between infection, onset of symptoms and questionnaire interview, and the limitations of the types of questions in the survey. The subtyping data of *C. jejuni* also revealed a high diversity of subtypes as found by other researchers of *C. jejuni* and discussed in Chapter Four.

Another study of *Campylobacter* isolates collected from clinical cases of campylobacteriosis over a year long period in Minnesota, USA, came to a similar conclusion (Hedberg *et al.* 2001). Researchers identified that from the 673 *Campylobacter* isolates, 74% of the 248 indistinguishable PFGE subtypes were represented by only one or two isolates. PFGE data

identified eight temporal clusters which involved 9% of the total number of isolates. Two outbreaks were identified by routine epidemiological methods but overall 87% of the cases could not be linked by PFGE subtype, time and/or geographical location.

Such findings led the researchers to question the validity of investigating sporadic cases of campylobacteriosis in an attempt to determine common causality to routes of infection (Michaud *et al.* 2005, Hedberg *et al.* 2001). The fact remains, however, that subtyping evidence has revealed many cases of campylobacteriosis are coming from, as yet, unidentified transmission routes and/or reservoirs in the environment. This is shown by the detection of subtypes of *Campylobacter* in cases of human infection that have not been identified in other environmental matrices (Champion *et al.* 2005). One of the methods to overcome the limitations of our knowledge in relation to these unidentified sources is to increase our data surveillance techniques. A large database of molecular and epidemiological information, therefore, may reveal commonalities between sources that have previously remained undetected.

5.4.4 Methods to reduce *Campylobacter* contamination

From the results in this study, twelve of the subtypes from chickens were not identified in any other environmental matrix besides chicken meat. As discussed above this may be due to limitations in the low numbers tested and represented in the database. It may also be, however, that some of these isolates represent subtypes that are found only in the chicken matrix as they are specifically adapted to colonisation of chickens and poorly adapt to colonisation of humans and other animals. If this were the case then these subtypes are of low pathogenic potential to humans and could be useful as controls in the study of infection and invasion of host intestinal cells. Comparative studies involving low and highly pathogenic subtypes of *Campylobacter* could identify virulence factors and survival mechanisms that confer an advantage to a highly pathogenic *Campylobacter* subtype. These studies could potentially lead to the design of genetic markers for identification of subtypes that carry specific virulence genes and therefore are likely to be pathogenic (Champion *et al.* 2005).

The implications for the poultry industry would include the ability to concentrate on eradication of subtypes known to be pathogenic to humans, such as the three subtypes identified in five of the ten chicken carcasses analysed in this study. This is of particular concern if those subtypes are detected in specified areas of the processing chain, where their genomic repertoire has

enabled them to survive the hostile environment so they can cross-contaminate chicken carcasses traveling through the production line.

This scenario was suggested by the research of Newell *et al.* (2001) who observed different subtypes on carcasses after processing which were not present prior to slaughter. This included subtypes identified in processed carcasses when the initial flock had tested negative for *Campylobacter* prior to slaughter or transport to the abattoir. Cross-contamination attributable to re-using *Campylobacter* contaminated crates for the transport of chickens to the abattoir was also identified in several studies as a risk factor (Slader *et al.* 2002, Hiett *et al.* 2002, Newell *et al.* 2001). The campylobacters identified in the crates were subsequently identified at low levels on processed carcasses from birds that had tested negative for *Campylobacter* prior to slaughter or had tested positive for different *Campylobacter* subtypes.

Certain subtypes may be capable of better survival in the abattoir/crate environment than the subtype identified in the original flock. It would also be important, however, to investigate the colonisation potential of those subtypes that contaminate chicken products during abattoir transport and processing. Gaynor *et al.* (2004) reported a reduction in colonisation potential of laboratory strains exposed to atmospheric oxygen, and therefore exposure to non-host environments may have affected the ability of these abattoir/crate subtypes to re-colonise a host lowering their pathogenic potential.

As observed by Lindmark *et al*. (2004) large abattoirs can distribute chickens to retailers on a nation wide basis allowing for the wide dissemination of *Campylobacter* clones that are virulent to humans. The reduction of *Campylobacter* incidence on retail poultry products can be targeted at various control points during the processing of the chicken from the farm to the consumer's table. The high prevalence of campylobacters on chicken meat products at point of sale suggests that interventions to lower numbers on the chicken carcass during abattoir processing should be accompanied by efforts to reduce *Campylobacter* colonisation of chickens at the farm level. Many researchers have concluded that prevention of *Campylobacter* colonisation on broiler farms is the most effective way to prevent the contamination of poultry meat (Rivoal *et al.* 2005).

Rosenquist *et al.* (2003) have shown by a quantitative risk assessment model that a 2-log reduction of *Campylobacter* numbers on chicken carcassses could reduce the incidence of

campylobacteriosis associated with chicken consumption by 30 times. A similar reduction in the number of campylobacteriosis cases could be obtained by reduction of the *Campylobacter* flock prevalence by 30 times (e.g. from 90% to 3%). Conversely, the introduction of logistical slaughter policies, whereby *Campylobacter*-negative flocks are slaughtered prior to positive flocks was expected to have only a limited influence (by a factor of 1.16) on campylobacteriosis incidence in humans. This was most likely due to the low numbers of *Campylobacter* transferred to the negative carcasses via cross-contamination. It was, therefore, considered more efficient to concentrate on strategies to reduce *Campylobacter* numbers on carcasses.

Competitive exclusion by intestinal microflora of the chicken that can outcompete campylobacters preventing colonisation of the intestinal tract, or vaccination of chickens are thought to be some of the most efficient potential strategies for reducing *Campylobacter* colonisation. Competitive exclusion has been successfully developed for *Salmonella* and shows promise for *Campylobacter* (Chen and Stern 2001, Stern 1994). Chen and Stern (2001) have used *C. jejuni* strains identified only in chickens to determine their potential to exclude *C. jejuni* isolates from humans. They found that those strains which had superior colonizing abilities were predominant, independent of when the chicken was exposed to the strain or whether they were pathogenic to humans. They suggest that research requires identification of non-pathogenic campylobacters with strong colonisation potential to further test the competitive exclusion hypothesis. As has been discussed, the existence of strains that are truly host specific to chickens is still open to debate.

To date, unlike *Salmonella* vaccination, the task of developing an effective vaccine to prevent *Campylobacter* colonisation of chickens (Sahin *et al.* 2003) has proved difficult (Rice *et al.* 1997, Widders *et al.* 1996). New vaccine strategies are continuing to be developed, including immunization with an avirulent *Salmonella* vaccine strain which carried a *C. jejuni* gene expressing a highly immunogenic protein to protect against colonisation by *Campylobacter* (Wyszynska *et al.* 2004).

5.4.5 Conclusions

Twelve of the subtypes in chicken carcasses were not implicated in any of the human cases of campylobacteriosis thus negating the third hypothesis that all subtypes of *C. jejuni* identified on chicken carcasses are also identified in human faecal specimens. Furthermore, these twelve subtypes were not identified in any other matrix. Three subtypes were identified in human clinical samples and two of those subtypes were identified in three and four human clinical samples each. Moreover, the three subtypes identified in human cases of campylobacteriosis were carried by five of the ten chickens analysed for multiple subtypes.

In addition, it was demonstrated that in the four samples which carried minor and/or intermediate subtypes, these non-dominant subtypes did not correlate with any isolates from human cases. In the fifth sample, which carried two *C. jejuni* subtypes that were both close to 50% prevalence, one of these subtypes was identified in three isolates from human cases. This subtype (Sm0030/Kp0056) was also identified in two other chicken carcasses tested in this study and collected at different time periods. In contrast to Schlager *et al.* (2002), the results suggest that the non-identification of minor/intermediate subtypes using the enrichment-PCR method from chicken carcasses may not be limiting the information relevant to tracking the source of an infection to chicken carcasses.

Analysis revealed a few human isolates that were distinct but clonally related to chicken isolates. For example, a human isolate was identified that had one band difference to Sm 0030 and two band differences to Kp0056. This tentatively increased to four the number of human isolates that correlated with the subtype Sm0030/Kp0056 isolated from three chickens.

A comparison of the results from this study with other published research concluded that it may be worthwhile to evaluate whether the employment of two isolation methods enhances the identification of diverse subtypes. Furthermore, if two methods reveal an increased diversity will this aid in the tracking of the source of an infection? It could also establish whether it is more efficacious to subtype one/two isolates from each method, rather than analyzing a large number of colonies derived from a single method.

6 Concluding discussion

6.1 Enrichment-PCR method

The ability to distinguish between *Campylobacter* species is important in the identification of *Campylobacter* sources and transmission routes (Wassenaar and Newell 2000). My thesis reports an enrichment-PCR assay that was developed for the detection of *C. jejuni* and *C. coli* from 13 environmental matrices: human, dairy cow, cattle, sheep, chicken, duck, possum and rabbit faeces; sheep, beef, pig liver; chicken meat, and river water. PCR was employed to identify campylobacters after an initial enrichment step.

Advantages of PCR-based assays over conventional plating methods and phenotypic testing for identification purposes include speed and cost-effectiveness (Olive and Bean 1999). For example, purification of individual colonies prior to identification by biochemical tests requires a 48 hour incubation of two consecutive subcultures. When coupled with antibiotic susceptibility assays, which require a further 48 hours of incubation, this can lead to a period of up to ten days for identification by the conventional method. Futhermore, there are few biochemical tests that differentiate campylobacters at the species level and the hippurate hydrolysis test that discriminates between *C. coli* and *C. jejuni* is known to misidentify *C. jejuni* based on the non-expression of the hippuricase enzyme (Waino *et al.* 2003). By comparison, PCR increases efficiency by identifying multiple species in one step, whereas clinical laboratories, using culture methods, often do not identify a *Campylobacter* isolate to the species level (Lawson *et al.* 1999). This results in a lack of data relating to the prevalence of individual *Campylobacter* species and mixed infections.

The initial step in the development of an enrichment-PCR assay required the design of specific PCR primers for *C. jejuni* and *C. coli* and optimisation of the multiplex PCR. The specificity of the PCR assay was confirmed by the lack of cross reactivity and non-specific amplicons when the multiplex PCR was tested against a range of *Campylobacter* species and other bacteria. The confirmation of *C. jejuni* and *C. coli* detection required the presence of two amplicons, one based on the thermotolerant group of campylobacters and the second being specific for either *C. jejuni* or *C. coli*. This allowed for confirmation of identity at the genus and species level.

The variation in numbers of campylobacters in environmental matrices necessitated the use of enrichment techniques to overcome environmental stresses and facilitate growth of the target organisms. Comparative studies of direct plating and enrichment of samples including human, cattle and sheep faeces have reported lower recoveries from direct plating compared with enrichment techniques. (Maher *et al.* 2003, Madden *et al.* 2000, Stanley *et al.* 1998c, 1998b, Atabay and Corry 1998). Waage *et al.* (1999) noted that direct PCR of food samples produced variable results in comparison to enrichment prior to PCR. They recommended the use of an enrichment step for food analysis to increase the sensitivity of the PCR method and overcome the non-reproducibility of results. In developing a single method that would detect *Campylobacter* in a diverse range of matrices, many factors were taken into consideration, some of which are outlined below.

Many environmental matrices contain substances that inhibit the PCR. Humic substances in water and components in food and faecal samples, such as complex polysaccharides, as well as the blood in enrichment media can all contribute to inhibition (Maher *et al.* 2003, Waage *et al.* 1999, Waegel and Nachamkin 1996, Rossen *et al.* 1992). Enrichment broths can aid dilution of inhibitors and washing of enriched cells prior to PCR can remove remaining inhibitory compounds.

Consideration of factors such as these led to the conclusion that an initial enrichment of the sample would increase the reproducibility of results across all matrices. Selection of a suitable enrichment medium is difficult and, as noted by Madden *et al.* (2000), the choice of broth will take into account overall rate of recovery and ability to enrich different species and subtypes of *Campylobacter*. A trial of five different enrichment broths in combination with all matrices was performed and m-Exeter broth was determined to be the optimal broth.

The incubation temperature of 42°C is selective as it is optimal for thermotolerant campylobacters (Griffiths and Park 1990) and inhibitory for other microflora. In addition, a pre-incubation period of four hours at 37°C was incorporated into the method as it has been shown to be efficacious in the recovery of injured *Campylobacter* cells (Waage *et al.* 1999, Humphrey 1986). Antimicrobials in the enrichment medium reduced competition from fungi and other bacteria but may also inhibit the growth of injured campylobacters (Mason *et al.* 1999, Humphrey and Cruickshank 1985).

Sensitivity tests of the enrichment-PCR assay showed that in most matrices less than ten cells per sample of either *C. jejuni* or *C. coli* could be detected in the sample. Rabbit faeces was the only matrix where sensitivity was greater than ten cells (range 3-32 cells of *C. coli*), but this was comparable with the range determined by the conventional method for *C. coli* cells in the same matrix. The enrichment-PCR method had similar sensitivity levels to the conventional plating method for identifying *Campylobacter*, but had the advantage of reducing the time required for identification from a maximum of ten days by the conventional method to 4-5 days. In food laboratories, due to a limitation of resources, only a few colonies (five or less) per plate are tested for confirmation by phenotypic techniques. The comparative assay performed in this study showed the advantages of PCR detection where a higher proportion of cells present in the enrichment were represented in the sample, allowing increased detection of both *C. jejuni* and *C. coli* in the same sample.

Scates *et al*. (2003) compared the diversity of subtypes isolated when employing two incubation temperatures (37°C versus 42°C) with the same broth and noted that although the prevalence and *Campylobacter* species identified were similar for both temperatures, a different range of genotypes was identified. They recommended employing two different incubation temperatures to increase the diversity of subtypes from a matrix. It should be noted, however, that Scates *et al*. (2003) did not employ an initial incubation of 37°C for four hours prior to incubating at 42°C for a further 44 hours, as employed in the current study. This initial 37°C incubation is incorporated to aid recovery of those *Campylobacter* cells that are injured or stressed. It would, therefore, be interesting to re-evaluate the diversity of genotypes isolated at the two temperatures when an initial 37°C recovery period is employed prior to further incubation at 42°C.

The time period for the enrichment-PCR method is longer than the rapid methods of direct PCR and real-time quantitative PCR (RTQ-PCR). Also, samples were enriched to increase sensitivity, which negates quantification of the initial concentration of *Campylobacter* cells. It would be possible, however, to modify the multiplex PCR developed for this study to incorporate a RTQ-PCR assay by designing probes for each of the PCR primer sets. This would require validation of the RTQ-PCR against all of the matrices included in the current study, but would provide concurrent data on the concentration of target campylobacters in samples, in addition to their prevalence in matrices.

The main benefits of the enrichment-PCR method reside in the employment of a single enrichment broth for detection of *C. jejuni* and *C. coli* in 13 environmental matrices. Most assays have been validated against a small range of matrices, such as poultry carcasses (Hong *et al.* 2003), bovine faeces (Inglis and Kalischuk 2004, 2003), water (Kirk and Rowe 1994) and human faeces (Maher *et al.* 2003), although Yang *et al.* (2003) have developed a RT-PCR method for the detection of *C. jejuni* in poultry, milk and environmental waters.

Scates *et al*. (2003) noted that identification of campylobacters throughout the food chain requires use of the same method with each matrix to allow genuine comparisons that aid epidemiological studies. This is because the presence/absence of a subtype in a matrix is partially reliant on the method employed. Each method has its own intrinsic biases that may allow the growth of one subtype over another.

The enrichment-PCR assay, therefore, allows the efficient identification of potentially pathogenic campylobacters in a wide range of environmental matrices. Employment of a single enrichment broth for 13 matrices will assist the large surveys required to better understand the transmission of *Campylobacter* through the environment to humans. In conclusion, the first hypothesis was confirmed, in that a robust enrichment-PCR assay was developed to detect and identify pathogenic *Campylobacter* from a range of environmental matrices.

6.2 Prevalence of *Campylobacter* in chicken carcasses and water.

Validation of the enrichment-PCR was accomplished by application of the method to field studies to test the prevalence of *Campylobacter* in two environmental matrices: chicken meat and river water. To this end, whole chicken carcasses were sampled at the point of purchase to the consumer. Sampling from retail outlets allowed a comparison of rates of *Campylobacter* isolation with previous New Zealand and international studies. A river system that flowed through land stocked by sheep, cattle and dairy cows was chosen as the site for testing river water for *Campylobacter* prevalence. The multiple inputs of *Campylobacter* from animals and wildlife received by this river system also made it an ideal site for determining if the enrichment-PCR was able to identify multiple subtypes in an individual water sample.

The prevalences of *C. jejuni* and *C. coli* in retail chickens were lower than those obtained from previous studies in New Zealand, but similar to international studies in Denmark, Finland and

the Netherlands. Differences between New Zealand studies may be due to this survey obtaining retail chickens from a major supplier for the country who is expected to have improved techniques that minimise the contamination of chicken carcasses during processing. Another point of difference is that this survey was conducted on whole fresh chickens rather than chicken portions, which, due to the increased handling, might be expected to be exposed to more cross contamination events than the prepackaged whole chicken. Subsequent surveys using the enrichment-PCR method have shown high prevalences of *Campylobacter* in a variety of environmental matrices which included minced chicken, sheep liver and ruminant faeces (Wong *et al*. 2005, Devane *et al*. 2005).

As has been noted internationally, there are no standardised methods for *Campylobacter* isolation, which has limited the ability to compare data between surveys (Rosef *et al.* 2001, Corry *et al.* 1995, Humphrey *et al.* 1995). This lack of standardised methods was part of the rationale behind development of the enrichment-PCR method for a wide range of environmental matrices. The wide variation in prevalence identified in chicken may be due to differences in sampling geography (i.e. local surveys compared to nationwide surveys), differences in methods of isolation, and differences in cuts of meat surveyed.

The prevalence of *C. jejuni* in river water was similar to international findings and previous studies of New Zealand rivers. The low prevalence of *C. coli,* although supported by some international literature, is also in contrast to other overseas studies where *C. coli* has been identified at higher prevalences than *C. jejuni*. This discrepancy between studies may be a reflection of the differences in the animal types contributing to the *Campylobacter* load in water. For example, pigs are known to harbour a higher prevalence of *C. coli* compared with *C. jejuni*, therefore the siting of a pig farm near a river system could affect the *Campylobacter* species identified in nearby waterways (Guevremont *et al.* 2004, Moore and Madden 1998). As expected from overseas studies the identification of *Campylobacter* followed seasonal trends with lower prevalence in warm summer water with high sunlight levels in comparison to winter conditions.

In conclusion, this work supports the hypothesis that a robust enrichment-PCR assay was developed to detect and identify pathogenic *Campylobacter* from chicken and water samples.

6.2.1 Detection of multiple subtypes by the enrichment-PCR method

The typing of bacterial isolates from various sources and determining their relative contribution to human infection is a prerequisite for the investigation of the transmission routes of a pathogen. It also allows the detection of changes in infectious disease aetiology. Furthermore, the distribution of virulence determinants in *C. jejuni* subtypes might be more accurately represented if dominant and minor subtypes from potential reservoirs are isolated from the same sample. Recognition of the importance of multiple subtypes of campylobacters present in a sample is only beginning to emerge.

Multiple subtypes identified on chicken carcasses add to the knowledge about the host specificity of *C. jejuni* subtypes and whether all subtypes identified in chickens contribute to human infection. Therefore, the identification of a single isolate per sample limits the information available to trace the source of a campylobacteriosis incident. As most cases of campylobacteriosis are recognised as being sporadic, this information may suggest that at least some of these sporadic cases are not recognised as being linked because not all of the available subtypes have been identified from either the clinical faecal specimens or the suspected transmission vehicle. This has become more relevant as evidence grows of multiple infections of *Campylobacter* in animal hosts. Flocks of chickens have been shown to carry multiple subtypes (Hiett *et al.* 2002) and evidence is emerging that individual chickens harbour multiple subtypes of *C. jejuni* (Schouls *et al.* 2003, Thomas *et al.* 1997). In addition, co-infection in human cases by two subtypes of the same *Campylobacter* species has been observed, albeit as a rare event (Steinbrueckner *et al.* 2001, Richardson *et al.* 2001).

In a study of *E. coli* strains from human faeces, Schlager *et al.* (2002) discussed the importance of multiple isolations from a single stool sample to identify both dominant and minor strains. They described a dominant strain as a clone that was represented by >50% of typed isolates in a sample. A minor strain was defined as a clone that represented <10% of typed isolates in a sample. They used a binomial formula to determine the number of randomly selected colonies required to achieve a 90% probability of identifying a minor clone. Schlager *et al.* (2002) caution that basing a study on the isolation of dominant clones of a bacterial species may exclude significant information, especially where the study is examining the frequency of virulence factors in a bacterial population.

The second hypothesis in this study stated that chicken carcasses carry multiple subtypes of *C. jejuni*. Prior to testing this hypothesis it was important to confirm that the enrichment-PCR method was capable of detecting multiple subtypes in a sample and that certain strains did not dominate the enrichment process. Whether a particular subtype is dominant in an individual sample may be dependent on various factors including its ability to compete with other microflora, and this capacity will be partly based on its genomic repertoire of survival strategies (Moen *et al.* 2005, Murphy *et al.* 2003). Another factor is the subtype's concentration in the water matrix at the time of sampling, including the numbers of injured cells of that particular strain.

Validation of this method required testing on a matrix known to carry multiple subtypes of *Campylobacter*. The matrix chosen was river water flowing through farmland as it was expected to have multiple inputs of campylobacters from varied sources including farm animals, feral animals and birds. Thus a river water sample was considered likely to contain more than one subtype of either *C. jejuni* or *C. coli*.

In all of the water samples that tested positive for *C. jejuni* (5 of 6), more than one subtype was identified, with a maximum of three subtypes found in one sample. One subtype was identified as dominant in each sample but, importantly, each subtype that was identified as being >85% of the typed colonies in one sample, was also identified as a minor subtype (6.7-8.3%) in another. This suggests that these subtypes were not selected preferentially over other strains, and that the method allowed for the detection of a wider range of subtypes when they were present. This suggestion, however, requires further confirmation to establish that the method is not selecting a limited range of subtypes. This could be achieved by testing a larger sample size from water and analysing the number of distinct subtypes identified.

Identification of multiple subtypes of *C. jejuni* in individual river water samples validates the ability of this method to test the second hypothesis: that chicken carcasses carry multiple subtypes of *C. jejuni*. In addition, the results supported the proposal that the enrichment-PCR method is facilitating the identification of different *C. jejuni* PFGE subtypes and indicated that an overlap of *C. jejuni* subtypes will be identified when sampling at different time periods. It is reasonable to assume, therefore, that the isolation regime of selecting one colony per sample over an extended temporal survey will reveal a variation of *Campylobacter* subtypes. This

would result in an accurate assessment of the *Campylobacter* subtypes present in an environmental matrix. The greater the number of *Campylobacter* subtypes detected, the higher is the likelihood of establishing potential transmission routes of *C. jejuni* and *C. coli* from the environment to humans. This assessment of a single colony per sample, in contrast to multiple isolates, may not hold true when investigating sporadic cases of campylobacteriosis where the aim is to establish a source of infection. The issue of isolate numbers from a single sample will be discussed further in the proceeding section.

6.2.1.1 Identification of multiple subtypes of C. jejuni in chicken carcasses

In this study multiple subtypes of *C. jejuni* were identified on individual chicken carcasses using the enrichment-PCR method. Subtyping was determined by PFGE typing using *Sma*I as the initial enzyme and *Kpn*I as the secondary enzyme for further discrimination of subtypes with the same *Sma*I profile. An average of 23 colonies were analysed by *Sma*I PFGE per sample and, based on the binomial formula of Schlager *et al.* (2002), this would achieve a 91% probability of identifying a minor clone. Although the cutoff for determining an indistinguishable subtype was set at 90% (Nadeau *et al.* 2003, de Boer *et al.* 2000), the average cutoff for *Sma*I digests was 99.6% and 98.1% for *Kpn*I digests demonstrating that technical reproducibility was high.

Five of the ten carcasses revealed multiple subtypes. Fifteen distinguishable subtypes were identified from the ten carcasses, and this included the identification of subtype Sm0030/Kp0056 in three different chicken samples. In the five chicken carcasses that were identified with multiple subtypes, two subtypes were identified in three carcasses and three subtypes in two carcasses. The second hypothesis was upheld, therefore, as chicken carcasses were observed to carry multiple subtypes of *C. jejuni*.

It was interesting to note that the literature reported differences in the numbers of subtypes identified in individual samples by various methods. Lindmark *et al*. (2004), using enrichments, did not identify more than one subtype per chicken carcass when typing up to five isolates per sample. In comparison, Dickins *et al*. (2002) employed direct plating from chicken carcasses to identify three to five subtypes in 22.6% of the *Campylobacter* positive carcasses (n = 39) by testing a mean of 6.2 isolates per sample.

Two other studies of direct plating from chicken faeces also revealed multiple subtypes of between three and four per sample when four and five isolates per sample (respectively) were typed (Schouls *et al.* 2003, Thomas *et al.* 1997). While faecal studies are not directly comparable to chicken carcasses, partly due to the generally higher numbers of *Campylobacter* in faeces, these two studies lend support to the case for investigating whether identification of multiple subtypes is aided by direct plating compared with enrichment. Studies using direct plating have noted higher numbers of subtypes carried by samples when on average less than seven colonies per sample were tested. In contrast, a maximum of three subtypes were identified in this study using the enrichment assay and testing up to 25 isolates per sample and only one subtype was identified in the enrichment study of Lindmark *et al*. (2004). It should also be noted that geographical differences between countries may also be responsible for less subtypes being identified per individual sample. For example, this study reports the first investigation into the presence of multiple subtypes in chicken carcasses in New Zealand.

It is probable, given the selective nature of the enrichment-PCR method that growth characteristics of each environmental *Campylobacter* subtype in the enrichment broth will vary. The degree of this variation is unknown and consequently, if the initial sample contained equal numbers of two subtypes, it is possible that one may grow faster and be identified by subtyping as being dominant due to its growth characteristics. The same premise will hold true for direct plating where injured/stressed cells have a lower probability of recovery in comparison to enrichment (Dickins *et al.* 2002). In reality, it is probable that the concentrations of each subtype in the original sample will be variable as was shown in this study, where the dominant subtype in a water sample was identified as a minor subtype in another water sample. It is recognised that the problem of dominant strains may be overcome by employing more than one procedure for each sample to enable identification of individual strains that vary under different growth conditions (Kramer *et al.* 2000).

None of these factors undermine the use of the enrichment-PCR as a tool for the long term isolation of campylobacters from a wide range of environments. It does suggest, however, that if studies are time limited and sample numbers are small then enrichment should be combined with direct plating or enrichment should employ two incubation temperatures (37°C and 42°C) as suggested by Scates *et al.* (2003). This may achieve a higher diversity of subtypes from individual samples with less input of labour, as fewer colonies per sample may need to be typed. In the case of direct plating the multiplex PCR developed in this study could be used to confirm

colonies as *C. jejuni* or *C. coli* by PCR identification directly from the plate. A comparative study of the diversity of subtypes isolated by enrichment versus direct plating may be worthwhile to establish the benefits of either method and to determine if it is better to use both methods and subtype only one or two colonies isolated from each.

6.3 Comparison of *C. jejuni* isolates from chicken carcasses and human clinical specimens

The high diversity of genotypes present in the *Campylobacter* population is well documented and the polymorphic nature of *Campylobacter* adds to the complexity when trying to deduce the source of an infection (Schouls *et al.* 2003, Duim *et al.* 1999). Analysis of DNA sequence data showed that this diversity is due to a high frequency of both intra- and inter-species recombination in *C. jejuni* (Suerbaum *et al.* 2001, Duim *et al.* 1999). Since multiple subtypes of *C. jejuni* are present in chickens it has been suggested that the subtypes causing disease in humans may be minor strains in chicken microflora and therefore are not identified by conventional bacteriological techniques where only a single colony is typed (Schouls *et al.* 2003).

The third hypothesis for this study was that all subtypes of *C. jejuni* found on chicken carcasses were also found in human faecal specimens. Therefore, if this hypothesis was confirmed it would imply that non-dominant subtypes in chicken meat are important in the aetiology of campylobacteriosis. This would suggest that isolation techniques need to be directed toward identifying both dominant and non-dominant subtypes in a sample.

In this study twelve of the 15 *Sma*I/*Kpn*I subtypes identified in chicken carcasses were not implicated in any of the human cases of campylobacteriosis identified, to date, in New Zealand. This finding contradicts the third hypothesis. Furthermore, these twelve subtypes were not identified in any other matrix, besides chicken carcasses. Three subtypes were identified in human clinical samples and two of those subtypes were identified in three and four human clinical samples each. These three subtypes, which have been implicated in human cases of campylobacteriosis, were identified in five of the ten chicken carcasses analysed for multiple subtypes in this study.

In addition, it was demonstrated that in the four samples that carried minor and/or intermediate subtypes, the non-dominant subtypes did not correlate with any isolates from human cases. In the fifth sample, which carried multiple *C. jejuni* subtypes that were both close to 50% prevalence and could be said to be co-dominant, one of these subtypes was identified in three human cases. This subtype (Sm0030/Kp0056) was also identified in two other chicken carcasses which were independent samples, having been collected at different times. In contrast to the suggestions of Schlager *et al.* (2002) and Schouls *et al.* (2003), these results imply that the non-identification of minor/intermediate subtypes using the enrichment-PCR method from chicken carcasses may not be limiting the information relevant to tracking the source of an infection to chicken carcasses. Confirmation of this suggestion regarding non-dominant subtypes would require a larger dataset of multiple subtypes collected from a large number of chickens and compared with human isolates from the same geographical area and timeframe.

6.3.1 Genotypic plasticity of *Campylobacter*

During this investigation, the question of the relevance of clonally related subtypes identified in the same sample was identified as warranting further research in relation to the information required for establishing the source of an outbreak (Steinbrueckner *et al*. 2001). Sample CPH012693 carried three *C. jejuni* strains, two of which were clonally related by the criteria of Tenover *et al.* (1995). One of the clonally related subtypes was identified in 82% of isolates for that sample; the other was intermediate in prevalence (12%) suggesting it would have a low probability of being identified. Therefore, if this intermediate subtype was identified in a human case it may not be correlated with the dominant chicken subtype, which highlights the importance of examining identical and closely related banding patterns.

Another example was Sm0030/Kp0056, the subtype identified in three chicken carcasses. The PFGE profile of a human isolate had one band difference to Sm0030 and two band differences to Kp0056 suggesting it was clonally related to the chicken subtype. This tentatively increased the number of human isolates to four for the subtype Sm0030/Kp0056, and raised the question as to whether, in an outbreak investigation, the two subtypes would have been recognised as clonally related. Further genotypic and phenotypic characterisation of the chicken subtype Sm0030/Kp0056 and the clonally related human isolate may be required to determine their degree of relatedness. The genotypic plasticity inferred by these isolates and their putative relatedness must be kept in context as Sm0030/Kp0056 was isolated from two other chicken

samples in this study that were collected at different times, the closest interval between the three samples being six weeks. Based on the times of collection, this subtype has shown stability as it was isolated from different chicken carcasses over a four month period. Furthermore, its isolation from three human clinical samples and a variety of matrices suggests it is stable in the environment. This does not, however, negate mutational or recombinational events occurring during host passage which could have led to the genetic divergence noted in the subtypes above.

The passage of *C. jejuni* in the laboratory has, in general, revealed few changes as shown by genotypic typing schemes (Dickins *et al.* 2002, Wassenaar *et al*. 1998). A new study (Gaynor *et al.* 2004) has shown, however, that there maybe changes in colonisation potential of a susceptible host during laboratory passage that are not identified by genotyping schemes such as PFGE and MLST. The comparative study of Gaynor *et al.* (2004) investigated the differences between the genome-sequenced variant of strain NCTC 11168 (designated 11168-GS) and the original strain (11168-O) that had been frozen since it was first isolated from a patient by Martin Skirrow in 1977. It was noted that 11168-GS had reduced potential to colonise one-day–old chicks in comparison to the parent strain 11168-O which retained its ability to colonise.

The colonisation differences observed between the two variant strains of NCTC 11168 provided a useful platform for the study of Gaynor *et al.* (2004) to assess virulence determinants significant in host colonisation. The study employed micro-array based transcriptional profiling to screen the bacterial variants and determine minor genomic differences that had occurred but were not detected by high resolution genotypic techniques such as MLST. These minor genomic differences translated into differences in gene expression when the two strains were grown under microaerophilic and anaerobic conditions which affected virulence-associated phenotypes such as motility, and invasion and translocation into host cells. Targeted sequencing revealed single nucleotide polymorphisms in genes encoding each sigma factor (a protein component of RNA polymerase that is important for the initiation of transcription). Furthermore, differences in expression were shown in genes associated with respiration and metabolism, affecting adaptation to environments with varying oxygen tension. The authors suggested that during laboratory passage 11168-O had adapted to survive exposure to aerobic environments, which compromised its ability to readapt to the anaerobic environment of the host intestine. It is a new finding that adaptation to different oxygen environments is an important factor in colonisation potential. The results of Gaynor *et al*. (2004) suggest caution when applying genotyping

techniques to the identification of passaged laboratory strains relevant to the study of virulence-associated phenotypes.

A study that employed transcriptional profiling investigated the expression of genes necessary for colonisation of one-day-old chicks by *C. jejuni* (Woodall *et al.* 2005). The study identified 59 genes that were differentially expressed *in vivo* in comparison with *in vitro* expression. These genes included those that regulate electron transport allowing *C. jejuni* to adapt to the low oxygen conditions found in the chick caecum. These findings supported Gaynor *et al*. (2004) in suggesting that adaptation to different oxygen environments is important for colonisation potential.

The observations of Gaynor *et al*. (2004) are also relevant to the genetic stability of subtypes during passage through the host (human or animal) and whether the virulence potential of a strain is compromised or enhanced by genetic events that occur *in vivo*.

Based on the observation that the minor and intermediate subtypes in this study were not prevalent in subtypes important in human infection, it may suggest that factors leading to recombination events within a chicken intestine may lead to avirulent campylobacters which do not colonise the human intestine as successfully. Confirmation of this hypothesis, however, also requires understanding of the direction of genetic change between clonally related subtypes, because the converse could also be true. The direction of genetic change may be from the minor to dominant subtype, which becomes more successful in both chicken and human colonisation. Investigating differences in virulence potential and the direction of genetic change between clonally related subtypes could involve monitoring the population changes that occur when a dominant and non-dominant subtype identified in the same chicken/chicken product or in a flock of chickens are inoculated individually into different one-day-old chicks.

From the methods used in this study, the direction of genetic change cannot be ascertained between a minor and dominant subtype. Schouls *et al*. (2003), employing MLST to investigate the host range of *Campylobacter* subtypes, recognised that inter- and intra-recombination events were approximately 50 times more frequent than mutational events in the *Campylobacter* genome. MLST can be used to track the lineage of clonal complexes and therefore could be useful in exploring the changes within clonally related subtype populations (Feil *et al*. 2004, Suerbaum *et al.* 2001, Dingle *et al.* 2001a). From the findings of Gaynor *et al*. (2004), however,

it may be necessary for MLST studies to target those genes that have shown differences during transcriptional profiling of (a)virulent strains. It is also worth noting that the definition of dominant and minor may be dependent on the isolation method employed and/or the initial numbers of each respective subtype in the original sample. Therefore future comparisons of multiple subtypes in a sample should include quantitative studies such as those afforded by real-time PCR.

More research is required to investigate whether the identification of subtypes that are clonally related to an outbreak subtype are important in establishing the source. For example, if only one of two clonally related subtypes in a chicken sample was detected but the other subtype was identified in a clinical isolate within the same timeframe and locality, would the epidemiological information link the two subtypes? The advent of computer-assisted analysis allows better tracking of subtypes that are distinguishable but clonally related. This will, however, require recognition by public health researchers that these related subtypes may be relevant to the identification of the source of an outbreak.

6.3.2 Chicken as a vehicle for transmission of *Campylobacter* to humans

The large number of subtypes from chickens (12 of 15), that were not identified in human cases raises the question as to whether those subtypes are adapted to grow in chickens, as they were also not identified in any other matrix than chickens. In contrast, five of the ten chicken carcasses did carry three subtypes of *Campylobacter* identified in human cases of campylobacteriosis, suggesting that it may be a small proportion of subtypes identified on chicken carcasses that predominate in human cases attributed to chicken consumption. Further investigation of multiple isolates from a larger sample size of chicken carcasses would be required to confirm this proposal. Validation of this suggestion would emphasise the need to study these pathogenic campylobacters for virulence factors that increase successful infection in humans over the majority of other subtypes carried by chicken carcasses.

The chicken carcass isolates studied in this research were collected from a distinct geographical area and within a seven month interval. The human isolates (n = 376) used for comparison with these chicken isolates were collected nationwide, although 61 of these isolates originated from the same defined area and within a one year time period, which included the same seven month period as the chicken sampling. The prevalence of *Campylobacter* (28.5%) detected in the chicken carcasses from this defined region was lower in comparison with previous New Zealand

studies (54-60%), however one could still have expected a higher correlation of the 15 chicken subtypes with 376 human isolates.

Identification of non-dominant chicken subtypes, in this study, did not clarify the importance of whether chicken acts as a reservoir and vehicle for the transmission of *Campylobacter* to humans. The lack of correlation between non-dominant subtypes in chicken carcasses and human cases, particularly the 61 cases from the same region and timeframe, is unexpected when it is generally accepted that 40-70% of cases are attributed to chicken consumption. Another factor, however, that may have affected this outcome was the rural nature of the region where this study was conducted. The fact that five of the ten chickens were contaminated with subtypes implicated in human campylobacteriosis but only one of those human cases was identified within the study region may suggest other routes of transmission of campylobacters for this area. Human cases were mainly derived from abattoir workers, farmers, their families and farm workers, and from the largest town in the area, which had a population of 14,000. Subsequent analysis of human cases and subtypes isolated from the environment revealed that the highest similarity to human cases was with subtypes isolated from ruminant animals (sheep, dairy cows and cattle) (Devane *et. al*. 2005, Baker *et al.* 2002). The higher similarity between human and ruminant isolates may suggest that the close contact between farm/abattoir workers and the animals they work with is a more likely route of *Campylobacter* infection in comparison with chicken consumption for this rural community. This premise is further supported by a review of *Campylobacter* infection in poultry workers, which concluded that there is an increased risk (three fold) of poultry workers developing campylobacteriosis compared to the general population, particularly during their first months on the job (Wilson 2004).

A study in Finland typed human isolates (n = 176) from two geographically distinct rural and urban areas and found that out of the 69 PFGE subtypes identified in the two areas, only nine were identified in both regions. This suggests geographic differences for sources of infection between these two areas (Hänninen *et al.* 1998). In the Finland study, isolates of *C. jejuni* from chicken faeces (n = 48) and chicken meat (n = 25) were collected at the same time as the human isolates, and subtypes compared. Although overall subtype diversity was high for the human isolates, there were five predominant subtypes identified in the urban area comprising 42% of isolates, and another five predominant subtypes (three of which were also in the urban predominant group) that comprised 44% of isolates from the rural area. Interestingly, four of the five predominant subtypes identified in the urban area were found in chickens and only two of

the five predominant in the rural area were identified in the chicken isolates. This supports a possible distinction between routes of infection in rural and urban environments.

The results of this study do not support the suggestion that the non-identification of minor subtypes in chicken carcasses is preventing identification of campylobacteriosis outbreaks attributed to chicken consumption. The small sample number of chicken carcasses, however, may be a limiting factor in the information generated by this study and larger surveys are required to clarify these findings. If it is to be proven that the number of outbreaks due to *Campylobacter* is underreported, the most likely approach to confirm this supposition would require a concerted effort to combine epidemiological information with subtyping data. This would have to be undertaken so as to overcome the delays between identification of indistinguishable subtypes and interviews with campylobacteriosis sufferers.

6.4 Conclusions

In accordance with the first hypothesis, a robust enrichment-PCR assay was developed to detect and identify pathogenic *Campylobacter* from 13 environmental matrices that included human, duck and animal faeces, meat products and river water. Application of the enrichment-PCR method to water and chicken carcass samples identified multiple subtypes of *C. jejuni* in individual samples in each matrix, which confirmed the second hypothesis. The third hypothesis was repudiated in that not all of the subtypes of *C. jejuni* identified on chicken carcasses were identified in human faecal specimens. This latter point has implications for host specificity suggesting that there may be subtypes that are adapted to colonisation of chickens but have low potential for pathogenicity in humans. These subtypes could be further investigated to determine if they lack virulence factors in comparison to human isolates. Such a finding could have implications for the design of PCR markers to identify virulent subtypes that are of infectious significance to the consumer.

Results suggest that the non-identification of minor/intermediate subtypes carried by chicken carcasses was not hindering the identification of a source of *Campylobacter* infection attributed to chicken consumption in this study. However, a larger number of chicken carcasses would need to be surveyed for multiple *C. jejuni* subtypes to confirm this suggestion. Although there was a low correlation between chicken isolates and subtypes identified in human clinical samples, five of the ten chickens carried a pathogenic subtype of *C. jejuni*. Correlation of

human and chicken isolates of *C. jejuni* suggested that PFGE subtype analysis needs to include recognition of subtypes that are clonally related as observed genomic plasticity during host passage can lead to small changes in PFGE subtype. Non-recognition of these minor changes may hinder identification of an outbreak source. Investigation of the direction of genetic change between clonally related subtypes identified in the same sample may provide further insight into adaptation of the bacterium to its environment and whether colonisation in an animal/bird host enhances virulence in humans. From the results of this study it is suggested that a comparative study between direct plating and enrichment methods is conducted to ascertain whether the use of two methods is more efficient and would reveal a higher correlation of environmental subtypes with human isolates.

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9 Appendix I

Media were sterilised at 121°C, 103.4 kPa for 15 minutes.

Brain Heart Infusion (BHI) Broth

Typical formula	per 500 ml
Beef Heart infusion (Difco)	12.5 g
Calf brain infusion	10.0 g
Protease peptone	5.0 g
NaCl	2.5 g
Na_2HPO_4:$12H_2O$	1.25 g
Glucose	1.0 g

Brain Heart Infusion (BHI) Broth containing 20% Glycerol

Brain Heart Infusion Broth (Merck 1.10493)	3.7 g
Glycerol (BDH # 10118 4K)	20 ml
Deionised water	80 ml

Weigh the required amount of broth into a Schott bottle and add the water. Mix thoroughly to dissolve broth, microwaving if necessary. Autoclave at 121°C for 15 minutes. Allow to cool and check the pH is 7.4 $\pm$ 0.2. Store at 2-8°C for up to 3 months.

Brucella Broth

Brucella broth (Difco 0495-17-3)	28 g
Distilled Water	I L

pH = 7.0 ± 0.2 at 25ºC

Mix thoroughly to dissolve and dispense 500 ml into screw capped bottles. Autoclave at 121°C for 15 min. Temper to 50°C in a waterbath. Aseptically add Antibiotic Supplement SR117E

(Oxoid) 1 vial per 500 ml (reconstitute by adding 2 ml of a 50:50 solution of acetone and sterile distilled water per vial).

m-Exeter medium

Nutrient broth No. 2 (Oxoid) made according to instructions per one litre volumes. After autoclaving, add the following per litre: 50 ml lysed horse blood, 5 ml filter-sterilised solution containing 4% sodium metabisulphite, 4% sodium pyruvate and 10% iron sulphate solution, (aseptically dispense solution in 5 ml amounts and store in the freezer) 15 mg cefaperazone, (add 2ml litre^{-1} of filter sterilised stock solution, 7.5 mg ml^{-1}) 2 vials Oxoid supplement SR117E.

Each vial of supplements supplies 2500 i.u. polymixin B, 5 mg rifampicin, 5 mg trimethoprim and 50mg actidione. These components vary from the "Exeter" formulation by the inclusion of actidione, but it provides the convenience of the commercial availability of the antibiotic supplement.

m-Exeter agar

Add 15 g of agar to a litre of nutrient broth No. 2 and boil to dissolve before autoclaving. Proceed as above for m-Exeter medium.

Nutrient broth No.2

Nutrient broth No 2 (Oxoid CM67)	25g
Distilled water	1 L

Mix thoroughly to dissolve and dispense 500 ml into screw capped bottles. Autoclave at 121°C for 15 min. Temper to 50°C in a waterbath. Aseptically add Antibiotic Supplement SR117E (Oxoid) 1 vial per 500 ml (reconstitute by adding 2 ml of a 50:50 solution of acetone and sterile distilled water per vial).

Preston Enrichment (PE) broth

Nutrient broth No 2 (Oxoid)	25g
Ferrous sulphate (BDH)	0.25g
Sodium metabisulphate (BDH)	0.25g
Distilled water	900 ml

Mix thoroughly to dissolve and dispense 450 ml into screw capped bottles. Autoclave at 121°C for 21 min. Temper to 50°C in a waterbath. Aseptically add the following:

Sodium pyruvate (BDH)	0.25g
Lysed horse blood	50 ml

Antibiotic Supplement SR117E (Oxoid) 1 vial per 450 ml (reconstitute by adding 2 ml of a 50:50 solution of acetone and sterile distilled water per vial).

Adjust pH if necessary to pH 7.5 ± 0.2.

TBSKY Broth and Media

TBSKY for cultivation of anaerobic *Campylobacter* species.

Trypticase Soy Broth	15 g
Brain Heart Infusion Broth	18.5 g
Yeast Extract (Oxoid LP0021B)	10 g
Hemin solution	10 ml
Distilled Water	Make to one litre

For agar plates: add 15 g l^{-1} agar

Autoclave 121^{o}C, 20 min.

After sterilising add 1ml/l Vitamin K solution (filter sterilised), and 5% defibrinated sheep's blood.

Hemin Solution (1%)

Hemin (Sigma #H2250)	50 mg
Dipotassium phosphate (anhydrous)	1.74g in 100 ml of distilled water

Boil to dissolve, aliquot and store at –20^{o}C.

Vitamin K Solution (water soluble Vit. K3)

Menadione sodium bisulfite (Sigma #M5750)	4 mg
Distilled Water	10ml

TSBKY agar plates were made by addition of 15g l^{-1} agar to the TSBKY broth.

Tryptic Soy Broth

Tryptic Soy broth (Difco 0370)	28 g
Distilled Water	I L

pH = 7.0 ± 0.2 at 25ºC

Mix thoroughly to dissolve and dispense 500 ml into screw capped bottles. Autoclave at 121°C for 15 min. Temper to 50°C in a waterbath. Aseptically add Antibiotic Supplement SR117E (Oxoid) 1 vial per 500 ml (reconstitute by adding 2 ml of a 50:50 solution of acetone and sterile distilled water per vial).

10 Appendix II

All solutions are prepared from Milli-Q water and sterilised (103.4kPa, 121°C) even if made using sterile Milli-Q water.
RT = room temperature

10.1 General reagents

Bovine serum albumin (BSA) preparation (2 mg ml^{-1})

Weigh out 100 mg BSA (Albumin, Sigma A-4503 from Global Science) into a Falcon tube
Add 50 ml sterile dd H_20 (2 mg ml^{-1})
Dissolve by shaking. Filter sterilise through a 0.2 µm filter
Dispense aseptically in 1ml aliquots into sterile 1.5 ml tubes
Store in freezer at -20ºC

Buffered Peptone Water 1% (BPW)

Peptone Water (Merck # 1.07228)	25.5 gram
Distilled Water	1 litre
pH	7.2

Autoclave at 121°C for 21 minutes

0.5 M EDTA, pH 8.0

	Per L
$Na_2EDTA.2H_2O$	186.1 g
10 N NaOH	~50 ml
Milli-Q water	made up to 1000 ml

1. Mix the EDTA with 800 ml of water.
2. Add 10 N NaOH slowly, checking the pH with a pH meter until the pH is 8.0. N.B. EDTA will not dissolve until close to pH 8.0.

3. Make the volume up to 1000 ml and then dispense 500 ml volumes in 1 L bottles.
4. Autoclave at 121°C for 15 min.

10 N NaOH

	Per L
NaOH	400 g
Milli-Q water	made up to 1000 ml

RT

1. Carefully dissolve NaOH in 800 ml of water.

CAUTION: extremely exothermic reaction. Perform in fume cupboard and cool the solution.

2. Cool to RT and make the volume up to 1000 ml.
3. Dispense 500 ml volumes in 1 L bottles and autoclave at 121°C for 15 min.

PBS

(Phosphate Buffered Saline)

Prepared from Oxoid tablets which are pH 7.3, 0.16 M NaCl (8 g l^{-1} = 0.8%), 0.003 M KCl, 0.008 M Na_2HPO_4 and 0.001 M KH_2PO_4.

Oxoid PBS Tablets	10
Milli-Q water	1 L

RT

1. Dissolve the tablets in the water.
2. Dispense into either 500 ml volumes in 1 L bottles or 100 ml volumes in 100 ml bottles.
3. Autoclave at 121°C for 15 min
4. Dispense 500 ml volumes in 1 L bottles and autoclave at 121°C for 15 min.

10 x TBE Buffer

(Tris-borate-EDTA buffer)

	Per litre
Tris-HCl (1M final conc.)	121.1 g

Boric Acid (1M final conc.)	61.83 g
0.5M EDTA (2mM final conc.)	4 ml
Milli-Q water	made up to 1000 ml

or purchase 10 x TBE powder from USB # 70454 which makes 200ml aliquots when reconstituted.

TE Buffer

(10 mM Tris:1 mM EDTA, pH 8.0)

	Per L
1 M Tris, pH 8.0	10 ml
0.5 M EDTA, pH 8.0	2 ml
Milli-Q Water	made up to 1000 ml

RT

1. Mix the solutions and dispense 500 ml volumes into 1 L bottles.
2. Autoclave at 121°C for 15 min.

1 M Tris, pH 8.0

	Per L
Tris -HCl	157.6 g
Milli-Q water	made up to 1000 ml

RT

5. Dissolve the Tris-HCl in 800 ml of water.
6. Let the solution come to RT.
7. Adjust the pH to 8.0 and then make the volume up to 1000 ml. Stir for at least one hour before rechecking the pH.

10.2 Hippurate reagents:

Sodium Hippurate

Prepare a 1% solution of Hippuric acid (sodium salt) Sigma #H9380 in distilled water. Dispense in 0.5 ml volumes in screw-capped tubes. eep frozen at –20ºC until needed.

Ninhydrin Solution

Prepare a 3.5% solution of ninhydrin (0.35g $10ml^{-1}$) in a 1:1 mixture of acetone and butanol.

10.3 Gel electrophoresis reagents

2% Agarose gel

1 g	Agarose	2%
5 mls	10 x TBE	1 x TBE
45 mls	dd H_20	
2.5 µl	Ethidium Bromide (10 mg ml^{-1} stock)	0.5 µg ml^{-1}

Prior to heating allow agarose to swell in water for 15 minutes.
Gradually heat in microwave until all agarose has dissolved.

DNA Ladder

Make stock solution:

1kb plus DNA (1.0 µg $µl^{-1}$) (Gibco # 10787-018, Life Technologies)	30 µl
1 x TBE Buffer	150 µl
Loading Dye	40 µl

Aliquot into Eppendorf tubes and store at –20ºC

Ethidium bromide (10 mg ml^{-1})

Weigh 10 mg Ethidium bromide (Sigma E-8751) into an Eppendorf tube. Add 1 ml of dd H_20. Store at 4ºC.

Gel-loading buffer –for agarose gels

0.25% bromophenol blue (Sigma #B0128)
30% glycerol (BDH #10118 4K) in water
Store at 4ºC

1 x TBE (working TBE)

200 ml	10 x TBE	0.09M
1800 ml	dd H_20	
100 µl	Ethidium Bromide (10 mg ml^{-1} stock)	0.5 µg ml^{-1}

or dilute 10 x TBE stock solution 1:10 in distilled water.

10.4 Reagents for PFGE analysis

EC Lysis Buffer

50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl

	Per 200 ml
1 M Tris, pH 8.0	10 ml
0.5 M EDTA, pH 8.0	20 ml
10% Sarcosyl	20 ml
Milli-Q water	150 ml

RT

1. Measure the Sarcosyl, Tris, EDTA, and water into a 500 ml Schott bottle and mix gently. Autoclave at 121°C for 15 min.

1.4% Megabase Agarose in TE Buffer

For making embedded DNA agarose plugs

	Per 20 ml
Megabase Agarose	0.28 g
Sterile TE Buffer	20 ml

1. Weigh the agarose into a 250 ml Schott bottle
2. Add the TE buffer and swirl gently to disperse the agarose. Leave at RT for 15 min.
3. Microwave for 20 sec, mix gently and repeat heating for 10 sec intervals until the agarose is completely dissolved.
4. Equilibrate to 55-60°C.
5. This agarose should not be reheated more than once as it quickly loses gel strength.

1.0% Megabase Agarose in TBE Buffer

For gel casting

	Per 100 ml
Megabase Agarose	1.0 g
0.5 xTBE Buffer	99 ml

1. Weigh the agarose into a 500 ml Schott bottle
2. Add the TBE buffer and swirl gently to disperse the agarose. Leave at RT for 15 min.
3. Microwave for 20 sec, mix gently and repeat heating for 10 sec intervals until the agarose is completely dissolved.
4. Equilibrate to 55-60°C .

0.5 x TBE buffer

	Per 2 litre
10 x TBE (USB, Cleveland, #70454)	100 ml
Milli-Q water	1900 ml

Made up immediately prior to use.

10.5 Phenol Chloroform reagents for DNA extraction

Ammonium acetate 7.5 M

Dissolve 57.75 g of Ammonium acetate into 80 ml of dd H_2O. Adjust volume to 100 ml. Sterilise by filtration through a 0.22 μm filter.

Lysozyme (50mg/ml)

Dissolve 50 mg lysozyme (Sigma L-7651) in 1 ml sterile dd H_20. Aliquot and store frozen at -20°C.

20 mg/ml Proteinase K Solution

	Per 20 ml
Proteinase K	0.40 g
Sterile Milli-Q water	20 ml

RT

1. Weigh the Proteinase K into a sterile 30 ml vial and add the measured water.
2. Mix to dissolve and then filter through 0.2 μm filter into a sterile 30 ml vial.
3. Dispense 400 μl volumes into sterile 1.5 ml tubes.

10% Sarcosyl

(N-Lauroylsarcosine, sodium salt. Available from Sigma L-9150)

	Per 100 ml
Sarcosyl	10 g
Sterile Milli-Q water	90 ml

1. Carefully add the sarcosyl to the water in a sterile container.
2. Dissolve by mixing gently and warming to 50-60°C.

Sodium Acetate 3M

Dissolve 12.3 g sodium acetate (Sigma S-2889) in 40 mls of dd H_20 and adjust pH to 5.2 with glacial acetic acid. Make up to 50 mls before autoclaving.

11 Appendix III

	Number Positive	Number Sampled	Proportion Positive (p)	Proportion Negative (q)	CIs for p Lower	CIs for p Upper
95% CI	0	10	0%	100%	0.0%	30.8%
	1	10	10%	90%	0.3%	44.5%
	2	10	20%	80%	2.5%	55.6%
	3	10	30%	70%	6.7%	65.2%
	4	10	40%	60%	12.2%	73.8%
	5	10	50%	50%	18.7%	81.3%
	6	10	60%	40%	26.2%	87.8%
	7	10	70%	30%	34.8%	93.3%
	8	10	80%	20%	44.4%	97.5%
	9	10	90%	10%	55.5%	99.7%
90% CI	0	10	0%	100%	0.0%	41.1%
	1	10	10%	90%	0.1%	54.4%
	2	10	20%	80%	1.1%	64.8%
	3	10	30%	70%	3.7%	73.5%
	4	10	40%	60%	7.7%	80.9%
	5	10	50%	50%	12.8%	87.2%
	6	10	60%	40%	19.1%	92.3%
	7	10	70%	30%	26.5%	96.3%
	8	10	80%	20%	35.2%	98.9%
	9	10	90%	10%	45.6%	99.9%
95% CI	0	15	0%	100%	0.0%	21.8%
	1	15	7%	93%	0.2%	31.9%
	2	15	13%	87%	1.7%	40.5%
	3	15	20%	80%	4.3%	48.1%
	4	15	27%	73%	7.8%	55.1%
	5	15	33%	67%	11.8%	61.6%
	6	15	40%	60%	16.3%	67.7%
	7	15	47%	53%	21.3%	73.4%
	8	15	53%	47%	26.6%	78.7%
	9	15	60%	40%	32.3%	83.7%
	10	15	67%	33%	38.4%	88.2%
	11	15	73%	27%	44.9%	92.2%
	12	15	80%	20%	51.9%	95.7%
	13	15	87%	13%	59.5%	98.3%
	14	15	93%	7%	68.1%	99.8%
90% CI	0	15	0%	100%	0.0%	29.8%
	1	15	7%	93%	0.0%	40.2%
	2	15	13%	87%	0.7%	48.6%
	3	15	20%	80%	2.4%	56.1%
	4	15	27%	73%	4.9%	62.7%
	5	15	33%	67%	8.0%	68.8%
	6	15	40%	60%	11.7%	74.4%
	7	15	47%	53%	15.9%	79.5%
	8	15	53%	47%	20.5%	84.1%
	9	15	60%	40%	25.6%	88.3%
	10	15	67%	33%	31.2%	92.0%
	11	15	73%	27%	37.3%	95.1%
	12	15	80%	20%	43.9%	97.6%
	13	15	87%	13%	51.4%	99.3%
	14	15	93%	7%	59.8%	100.0%

Table 18: Sampling Attribute Plan

Sampling Attribute Plan employed to calculate the sample size required to determine 95% confidence levels in the sample size for *Kpn*I digestion of 25 chicken isolates (refer to Chapter 4 for interpretation).

12 Appendix IV Subtyping systems

12.1 Description of phenotypic subtyping systems

Table 19 describes the phenotyping methods referred to in this thesis. References for this table were: Woodward and Rodgers (2002), McKay *et al.* (2001), Nielsen *et al.* (2000), Nielsen and Nielsen (1999), Ribeiro *et al.* (1996), Patton *et al.* (1991).

Table 19: Phenotypic subtyping systems

Subtyping Method	Target	Brief Description
Antibiotic Resistance Typing [also known as multiple drug resistance (MDR)]	Gene(s) that confer resistance to antimicrobial agent(s)	Bacteria are subjected to increasing concentrations of an antimicrobial agent to determine their susceptibility/resistance to the target antibiotic. A profile of the organisms susceptibility to various agents is used to compare with other isolates.
Penner Serotyping	Heat stable antigens on bacterial surface	Passive haemagglutination to differentiate *Campylobacter* strains on the basis of soluble heat-stable (HS) antigens
Laboratory of Enteric Pathogens (LEP)	Heat stable antigens on bacterial surface	Modification of Penner serotyping system which uses absorbed antisera in an effort to overcome cross reactivity associated with the Penner scheme
Lior serotyping	Heat labile antigens on bacterial surface	Slide agglutination procedure using live bacteria together with unabsorbed and absorbed antisera
Multi Locus Enzyme Electrophoresis (MLEE)	Isoenzymes i.e. alleles of the same enzyme	Protein extracts are electrophoresed through starch gels and screened for various enzymes. The different mobilities of each enzyme are dependent on allelic differences.
Plasmid Profile Analysis	Plasmids	The presence /absence of plasmids is ascertained for each bacterial isolate (based on a plasmid size library for each bacterial species)
Phage subtyping	Bacteriophage	The susceptibility to lysis by a panel of phage is ascertained for each bacterial isolate

12.2 Description of genotypic subtyping systems

Table 20 briefly describes the principles of genotyping methods referred to in this thesis. References for this table were Ribot (2002), Steinbrueckner *et al.* (2001), Fitzgerald *et al.* (2001a), Nielsen *et al.* (2000), Wassenaar and Newell (2000), Garaizar *et al.* (2000), Hernandez *et al.* (1996), Patton *et al.* (1991).

Table 20: Gentoypic subtyping systems

Genotypic Subtyping Method	Target	Brief Description
Pulsed-Field Gel Electrophoresis (PFGE)	Entire Genome	Restriction enzyme (RE) cleavage followed by DNA separation on agarose gel. Different DNA cleavage patterns are indicative of strain variation.
Denaturing Gradient Gel Electrophoresis (DGGE)	Entire genome or specific gene (e.g. flagellin)	RE Cleavage of DNA is followed by denaturing gradient gel electrophoresis which detects differences in the melting behaviour of small DNA fragments (200-700 bp) that differ by as little as a single base substitution
Restriction Fragment Length Polymorphism (RFLP)	Gene(s) specific (e.g. flagellin, *fla* typing)	PCR amplification of a specific gene(s) followed by RE cleavage and separation by electrophoresis. Different DNA patterns are indicative of strain variation.
Random Amplified Polymorphic DNA (RAPD)	Entire genome	PCR amplification using short random (non-specific) primers which amplify regions of the genome. The number and location of these sites varies for different strains of a bacterial species. Separation of the PCR products by electrophoresis generates different patterns which are indicative of strain variation.

Genotypic Subtyping Method	Target	Brief Description
Enteropathogenic repetitive intergenic consensus (ERIC) sequences	Entire genome	Similar concept to RAPD analysis but primers target specific repetitive sequences (ERICs) within the genome. Low stringency conditions are used for *Campylobacter* which do not contain highly conserved repetitive sequences that would allow high fidelity amplification.
Amplified Fragment Length Polymorphism (AFLP)	Entire genome	Restriction digestion of genomic DNA by two RE. PCR of the fragments by two primers based on the two RE sequences amplifies only those fragments flanked by both RE sites. One of the primers contains a fluorescent or radioactive label and PCR products are analysed on denaturing polyacrylamide gels. 80–100 bands are generated by this technique.
Multi Locus Sequence Typing (MLST)	Entire genome	Double stranded DNA sequencing of at least 7 conserved genes in an organism. Comparison of the allelic differences within each gene is indicative of strain variation.
Ribotyping/riboprinting	Multiple copies of ribosomal RNA gene (rRNA)	Cleaved genomic DNA is electrophoresed followed by Southern blot hybridisation with a probe specific for rRNA genes. NB *Campylobacter* contains only three copies of rRNA genes.