ANIMAL SOURCES OF HUMAN CAMPYLOBACTERIOSIS

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This thesis is dedicated to the memory
Of
My Beloved Father

ABSTRACT

New Zealand has one of the highest reported rates of *Campylobacter* infections in humans in the developed world. It is the single largest notifiable disease in all regions of the country. Consumption of poultry meat has been widely implicated both overseas and in New Zealand as the main cause of human infections. The potential contribution of other animals especially cattle and sheep is less well known. The present study was undertaken to fill this gap in knowledge.

Faecal samples from 300 cattle and 158 sheep were collected from local abattoirs and farms plus 50 samples from the sheep slaughterhouse environment and examined for the presence of thermophilic *Campylobacter* spp. *Campylobacter* spp. were isolated from 45% of the cattle, 44% of the sheep and 56% of the environmental samples. *C. jejuni* and *C. hyointestinalis* were the predominating species isolated from cattle followed by *C. coli* and *C. lari*. In sheep and in environmental samples from the sheep abattoir *C. jejuni* was the only species isolated. The isolation rate and the species of *Campylobacter* varied between beef and dairy cattle, bull and heifer calves, age of the heifer calves, and time of the year. The high isolation rate of *Campylobacter* from the cattle, sheep and their environment strongly suggests the possibility of these micro-organisms finding their way into milk and meat, as faecal contaminants at the farm and slaughter level. There is also the potential to contaminate the environment and water following disposal of abattoir effluents and run off from farms.

The species of the isolates from human diarrhoeal cases were found to be predominantly *C. jejuni* (95%) and *C. coli* (5%). Molecular typing of *C. jejuni* using *Sma* I generated pulsed-field gel electrophoresis (PFGE) profiles yielded 13 to 16 different patterns in the cattle, sheep and human isolates showing a large inter-species variation in the isolates even from the same sources. However, indistinguishable as well as closely related profiles (pulsotypes) were found across the isolates from cattle, sheep and humans. The results obtained from the PFGE typing strongly indicate that cattle and sheep may be important reservoirs of human campylobacter infections. It was also observed that a few closely related types mostly dominate the *C. jejuni* populations in

the host animal species. The possibility of faecal contamination from these animals at slaughter and thus *C. jejuni* entering the meat was studied.

Retail packs of beef (25), lamb (25) and chicken (50) mince purchased from local supermarkets were examined. A combined selective enrichment and PCR based method was evaluated to offer a rapid, sensitive and specific detection method for the identification of *C. jejuni* from meats. *C. jejuni* was detected by culture and PCR in 44% of the chicken, 16% of the lamb and 12% of the beef mince samples. These results lend credibility to our contention that faecal contamination of sheep and beef carcasses at slaughter has significant implications for food safety. The much higher rate of detection in chicken mince may be related to a higher prevalence of infection in chickens or to the method of processing which may facilitate spread between birds and / or between product.

The *C. jejuni* isolates from the animal and human sources were also examined for antibiotic resistance by the disc diffusion method to antibiotics commonly used for the treatment of campylobacter infections in humans. No resistance was detected in the cattle and sheep isolates. Two human isolates exhibited resistance to tetracycline with MICs of >128 μg/ml. All other human isolates were found susceptible to the antibiotics tested. The nil to negligible resistance detected in the animal and human isolates of *C. jejuni* suggest that it is not a major problem in New Zealand at the present time however, further work is required to examine the situation in more intensively farmed species and monitor any changes in human isolates over time.

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LIST OF ABBREVIATIONS

PFGE	Pulsed-field gel electrophoresis
PCR	Polymerase chain reaction
NASBA	Nucleic acid sequence based amplification
CCDA	Charcoal-cefoperazone-desoxycholate agar
CCVA	Campylobacter-cefoperazone-vancomycin amphotericin
CSM	Charcoal selective media
HS	Heat stable
HL	Heat labile
FBP	Ferrous metabisulphite pyruvate medium
OMP	Outer membrane protein
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
DNA	Deoxyribonucleic acid
MEE	Multilocus enzyme electrophoresis
REA	Restriction endonuclease analysis
RNA	Ribonucleic acid
HACCP	Hazard analysis critical control point
RFLP	Restriction fragment length polymorphism
ERIC	Enterobacterial repetitive intergenic consensus
RAPD	Random amplified polymorphic DNA
REP	Repetitive extragenic palindrome
GBS	Guillain-Barre syndrome
ELGA	Enzyme linked gel assay
CBF	Campylobacter blood free agar
TSI	Triple sugar iron agar
EDTA	Ethylene diamine tetra acetic acid
BSA	Bovine serum albumin
CHEF	Contour clamped homogenous electric field
TBE	Tris borate EDTA
MIC	Minimal inhibitory concentration
DIG	Digoxigenin
bp	Base pair

CHAPTER 1

1.1. INTRODUCTION

The epidemiology of foodborne diseases is rapidly changing. In the past two decades, newly recognised pathogens such as *Campylobacter jejuni, Escherichia coli O157:H7* and *Yersinia enterocolitica* have emerged as important public health problems worldwide. Well-recognised pathogens such as *Salmonella* have increased in prevalence or become associated with new food sources and some pathogens are becoming increasingly resistant to antimicrobial agents. Many pathogens, including *Salmonella* spp., *Escherichia coli O157:H7, Campylobacter* spp. and *Yersinia enterocolitica* have reservoirs in healthy food animals from which they spread to an increasing variety of foods (Todd, 1997).

These pathogens cause millions of cases of acute, sporadic illness and chronic complications as well as large and challenging outbreaks in many countries worldwide. In the past the main challenge of foodborne disease lay in preventing the contamination of animal-derived food for human consumption. It is likely that in the future, prevention of foodborne disease will be increasingly dependent on controlling contamination of feed and water consumed by the animals themselves (Buzby and Roberts, 1997).

Evolving trends in foodborne diseases are being driven by the same factors that have led to the emergence of other infectious diseases including changes in the demographic characteristics of populations, human behaviour, industry and technology and the shift towards a global economy, microbial adaptation, and the breakdown in the public health infrastructure (Kaferstein and Abdussalam, 1999).

Many of the re-emerging or newly-recognised pathogens are foodborne or have the potential to be transmitted by food and/or drinking water. The emergence of "new" foodborne pathogens can be expected because of changing production methods and processes, food handling practices and eating habits. Foodborne illness of microbial origin is a serious problem in the United States with 79% of outbreaks between 1987-92 being bacterial and with improper holding temperature and poor personal hygiene of food handlers contributing most to the disease incidence (Collins, 1997). The global economy also, has facilitated the rapid transport of perishable foods, increasing the potential for exposure to foodborne pathogens from other parts of the world (Altekruse and Swerdlow, 1996).

However, an even greater challenge to food safety will come from changes resulting directly in degradation of sanitation and in the transformation of the immediate human environment. Changes include the increased age of the human population, unplanned urbanisation and migration, and mass production of food due to population growth and changed food habits (Kaferstein and Abdussalam, 1999).

A pathogen may emerge as an important public health problem because of changes in the pathogen itself or in its transmission pathways. Alternatively there may be changes in host susceptibility to infection. Factors influencing host susceptibility within the population as a whole include increases in the number of immunocompromised patients, increased use of immunosuppressive agents, ageing of the population and malnutrition (Morris and Potter, 1997).

A better understanding of how the pathogens persist in animal reservoirs and enhanced methods of laboratory identification are important in successful long-term prevention. Sensitive and timely surveillance that combines rapid subtyping methods, cluster identification and collaborative epidemiological investigations can identify and halt large, dispersed outbreaks.

Campylobacter species have long been recognised as a cause of illness and abortion in animals (MacFadyean and Stockman, 1913). In the last two decades, awareness of the

important role of *Campylobacter* species in human disease has been increasing (Healing *et al.*, 1992). *Campylobacter jejuni* and *Campylobacter coli* are now recognised amongst the most common bacterial causes of diarrhoea in the world (Tauxe, 1992). More recently, there has been a growing appreciation that many *Campylobacter* species besides *C. jejuni* may also be human pathogens (Mishu *et al.*, 1992), but the incidence of infection and relative importance of these other species is unclear (ACMSF, 1993).

Campylobacter infection can result in serious illness that may last for more than a week (Ketley, 1995). The clinical signs in the majority of patients are characterised by acute abdominal pain often with fever and general malaise which progresses to a profuse diarrhoea (Humphrey, 1995). Bacteremia is infrequently reported. The disease is usually self limiting (Reina *et al.*, 1994), but a few patients develop late complications, which are probably triggered by an auto-immune response to specific campylobacter antigens, notably reactive arthritis (Highton and Priest, 1996), or more seriously, peripheral polyneuropathy known as the Guillain-Barre' syndrome (Kuroki *et al.*, 1991), and the Miller-Fischer syndrome (Salloway *et al.*, 1996) a neuropathy associated with ataxia, areflexia and ophthalmoplegia.

Between 1980 and 1995 surveillance data of food-borne disease in Australia shows that *Campylobacter* species were the most commonly reported pathogen (Crerar *et al.*, 1996) and similarly *Campylobacter* has been the commonest enteric pathogen isolated from humans in England and Wales since 1981 (Pebody *et al.*, 1997). Campylobacteriosis is a serious public health concern in all areas of New Zealand. It is the most common notifiable, foodborne disease and appears to be increasing in frequency. The incidence of human cases in this country is one of the highest in the industrialised world (Ikram *et al.*, 1994). The cost of the disease to the New Zealand society is estimated to be $16 million per year (Withington and Chambers, 1997).

In a recent national study of sporadic disease, campylobacter infection was mostly associated with consumption of raw milk or undercooked chicken (Eberhart-Philips *et al.*, 1997). Outbreaks of the disease have also been attributed to consumption of untreated water (Ikram *et al.*, 1994; Stehr-Green *et al.*, 1991; Brieseman, 1987) and raw

milk (Brieseman, 1984). Campylobacter organisms found in surface water most probably originate from wild and domestic animals (Park *et al.*, 1991), water run-off from farmland following heavy rainfall or effluent from sewage purification plants (Koenraad *et al.*, 1995; Jones *et al.*, 1990).

Analysis of the literature indicates that the majority of the cases of human campylobacteriosis are associated with poultry, however, no single source of food of animal origin can be excluded as a potential vehicle of infection for humans. Outbreaks of campylobacteriosis have also been associated with consumption of unpasteurised cows milk in many parts of the world as well as New Zealand, but there is little information about the intestinal carriage of *Campylobacter* spp. in cattle, including dairy cows.

An alarming trend found overseas has been the increase in resistance of *Campylobacter* spp. to antibiotics used in the treatment of human infections (Hoge *et al.*, 1998; Velazquez *et al.*, 1995). This has been attributed to the use of antibiotics in animals as growth promoters and in prophylaxis against diseases particularly in intensively housed species like pigs and poultry (Tollefson *et al.*, 1998). It has been demonstrated that the flow of resistance genes in the environment from animals to human beings is possible (Johnson *et al.*, 1994; Linton, 1986; Scoli *et al.*, 1980). In a recent study, low level resistance to erythromycin, ciprofloxacin and doxcycline was seen in *Campylobacter* isolates from people living in Auckland (Dowling *et al.*, 1998).

The traditional approach to the identification of *Campylobacter* species is based on biochemical tests, resistance patterns and growth temperatures. Variation in a single test may result in misidentification of the strain. The methods are also labour intensive and time consuming. Therefore a rapid method of identification of the organism would be of immense value.

1.2. OBJECTIVES OF THIS STUDY:

The overall objective was to determine whether or not cattle and sheep constitute a source of *Campylobacter* spp. for humans in New Zealand. The specific objectives to address this major question were therefore:

1) To determine the prevalence of *Campylobacter* spp. in the intestinal contents of cattle and sheep.

2) To determine if *Campylobacter* spp. are present in retail meat derived from these species in New Zealand.

3) To determine whether or not the animal isolates of *C. jejuni* are the same as human isolates by molecular typing using Pulsed-Field Gel Electrophoresis (PFGE) of genomic DNA.

4) To develop a rapid method of detection and identification of *C. jejuni* from meats by utilisation of the polymerase chain reaction (PCR).

5) To study the antimicrobial susceptibility patterns of *Campylobacter* isolates derived from animals and from human clinical cases of campylobacteriosis.

CHAPTER 2

2. REVIEW OF LITERATURE

2.1. GENERAL CHARACTERISTICS OF CAMPYLOBACTER

The genus name *Campylobacter*, derived from a Greek word for curved rod, was proposed by Sebald and Veron (1963), to include microaerophilic bacteria that were different from *Vibrio cholerae* and other vibrios.

Campylobacter are Gram-negative bacteria, 0.5 to 0.8 μm long and 0.2 to 0.5 μm wide with characteristically curved, spiral or S-shaped cells (Sebald and Veron, 1963). The motility of the bacteria is characteristically rapid and darting in a corkscrew fashion, a feature by which they can be distinguished from other motile bacteria (Karmali and Fleming, 1979). Microaerophilic characteristics require propagation in an atmosphere comprising 5% oxygen, 10% carbon dioxide and 85% nitrogen (Prescott and Munroe, 1982).

2.2. SPECIES OF THE GENUS CAMPYLOBACTER

The genus *Campylobacter* contains eighteen species and subspecies, most of which have been isolated from humans, but not all are associated with disease (Skirrow, 1994).

C. jejuni and *C. coli*, are common causes of bacterial diarrhoeal disease in humans in many countries around the world (Tauxe, 1992). *C. jejuni* has also been isolated from cases of bacteraemia, appendicitis and recently has been associated with the Guillain-Barré syndrome (Fujimoto *et al.*, 1992).

The type species, *C. fetus*, is an important cause of abortion in sheep and cattle but is infrequently recovered from apparently healthy humans. The typical manifestation of the

disease caused by *C. fetus* in humans is that of a low-grade bacteraemia, especially in the elderly or in patients receiving immuno-suppresive therapy. It may cause septic abortion, meningitis, salpingitis and diarrhoea (Lior, 1994).

C. lari was first described by Skirrow and Benjamin (1980), as a thermophilic *Campylobacter* species that is distinct from *C. jejuni* and *C. coli* in several ways, most notably in its resistance to nalidixic acid. *C. lari* is now recognised as a potential human pathogen frequently isolated from stools of diarrhoeal patients and also reported to cause fatal bacteraemia in immuno-compromised patients (Borczyk *et al.*, 1987; Tauxe *et al.*, 1985).

In 1981, Sandstedt and Weirup, reported the isolation of thermotolerant, catalase negative *Campylobacter* spp. from dogs with and without diarrhoea. *C. upsaliensis* is now implicated in cases of diarrhoea and bacteraemia in immuno-compromised and healthy humans (Goossens *et al.*, 1990).

Another thermophilic species, *C. hyointestinalis*, has been identified as a possible cause of proliferative enteritis in pigs (Gebhart *et al.*, 1983), and has been frequently isolated from other animals. It is suggested to be an important cause of diarrhoeal disease in homosexual men, immuno-compromised patients and the population at large (Edmonds *et al.*, 1987; Fennel *et al.*, 1986).

2.3. SOURCES OF INFECTION FOR PEOPLE

The presence of *Campylobacter* spp. as commensals in the intestinal tracts of a wide range of birds and mammals (Glunder *et al.*, 1992; Yogasundram *et al.*, 1989), including animals most widely used for food production (Stern *et al.*, 1992), and as pets (Whelan *et al.*, 1988), open up several pathways of infection to humans. *Campylobacter* spp. can survive in the environment for several weeks at temperatures around 4°C and can cause human infection when consumed in untreated water and milk (Skirrow, 1994). Infection can also be acquired by direct contact with infected animals and is usually associated with particular occupational groups.

Campylobacter enteritis is essentially a food borne disease and the principal vehicle of infection is raw or undercooked meat (Deming *et al.*, 1987). Any raw meat may be contaminated with *Campylobacter* and poultry products are by far the most important source (Bryan and Doyle, 1995; Harris *et al.*, 1986). Retail chickens have been found to have contamination rates of 60-88% with counts in the region of 10^3-10^5 cfu per chicken carcass (Oosterom *et al.*, 1983).

Neilsen *et al.* (1997), in a nation-wide survey in Denmark, found the intestinal carriage rate of *Campylobacter* in cattle to be 47%, chicken 36% and swine 46%. *C. jejuni* was found to be the predominant species, accounting for 83 to 91 % of the cattle and poultry isolates while *C. coli* accounted for 95% of the isolates from pigs. In human patients with campylobacter enteritis, 94% of the isolates were *C. jejuni* and 6% *C. coli*. Serotyping of the isolates revealed identical serotypes in chickens and cattle and also these serotypes overlapped with the serotypic distribution of human isolates.

Seventy four serogroups of thermophilic *Campylobacter* spp. were isolated from human and animal sources in Israel (Rogol and Sechter, 1987). Six of these serogroups were found frequently in chickens and four were common to cattle. *C. jejuni* accounted for 86.7% to 92.1% of these isolates.

Rosef *et al.* (1985), compared 42 serotypes from different animal species to human clinical isolates. The highest degree of similarity was observed in the poultry isolates followed by strains from wild birds, flies and pigs.

Jones *et al.* (1984), compared biotypes and serotypes of *Campylobacter* spp. isolated from patients with enteritis and from animal and environmental sources. They found that most of the human strains were *C. jejuni* and common serotypes were frequent among strains isolated from animal and environmental sources. However, they could be different strains that are of the same serotype and would be possibly differentiated by molecular methods.

2.4. ANIMAL CARRIAGE OF CAMPYLOBACTER SPECIES

As the recognition of the importance of *Campylobacter* spp. as a cause of diarrhoea in humans grew, investigations of carriage of *Campylobacter* in animals became more urgent. Aeschbacher and Piffaretti (1989), have indicated in a report that every animal strain of *Campylobacter* should be considered a potential human pathogen. However, the pathogenic potential of many *Campylobacter* strains has yet to be established so it is not possible to arrive at this conclusion with certainty.

Jones *et al.* (1999), studied the shedding of *Campylobacter* spp. by sheep at pasture in the UK. Overall shedding of campylobacters was between a third and half of the carriage rate of 92% determined at slaughter. Rates of shedding depended on the type of pasture, and time of year. The highest rates (100%) were observed at the time of lambing, weaning and movement onto new pasture and the lowest rates (0%) occurred when the sheep were fed on hay and silage. *C. jejuni* was the main species isolated and was found to survive for up to four days in sheep faeces.

Hald and Madsen (1997), sampled 72 healthy puppies and 42 healthy kittens for faecal campylobacter shedding in Denmark and found 29% of the puppies positive with a species distribution of 76% *C. jejuni,* 5% *C. coli* and 19% *C. upsaliensis.* Only two (5%) of the kittens excreted campylobacters and both were *C. upsaliensis.*

In Japan, Ono *et al.* (1995), examined 279 samples of caecal contents from slaughtered cattle and pigs. *Campylobacter* spp. were isolated from 31.3% of 176 cattle and 93.2% of the 103 pig samples.

Giacoboni *et al.* (1993), examined faeces from calves and adult cattle in Japan for *Campylobacter* and found 97% of calves and 46% of adult cattle positive. *C. jejuni, C. hyointestinalis* and *C. fetus* were isolated from 61.8, 26.5 and 26.5% of the calves respectively. However these three species were detected at a much lower rate (11.7 to 15%) in adult cattle.

Kakkar and Dogra (1990), studied the prevalence of *Campylobacter* spp. in the faeces of cattle, buffaloes, pigs and chickens in India, and found 63% of the samples were positive for *Campylobacter* spp. Thirty two isolates were fully identified; 23 were *C. jejuni,* 8 *C. coli* and one *C. lari.*

Turkson *et al.* (1988), cultured rectal swabs from 992 domestic animals and 97 human patients in Nairobi, Kenya. The isolation rate for thermophilic campylobacters was 55.1% from pigs, 51.5% from chickens, 47.2% from dogs, 29.4% from ducks, 6% from goats, 5.8% from cattle, 3.1% from humans and 2% from sheep.

Grau (1988), examined the prevalence of *Campylobacter* spp. in calves and cattle in Australia. *C. jejuni* was commonly detected in both rumen (74%) and faecal (54%) samples obtained at slaughter from four-week-old calves but was less frequently found in adult cattle. However there was a high prevalence (47 to 88%) of *C. hyointestinalis* in samples from the intestinal tracts of both calves and cattle.

Manser and Dalzeil (1985), found 259 (31%) of 846 samples of faeces and rectal contents of domestic animals were positive for *Campylobacter* spp. in the U.K. The highest rate of isolation was in pigs (66%) followed by cattle (24%) and sheep (22%).

Garcia *et al.* (1985), reported that many of the serotypes of *C. jejuni* isolated from cattle are similar to those found in human disease in Canada. This is further supported by Dilworth *et al.* (1988), who identified cattle as the reservoir of infection in two unrelated cases of human gastroenteritis. Biotyping and serotyping of isolates from both the humans and suspected cattle provided support for this connection.

Waterman *et al.* (1984), screened the faeces of healthy cows for *C. jejuni* in the U.K. Of 74 cows 13% were positive during the summer when they were on pasture and during winter when they were housed, 51% were positive.

In a study of the prevalence of enteric pathogens in the faeces of healthy beef calves in the United States, Myers *et al.* (1984), found *C. jejuni* in 28% of animals sampled.

Infection with unidentified *Campylobacter* spp. occurred in 20.5% of calves between 1 and 4 weeks of age.

The above studies show that campylobacters of the same species, biotype and serotypes found in diseased humans are commonly found in food-producing animals. What is not known is whether these classifications discriminate sufficiently and whether they are really the same organisms or whether there are animal species-specific strains.

2.5. MILK AND MEAT AS SOURCES OF INFECTION

The weight of published information indicates that campylobacteriosis is a zoonotic disease and contaminated foods are the principal source of sporadic infections in humans. Cross contamination and improper handling and cooking of foods of animal origin account for the majority of disease (Altekruse *et al.*, 1999).

Outbreaks of campylobacter enteritis have been prominently associated with consumption of unpasteurised milk. One of the first reports of the association between campylobacter enteritis and raw milk was provided by Blaser *et al.* (1979). In that outbreak, three members of a family became ill after consuming unpasteurised milk from a cow whose faeces cultured positive for *C. jejuni*. The organism was also recovered from the patients' stool specimens but no detailed comparison of the isolates from the different sources was reported.

Korlath *et al.* (1985), reported an outbreak of campylobacteriosis in students after a one-day field trip in which the activity involved hand milking of cows and drinking of raw milk. Both children (45%) and adults (12 %) were affected. *C. jejuni* was isolated from the stools of 13 children and one asymptomatic adult. Positive persons excreted the organism for two weeks.

A large outbreak of milk-borne *C. jejuni* infection in Scotland was reported by Porter and Reid (1980), who isolated the organism from the stools of 148 patients and from a milk filter. Potter *et al.* (1983), reported an outbreak associated with certified raw milk. Fifty individuals developed campylobacteriosis and both the human and milk isolates were of the same serotype.

Major outbreaks involving consumption of contaminated or inadequately pasteurised milk have been regularly recorded over the years (Fahey *et al.*, 1995; Pearson and Healing, 1992). Evans *et al.* (1996), reported an outbreak of milkborne campylobacteriosis in school children and adult helpers on a visit to a dairy farm after drinking raw milk. *C. jejuni* was isolated from most of the cases as well as from dairy cattle and bird faeces obtained at the farm.

Contamination of milk with *Campylobacter* spp. can be faecal in origin or could occur through a mastitis infection. Lander and Gill (1980), provided evidence by way of an experimental infection showing that such a condition could exist in cows. Raw milk from these cows could infect humans. Subsequently Morgan *et al.* (1985), reported the isolation of *C. jejuni* from a naturally occurring case of mastitis in association with human illness involving the same serotype.

Diker *et al.* (1987), studied the survivability of *C. coli* in unpasteurised milk. The organisms were found to survive for 2- 5 days at 37° C and for 18 days at refrigeration temperature.

Piazza and Lasta (1986), described the isolation of *C. jejuni* and *C. sputorum* from intestinal contents (1.7% and 6.9% respectively) and *C. jejuni* (3.2%) from swabbing of carcasses of clinically healthy cattle and pigs destined for human consumption.

Several reports describe the association of *Campylobacter* species and beef products. One of the earliest reports was from England describing a study in which the organism was isolated from 1.6% of more than 6000 meat samples (Turnbull and Rose, 1982).

Stern *et al.* (1985), using enrichment methods found that approximately 5% of all red meat samples from retail outlets throughout the U.S. yielded *Campylobacter* spp. Lammerding *et al.* (1988) also reported high levels of *Campylobacter* spp. in a variety of foods of animal origin in Canada.

Bolton *et al.* (1985), examined 730 samples of offal, mince meat and sausage meat from abattoirs and retail butchers shop for *Campylobacter* species. *C. jejuni* and *C. coli* were isolated from 30.6%, 10.5% and 6% of sheep, cattle, and pig offal samples respectively.

Bucci and Maini (1988), isolated over a two year period thermophilic *Campylobacter* spp. from 6% of 1680 stool specimens of patients with enteritis, 51.7% of 325 faecal specimens of healthy animals, 51.7% of 106 meat samples of 6 different species, and 32.1% of 194 samples of raw milk. Typing of the 286 isolates revealed 64% to be *C. jejuni*, 29.7% *C. coli* and 5.6% remained unclassified.

Tomancova *et al.* (1987), studied the survivability of *C. jejuni* in artificially contaminated meat. With standard packing the organisms survived six to seven days and in vacuum packaged meat about ten to eleven days.

Zanetti *et al.* (1996), studied the prevalence of thermophilic bacteria in 57 manually shelled eggs and 130 raw meat samples. No bacteria were found in the egg samples, but 16 strains of *C. jejuni* and 4 strains of *C. coli* were detected from the meat samples.

Thus food products of animal origin have commonly been reported to contain *Campylobacter* spp. and there is epidemiological evidence linking human disease with food source. However, there is a need to demonstrate, whether or not the strains in food are the same as those associated with human disease.

2.6. ISOLATION, IDENTIFICATION AND TYPING OF CAMPYLOBACTER

Campylobacter species represent a taxonomically heterologous group. Their identification can be difficult since strains have relatively fastidious growth requirements and are asaccharolytic, and since only a limited number of biochemical tests give adequate discrimination.

Resistance to various agents (antibiotics, chemicals and dyes), temperature tolerances and growth requirements are among the phenotypic tests used in characterising

campylobacters. However, no standard methods for the performance of such tests have been published, and therefore most workers use methods peculiar to their own laboratories.

The conventional approach to identification of *Campylobacter* spp. by culture methods is a labour intensive and time-consuming process. Recent developments in molecular techniques offer attractive alternatives for the detection of the organism based on identification of specific segments of the genome. The utilisation of the polymerase chain reaction (PCR) and nucleic acid sequence based amplification (NASBA) offer a rapid method of identification of the organism from clinical samples and foods.

2.6.1. ISOLATION

The isolation of *Campylobacter* species involves plating of specimens on selective media. These include the blood-containing Skirrow medium (Skirrow, 1977), and Campylobacter-cefoperazone-vancomycin-amphotericin (CVA), (Reller *et al.*, 1983), and the blood-free charcoal-cefoperazone-deoxycholate agar (CCDA), (Hutchinson and Bolton, 1984) and charcoal-based selective medium (CSM), (Karmali *et al.*, 1986). Highest yield of *Campylobacter* spp. from stool samples was obtained by either CCDA or CSM (Endtz *et al.*, 1991b; Gun-Monro *et al.*, 1987). Zanetti *et al.* (1996) found CCDA media to be better than both Butzler and Preston media for the isolation of *Campylobacter* species.

Also enrichment culture for 24 to 48 h at 42°C is recommended for the isolation of *C. jejuni* when only small numbers of organisms are present in meat and faecal specimens (Rogol *et al.*, 1985). A number of enrichment broths have been formulated to enhance the recovery of *Campylobacter* spp. such as Campy-thio (Blaser *et al.*, 1979), Campylobacter enrichment broth (Martin *et al.*, 1983) and Preston enrichment broth (Bolton and Robertson, 1982).

Most *Campylobacter* species require a microaerophilic atmosphere containing approximately 5% oxygen, 10% carbon dioxide and 85% nitrogen for optimal recovery (Prescott and Munroe, 1982). Several manufacturers produce microaerobic gas generator packs that are suitable for routine use.

Depending on the medium used *Campylobacter* spp. produce grey, flat, and irregular, spreading colonies on fresh media. As the moisture content of the media decreases, colonies may become round, convex, and glistening with little spreading (Nachamkin, 1995).

Phenotypic tests routinely used for identification of *Campylobacter* spp. include growth temperature studies, oxidase, presence of catalase, hippurate hydrolysis, nitrate reduction, production of H_2S, and antibiotic sensitivity by the disc diffusion method (Isenberg, 1992; Barret *et al.*, 1988; Morris and Patton, 1985).

2.6.2. SEROTYPING

Two serotyping systems have gained international recognition for *C. jejuni* and *C. coli*. The Penner and Hennessey (1980) scheme based on heat stable (HS) soluble (LPS) antigens is a passive haemagglutination assay, that currently recognises 65 serotypes in total, and comprises 47 antisera for *C. jejuni* and 18 antisera for *C. coli* (Penner *et al.*, 1983).

The Lior scheme based on thermolabile antigens (HL) is a slide agglutination method with about 130 serotypes covering *C. jejuni, C. coli* and *C. lari* (Lior *et al.*, 1982). A separate scheme for *C. upsaliensis* has been proposed by Lior and Woodward (1993).

A renewed interest in the use of the HS antigen typing system has arisen since HS antigens of particular serotypes have been implicated as possible pathogenic factors in the development of Guillain-Barre' syndrome and Miller-Fisher syndrome (Kuroki *et al.*, 1993; Yuki *et al.*, 1994).

The main disadvantage of these methods is the lack of commercially available, high quality antisera. Production of antisera to the large numbers of strains for either one or both systems would be too time consuming, costly and impractical for most clinical or reference labs. Therefore, most of the reports of the isolation and identification of campylobacters from various animal species and food sources do not include a comprehensive identification of all the isolates that are made. It is difficult to conclude from many published papers exactly what is the precise identity of the isolates made.

2.6.3. BIOTYPING

A biotyping scheme which was an extension of the scheme of Skirrow and Benjamin (1980), was proposed by Lior (1984), for the differentiation of campylobacters. By this scheme based on hippurate hydrolysis, production of H_2S on FBP mediums and resistance to nalidixic acid, *C. jejuni* was divided into 4 biogroups; *C. coli* into two and *C. lari* into two biogroups. This scheme has been widely used by various workers in discriminating *Campylobacter* spp. (Nicholson and Patton, 1993; Owen *et al.*, 1994; Jimenez *et al.*, 1994).

The Preston biotyping scheme uses 12 tests, including 10 resistotyping tests that determine resistance to antibiotics, dyes and chemicals. Fifty five biotypes of *C. jejuni* have been identified (Patton and Wachsmuth, 1992)

The biotyping scheme is simple and available to most laboratories, although it produces only a few markers among strains when used alone. In recognition of this, biotyping is suggested for use in conjunction with serotyping (Smith *et al.*, 1997; Tay *et al.*, 1995). However, its use in epidemiological studies to trace the source of infection is extremely limited.

2.6.4. PHAGE-TYPING

Grajewski *et al.* (1985), was the first to describe a phage-typing system for *C. jejuni* and *C. coli* based on a panel of 14 phages isolated from poultry faeces.

There are two phage-typing systems currently in use, both of which incorporate some or all of Grajewski phages. The first, proposed by Khakhria and Lior (1992), extends to 19 phages and typed 78% of 301 *C. jejuni* strains from worldwide sources examined. The second described by Salama *et al.* (1990), combined six of Grajewski phages with ten isolated in the UK.

Excellent results have been obtained when the three typing schemes, serotyping, biotyping and phage-typing have been combined, supporting the complementary use of phage-typing in conjunction with other typing schemes for the epidemiological analysis of *Campylobacter* infections but from a practical standpoint, the combination of all these methods may be too time consuming and costly for most labs (Patton *et al.*, 1991).

2.6.5. WHOLE CELL PROTEIN PROFILES

Total protein profile and outer membrane protein (OMP) profile analyses that have been valuable in studies of specific *Campylobacter* proteins, such as porins and flagellin. However, these two methods are complex and have been found to demonstrate only low to moderate discrimination between campylobacters (Patton and Wachsmuth, 1992).

The use of OMP profile analysis with SDS-PAGE could not discriminate between strains of *C. jejuni* and *C. coli* in two separate studies (Blaser *et al.*, 1983a; Logan and Trust, 1982). But 9 sub-types of *C. jejuni* could be distinguished on the basis of migration of major bands. However, total protein profile analysis by polyacrylamide gel electrophoresis (PAGE) has been used to identify and distinguish between *Campylobacter* species, including *C. coli* and *C. jejuni* and for resolving taxonomic problems at the species and subspecies level (Vandamme and DeLey, 1991).

2.6.6. PLASMID PROFILE ANALYSIS

Plasmids have been observed in about 30-50% of *C. jejuni* and *C. coli* isolates, but the instability of the plasmids may diminish their potential value in epidemiological investigations (Taylor, 1992).

Fraser *et al.* (1992), examined a number of *C. coli*, serogroup 20 (Lior) strains and found that 24 of 27 examined carried one or more plasmids, suggesting a high degree of genetic exchange in the strains of this serogroup.

Stanley *et al.* (1994), described the typing of human and canine isolates of *C. upsaliensis* by 16S rRNA genotyping and plasmid profiling. Plasmids were found in 60% of the strains, ranging in size from 1.5 to 100 KB, and gave 15 distinct plasmid profiles. All isolates from humans contained one or more plasmids, as did strains isolated from dogs with sporadic diarrhoea. The two commonest 16S ribotypes were divided into eight and nine serogroups by plasmid profiling and the authors concluded that a combination of 16S ribotyping with plasmid profiling would be valuable for detailed epidemiological studies of *C. upsaliensis*

Bopp *et al.* (1985), studied 31 *C. jejuni* strains from 11 outbreaks for plasmids and 19 possessed plasmid DNA. Four of the strains containing plasmids were sensitive to all the antimicrobial agents used. Tetracycline resistant strains were found to contain 38-megadalton plasmids and these plasmids shared common nucleic acid sequences.

2.6.7. MULTILOCUS ENZYME ELECTROPHORESIS (MEE)

MEE is based on the electrophoretic migration distance of enzymes present in bacteria. Enzyme mobility differences relate directly to allelic variation in the structural gene locus for each enzyme (Selander *et al.*, 1986). From this analysis, an estimate of the genetic relatedness among strains can be determined.

In an application of MEE to *Campylobacter* strains, 50 and 14 electrophoretic types were identified among 104 *C. jejuni* & 21 *C. coli* strains respectively, demonstrating a high degree of genetic diversity within these two species (Aeschbacher and Piffaretti, 1989).

MEE, though a highly sensitive technique for differentiation of epidemic-associated strains (Patton *et al.*, 1991), is highly complex and depends on the number of enzymes analysed and the use of appropriate electrophoretic parameters and, in comparison with other genetic methods, is relatively time consuming.

Methods that examine chromosomal DNA and detect minor changes in nucleotide sequences have been applied to *Campylobacters*. Restriction endonuclease analysis (REA), southern blot and hybridisation of DNA fragments produced by REA with rRNA or rDNA (ribotyping), PCR based DNA profiling and Pulsed field gel electrophoresis (PFGE) measure relatively stable chromosomal differences and do not depend on phenotypic expression of bacterial cell products.

2.6.8. RESTRICTION ENDONUCLEASE ANALYSIS (REA)

Analysis of restriction endonuclease digest patterns produced by high frequency cutting enzymes is referred to as chromosomal restriction-enzyme analysis (REA) or bacterial restriction-endonuclease analysis (BRENDA) and was applied to strains within various species of Campylobacter because of limitations of biochemical and other phenotypic methods (Bradbury *et al.*, 1984; Owen *et al.*, 1989; Owen and Hernandez, 1990).

Owen *et al.* (1990), has applied REA for the differentiation of *Campylobacter* strains, and this method was successfully used in three outbreaks investigated. Fraser *et al.* (1992), have examined a number of *C. coli* strains of serogroup 20 and reported that REA demonstrated the greatest degree of discrimination among the strains. The enzyme *Hha* I yielded the greatest differences between the strains. *Hha* I produced 16 different profiles each showing a distinct banding pattern among 27 *C. coli* strains. Some strains,

which displayed identical plasmid profiles, had different restriction profiles. A disadvantage of this method however, is the fact that chromosomal restriction endonuclease digests, in many instances, will generate large numbers of bands that may be difficult to interpret.

2.6.9. RIBOTYPING

Ribotyping is based on hybridisation of a rRNA or rDNA probe to restriction digested bacterial chromosomal DNA, measuring chromosomal differences. The discriminatory power of ribotyping is dependent on the choice of both enzyme and specific probe for hybridisation. The pattern of three to six restriction fragments or bands produced in most ribotyping experiments is much easier to interpret than REA patterns (Patton et al., 1991; Wachsmuth et al., 1991).

Owen *et al.* (1990), determined the DNA restriction endonuclease (*Hae* III and *Hind* III) total digests and 16S and 23S rRNA gene patterns for 18 isolates of *C. jejuni.* An excellent correlation was found between the genomic DNA fingerprint data and the Preston bacteriophage group. An *E. coli* 16S +23S rRNA probe was more sensitive than *C. jejuni* 16S rDNA probe.

In another study involving 72 strains of *C. jejuni, C. coli, C. upsaliensis* and *C. lari,* Owen *et al.* (1993), came to the conclusion that the choice of restriction endonuclease is of critical importance when examining different species of *Campylobacter. Hae* III ribopatterns were the most effective means of typing strains of different species and, when combined with *Pst* I ribopatterns offered a highly discriminating basis for molecular typing.

Fitzgerald *et al.* (1996), described a ribotyping scheme in which strains belonging to all 47 heat-stable serotypes of *C. jejuni* were examined for polymorphism around the 16S rRNA genes and complete typability was obtained.

Jackson *et al.* (1996), examined *C. jejuni* serogroup reference strains and a collection of outbreak-associated isolates for RFLPs using *C. jejuni* random chromosomal and 16sRNA gene probes. Both probes were able to differentiate between certain random isolates of the same serogroups but greater discrimination was obtained with RFLP than ribotyping. Genotyping also distinguished between related and unrelated strains when applied to outbreaks.

Iriarte and Owen (1996), examined 47 strains of *C. jejuni* by PCR-RFLP analysis of 23S rRNA genes. 83% of the strains, including those with different Penner serotypes and from different hosts had the same molecular profiles. The authors suggested that this could be because of the high degree of conservation within the 23S rRNA sequences, and the technique would be more useful for species-specific identification assays but not for subtypic discrimination within *C. jejuni.*

2.6.10. PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

The development of PFGE allows the analysis of a smaller number of large molecular weight chromosomal DNA fragments generated by appropriate digestion by rare-cutting restriction enzymes (Schwartz and Cantor, 1984). Yan *et al.* (1991), reported the investigation of *C. jejuni* and *C. coli* strains digested with *Sma* I enzyme and found that PFGE analysis can be an alternative method useful in epidemiological investigations for differentiating isolates of *C. jejuni* and *C. coli.*

During the last few years PFGE is being increasingly applied for the typing of *Campylobacter* organisms. On *et al.*(1999), used PFGE to demonstrate persistence of *C. sputorum* infection in cows over a period of time. In another study, On *et al.* (1998), examined *C. jejuni* strains from humans, water, poultry and cattle and demonstrated a clear link between human and the animal isolates and found the technique robust and accurate.

PFGE has been compared with other techniques for the epidemiological typing of *Campylobacter* spp. by various workers. Steele *et al.* (1998), compared PFGE to fatty acid profile typing, biotyping and serotyping and found PFGE the most discriminatory. Shi *et al.* (1996), compared PFGE and enterobacterial repetitive intergenic concensus (ERIC) and Slater and Owen (1998), restriction fragment length polymorphism (RFLP) analysis based on polymerase chain reaction technique and found PFGE a better discriminatory technique in both the cases.

The technique has been successfully applied in epidemiological studies pertaining to outbreaks of *Campylobacter* infections in humans (Hanninen *et al.*, 1998b) in Finland and in identifying the source of the transmission of infection in a small dairy herd (Hanninen *et al.* 1998a).

Some workers have utilised PFGE for discrimination within the species *C. upsaliensis* (Bourke *et al.*, 1996), *C. hyointestinalis* (Salama *et al.*, 1992a) and between two subspecies as done by Salama *et al.*(1992b), to differentiate between *C. fetus ssp. fetus* and *C. fetus ssp. venerealis.*

2.6.11. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

The analysis of random amplified polymorphic DNA by simple gel-electrophoretic procedures revealed that, for different organisms, highly diverse DNA banding patterns could be generated.

These DNA fingerprints allow discrimination between species and enable differentiation of isolates belonging to a single species. Also, PCR primers aimed at bacterial DNA repeat motifs proved to be highly useful in this type of assay.

Mazurier *et al.* (1992), described a protocol for the differentiation of campylobacters by RAPD fingerprinting and PCR without the need to purify DNA, using three different, randomly designed, 10-mer primers. Nine distinct RAPD profiles were obtained with one

of the primers and 10 other profiles with another primer. Distinct RAPD profiles were identified among strains belonging to the same serotype.

Giesendorf *et al.* (1993), reported the development of species-specific DNA probes by PCR fingerprinting of *C. jejuni, C. coli* and *C. lari.* PCR primers aimed at arbitrary sequences, in combination with primers directed against the repetitive extragenic palindrome (REP) or enterobacterial repetitive intergenic consensus (ERIC) motifs, and were shown to generate isolate-specific banding patterns. Analysis of these PCR fingerprints obtained from 33 isolates of *C. jejuni,* 30 isolates of *C. coli* and eight isolates of *C. lari* revealed that, besides generation of isolate specific fragments, species-specific DNA fragments of identical size were synthesised, which could be used as species-specific probes in southern blots.

This combination of PCR fingerprinting and probe hybridisation resulted in a highly specific identification assay and provides an example of species-specific test development without the prior need for DNA sequence information.

Madden *et al.* (1996), used the RAPD method of typing based on a 10-mer primer for sub-typing animal and human *Campylobacter* species and found it an effective method with high discrimination and reproducibility and, unlike serotyping, no untypable strains were found out of a total of 269 isolates.

Chuma *et al.* (1997), analysed the distribution of *C. jejuni* and *C. coli* in broiler chickens by using restriction fragment length polymorphism (RFLP) of the flagellin gene and found it a useful technique for the epidemiological studies of campylobacter contamination of broilers in different flocks and different growth cycles on the farm.

Nishimura *et al.* (1996), used PCR-based RFLP analysis based on the flagellin gene in typing 179 isolates from Japan and China and were successful in typing most (98.7%) of the isolates into 25 separate RFLP groups as compared to serotyping (61.7%). Also 11 isolates of HS- 019 strains, frequently isolated from Guillain-Barre′ syndrome (GBS), were found to show an identical RFLP pattern.

Fujimoto *et al.* (1997), used RFLP and RAPD techniques to analyse *C. jejuni* serotype 019 strains associated with GBS and other strains. Their data indicated that all the strains were closely related to one another whether or not they were associated with GBS.

2.6.12. POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction has been applied extensively to the detection of infectious agents. PCR allows amplification of a pre-selected region of DNA and can be a highly specific and sensitive detection technique. PCR has also been used for direct identification of *Campylobacter* species from complex substrates without prior isolation and purification of the organisms (Wegmuller *et al.*, 1993).

The difficulties in routine detection, isolation and identification of *Campylobacter* species make these organisms ideal candidates for PCR identification. One campylobacter gene, which has the potential to allow for organism identification at the level of species and at the narrower level of strain, is the flagellum gene. The flagella of C. *jejuni* and *C. coli* are composed of two subunit flagellins, the products of the *fla A* and *fla B* genes. These genes have been cloned and sequenced and shown to contain highly variable regions that could be used for strain-specific detection. They also contain other regions that are highly conserved among *C. coli* and *C. jejuni* strains. Therefore these genes are potentially useful for the detection of the *Campylobacter* species most commonly associated with human diarrhoeal disease.

Oyofu *et al.* (1997), described the development of a routine detection assay for *C. jejuni* and *C. coli* in clinical specimens by using the PCR. An oligonucleotide primer pair from a conserved 5' region of the *fla A* gene of *C. coli* was used to amplify a 450bp region by PCR. The primer pair specifically detected four strains of *C. coli* and 47 strains of *C. jejuni*, but it did not detect strains of *C. fetus, C. lari, C. upsaliensis, C . cryoaerophilia* and *C. hyointestinalis.* In stools seeded with *C. coli* cells, the probe could detect between 30-60 bacteria per PCR assay. The assay was also successfully used to detect *C.*

coli in rectal swab specimens from experimentally infected rabbits and *C. jejuni* in human stool samples.

Birkenhead *et al.* (1993), used the PCR method for detection and typing of campylobacters. The *fla* gene was amplified. Primers were chosen which amplified 1.3kb of the *fla* gene in *C. jejuni* and *C. coli.* The *C. upsaliensis* amplimer was approximately 1.7kb in size and was easily distinguishable. Other species of campylobacter failed to yield amplimer. The amplimer was digested with *Alu* I, which demonstrated considerable restriction fragment length polymorphism and suggested that it might allow the development of a rapid and novel typing scheme.

Waegel and Nachamkin (1996), described a PCR based on primers from *fla A* gene for detection of *C. jejuni* from stool samples, which required the faeces to be purified by column chromatography before subjecting it to PCR.

Genes for 16S r RNA have often been used as target sequences in PCR assays for identification of fastidious bacteria including campylobacter species. rRNA genes have a typical mosaic structure of phylogenetically conserved and variable regions. The latter may vary considerably among different bacterial species and therefore are excellent targets for species or even subspecies-specific primers.

Eyers *et al.* (1994), reported the use of a 23 S rRNA fragment as a target for PCR amplification and the primers used detected all thermophilic campylobacter species and also allowed discrimination among the thermophilic species. The rRNA, an essential part of prokaryotic and eukaryotic ribosomes, is genetically stable and consists of conservative and variable regions. The latter may vary considerably among different bacterial species and therefore can be targets for specific oligonucleotide probes.

Giesendorf *et al.* (1992),. described a method for rapid detection and identification of *Campylobacter* in chicken products using PCR after a short enrichment culture. They use a 16S rRNA gene sequence of *C. jejuni* as a primer and probe combination. With this

primer set and probe, 426-bp fragments from *C. jejuni*, *C. coli* and *C. lari* could be amplified. The detection limit of the PCR was 12.5 bacteria.

Docherty *et al.* (1996), described the magnetic immuno-PCR assay for the detection of campylobacter in milk and poultry products in which the target bacteria are captured from the food sample by magnetic particles coated with a specific antibody. The bound bacteria are then lysed and subjected to PCR.

Van Camp *et al.* (1993), also reported the development of a method utilising PCR amplification and hybridisation analysis for detection of enteropathogenic *Campylobacter* species based on 16S rRNA genes but concluded that it was not possible to differentiate between the species on the basis of 16S rRNA as *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* form a tight taxonomic cluster.

Wegmuller *et al.* (1993), described a PCR method designed to sensitively detect and identify *C. jejuni* and *C. coli*, without the need of isolating and culturing strains. The intergenic sequence between the flagellin genes *fla A* and *fla B* was amplified and characterised with a triple primer or seminested primer approach. A total of 50 bacterial strains, 27 of *C. jejuni* and *C. coli* and 23 of other species were tested, giving no false positive or false negative results. The detection limit as determined by ethidium bromide staining of amplification products on agarose gels was 10 bacteria or less in artificially contaminated water, milk and soft cheese samples with the seminested primer PCR assay. As an application of the PCR system, 93 samples of milk and dairy products were screened for the presence of *C. jejuni* and *C. coli* out of which 6 positive samples were identified while none were found positive with a conventional culture method.

Hum *et al.* (1997), evaluated the PCR assay and traditional phenotypic methods for the identification and differentiation of *C. fetus* subspecies and found agreement between strain identities suggested by both the assays to be 80.8%. The PCR was found to be a reliable, rapid technique for the species and subspecies identification of *C. fetus*.

Mahendru *et al.* (1997), used PCR with primers based on *fla A* gene and were successful in identification of 15 *C. jejuni* strains isolated from human faeces. RFLP of the amplified product classified the 15 strains into 5 types.

Wesley *et al.* (1997), used a multiplex PCR to distinguish *C. jejuni* and *C. coli* associated with reproductive failure in pigs. They found it useful in identifying the *Campylobacter* species from atypical cases and suggested that multiplex PCR, in conjunction with other conventional assays, may be useful for verifying unusual instances of campylobacteriosis.

Linton *et al.* (1997), used the PCR for detection and identification to species level of *C. jejuni* and *C. coli* isolates direct from diarrhoeic samples using three different sets of primers and a simple technique to purify DNA. The results concurred with culture and phenotypic identification to species level. The PCR offered a rapid method to define the occurrence and species prevalence.

Winters *et al.* (1997), described a method for the rapid detection of *C. jejuni* from chicken washes using a nested PCR and detected *C. jejuni* in 80% of four groups of chicken. PCR methods have the potential to be very fast and sensitive and can be designed to be group-reactive or quite specific depending on what is required in a particular investigation and on which primers are chosen. They are also able to detect non-cultivable organisms.

2.6.13. NUCLEIC ACID SEQUENCE BASED AMPLIFICATION (NASBA)

An alternative approach to PCR is NASBA, a technique to selectively amplify RNA. Isothermal nucleic acid amplification of RNA in NASBA is achieved through the concerted action of avian myeloblastosis virus reverse transcriptase (AMV-RT), T7 RNA polymerase and RnaseH (Compton, 1991).

The reaction starts with a non-cyclic phase, in which a downstream primer, containing a tail sequence of the T7-promoter sequence, anneals to the single stranded target sequence. Through the action of AMV-RT, cDNA is formed. The RNaseH hydrolyses the RNA from the RNA-DNA hybrid, which results in a single strand of DNA to which the upstream primer can anneal. The AMV-RT synthesis, through its DNA polymerase activity, produces the double-stranded DNA intermediate with a transcriptionally active promoter sequence. TheT7 RNA polymerase generates from these, intermediate single-stranded RNA copies (100-1000) which serve as a template in the now cyclic phase of NASBA.

Major advantages of NASBA over PCR are that NASBA is performed isothermally which precludes the use of a special thermocycler, and that no separate RT step is required for RNA amplification (Kievits *et al.*, 1991).

Uyttendale *et al.* (1994), evaluated the NASBA technique for the specific identification of *C. jejuni, C. coli* and *C lari.* A set of primers and a probe were chosen from the 16S rRNA sequence of campylobacter. The probe was hybridised in solution with the amplified nucleic acids of 12 *Campylobacter* species and 9 other Gram-negative bacteria. The probe was shown to hybridise specifically to the amplified single-stranded RNA of *C. jejuni, C. coli* and *C. lari* in an enzyme-linked gel assay (ELGA).

More recently Uyttendale *et al.* (1995), reported the utilisation of the NASBA amplification of RNA and a non-radioactive in-solution hybridisation combined with a short selective enrichment for the detection of *C. jejuni* in foods. Interference by food components was eliminated by centrifugation following the enrichment step. The prevalence of high numbers of indigenous flora affected the sensitivity of the assay. However, identification of *C. jejuni* was possible up to a ratio of indigenous flora to *C. jejuni* of 10,000:1. The base of enrichment culture and NASBA shortened the analysis time from 6 days to 26 hours.

2.7. ANTIBIOTIC RESITANCE AMONG CAMPYLOBACTER SPECIES

Erythromycin has long been regarded as the antibiotic of choice for treating campylobacter infections in humans. Although the use of this antibiotic may reduce the severity of the symptoms in children and curtail the convalescent carriage state, it generally does little to alter the course of the illness (Mandal *et al.*, 1984).

Gaudreau and Gilbert (1997), compared the disc diffusion and agar dilution methods for antibiotic susceptibility testing and came to the conclusion that disc diffusion is a reliable, easy and inexpensive susceptibility method for *C. jejuni* and *C. coli* for erythromycin, ciprofloxacin and tetracycline.

The potential problem of antibiotic resistance has been illustrated by a study in Thailand (Taylor *et al.*, 1987), where erythromycin treatment, in acute diarroheal disease among 100 infants in an orphanage had no effect on the duration of diarrhoea. *Campylobacter* strains isolated from the children before treatment showed a high level of resistance to erythromycin (53% for *C. jejuni* and 91% for *C. coli*). In developed countries erythromycin resistance is commonly reported at a 2% level for *C. jejuni* and 10% for *C. coli* (Doyle and Jones, 1992). The report of such high rates of erythromycin resistance in Thailand is disturbing and may indicate what could happen if antibiotics become indiscriminately used in developed countries for the treatment of campylobacter infections (Taylor *et al.*, 1987).

Dowling *et al.* (1998), examined 202 Campylobacter isolates from the Auckland area by the disc diffusion method and found 1% (2) resistant to doxcycline, 1.5% (3) to erythromycin and 2.5% (5) to ciprofloxacin. All the isolates were susceptible to gentamicin. Unpublished studies by the author earlier, had detected 2- 11% of the isolates resistant to erythromycin.

Gaudreau and Gilbert (1998), compared the antimicrobial resistance of *C. jejuni* strains isolated from humans in Canada during 1985-87 and 92-93 to isolates from 1995-97, and found an increase in resistance to tetracycline, nalidixic acid and ciprofloxacin over the

years. Resistance to erythromycin was not observed in any of the strains. Thermophilic *Campylobacter* spp. are associated with a proportion of cases of travellers diarrhoea and are thus likely to be treated with broad spectrum antibiotics. It is in this context that resistance to trimethoprim-sulphamethoxazole and tetracycline becomes clinically important. Also fluoroquinolones are now popular and ciprofloxacin is effective in treating erythromycin-resistant campylobacter infections (Dupont *et al.*, 1987).

The use of antibiotics in animal husbandry could have serious consequences for effective treatment of human infections. Treatment of food producing animals with antimicrobial agents that are important in human therapy may present a public health risk by transfer of antibiotic-resistant, zoonotic pathogens from animals to humans. Resistant bacteria can diminish the effectiveness of antibiotics and demand the use of more expensive or less safe alternatives (Tollefson *et al.*, 1998).

A recent study of antibiotic resistance in Australian isolates of *Campylobacter* spp. from humans and animals to doxycycline (D), erythromycin (Er) and enrofloxacin (Enr) was performed (Korolik *et al.*, 1996). Of the human strains 29% were Er^R, 11% were D^R and one strain showed intermediate resistance to Enr. In the chicken isolates 30% were Er^R, 12% D^R and two strains showed intermediate resistance to enrofloxacin.

Hoge *et al.* (1998), examined the trends in antibiotic resistance of *Campylobacter* species isolated from indigenous persons and travellers in Thailand for up to 15 years and found that ciprofloxacin resistance increased from zero before 1991 to 84% in 1995, of isolates from both groups of people. Jacobs-Reitsma (1997), screened 617 *Campylobacter* isolates from poultry for quinolones and found 29% to be resistant. Quinolone treatment of campylobacter colonised broiler flocks was found to induce quinolone resistance under experimental conditions.

Aarestrup *et al.* (1997), in a study to determine the antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. from humans, pigs, cattle and broilers in Denmark found that all isolates were susceptible to apramycin, neomycin and gentamicin. Only a few *C. jejuni* isolates were resistant to one or more antimicrobial agents. Resistance to

tetracycline was more common among *C. jejuni* isolates from humans (11%) than among isolates from animals (0 to 2%). Among *C. coli* isolates especially from pigs there was a high level resistance to macrolides and streptomycin.

Sjogren *et al.* (1997), examined 1659 isolations made of *C. jejuni* and *C. coli* from Swedish patients with diarrhoea from 1992 to 1995 for norfloxacin resistance by the disc diffusion method. 18.6% of the isolates were found to be resistant.

Huysmans and Turnidge (1997), in Australia examined 100 clinical human isolates of thermophilic *Campylobacter* spp. including 79 strains of *C. jejuni*, 19 of *C. coli* and 2 of *C. lari* by the disc susceptibility method. They found no resistance to quinolones in any of the species except *C. lari.* Resistance to erythromycin, chloramphenicol or gentamicin was not found in any isolate, while nine isolates were resistant to tetracycline.

Gaunt and Piddock (1996), examined 2209 isolates of *Campylobacter* spp. collected over a 1 year period from UK public health labs and found 4.1% of the isolates to be resistant to ciprofloxacin although none of the 91 patients involved had taken a quinolone.

Tadano *et al.* (1996), studied the susceptibilities of 600 clinical isolates of *C. jejuni* in Tokyo hospitals over a 6-year period to six antibiotics. The resistance rates were, norfloxacin (7.5%), ofloxacin (7.5%), ciprofloxacin (7.3%), nalidixic acid (10.3%), Erythromycin (0.6%) and tetracycline (43.2%). It was observed that the resistance to fluoroquinolones had increased significantly since 1993 but susceptibility to erythromycin had remained at the same level during the past 6 years.

Sicinschi (1996), studied the susceptibility to antibiotics of 39 *C. jejuni/coli* isolates using the disc diffusion method. All strains were resistant to carbenicillin, rifampicin, polymyxin M sulphate, ketokonazole, cephalotin, and cephazolin. All the strains were sensitive to ciprofloxacin, chloramphenicol, gentamycin, kanamycin, streptomycin and tobramycin. Lekowska-Kochaniak *et al.* (1996), found almost all of 47 human and 52 poultry isolates to be susceptible to erythromycin, chloromphenical, gentamicin and

nitrofuratoin. Koenraad *et al.* (1995), tested the *in vitro* susceptibility of 209 *Campylobacter* strains isolated from sewage and poultry abattoir drain water to the quinolones, nalidixic acid, flumequine, ciprofloxacin and enrofloxacin. They found resistance ranging from 28% for enrofloxacin to 50% for nalidixic acid.

Gomez-Garces *et al.* (1995), reported that resistance of *C. jejuni* strains to fluoroquinolones is frequent in Spain and reaches nearly 50%, but some 12 other oral antimicrobial agents studied like macrolides, amoxicillin, clindamycin and fosfomycin displayed good *in vitro* activities. Velazquez *et al.* (1995), in Spain, studied 102 clinical *C. jejuni* isolates and found *in vitro* resistance to erythromycin (1.9%), three fluoroquinolones (31.3%- 34.3%), tetracycline (43%), kanamycin (4.8%), and ampicillin (18.6%).

Coker and Adefeso (1994), found that in Nigeria resistance to erythromycin in clinical isolates of *C. jejuni* was 79.2% as compared to an earlier study wherein 82% sensitivity was observed (Coker *et al.*, 1989). Schwartz *et al.* (1993), examined 30 *C. jejuni* strains isolated from stools of Israeli children with enteritis for antimicrobial susceptibility to eight agents. It was found that all the isolates were sensitive to ciprofloxacin, ofloxacin, furazolidone and erythromycin, but 70% of the strains were found to be resistant to tetracycline.

Endtz *et al.* (1993), studied the in vitro susceptibilities of both quinolone susceptible and resistant *C. jejuni* to three new macrolide antibiotics, azithromycin, clarithromycon and roxithromycin. They found no difference in their activity against the quinolone susceptible and resistant strains and suggested that these new antibiotics might replace erythromycin for the treatment of campylobacter infections.

Ansary and Radu (1992), studied six *C. jejuni* isolates for the occurrence of plasmids in association with antibiotic resistances as well as conjugal transfer. All isolates seemed to carry three similar plasmids and multiple resistance to three of the antibiotics tested was observed with resistance to tetracycline most common. Conjugal transfer of donor resistances was observed with erythromycin, neomycin, chloramphenical, kanamycin and tetracycline.

Rautelin *et al.* (1991), compared the *in vitro* susceptibilities of human *Campylobacter* strains isolated in 1978-80 and during 1990 to ciprofloxacin, norfloxacin, erythromycin, gentamycin, and doxycycline. Results indicated that the susceptibility to erythromycin, gentamicin and doxycycline had remained the same during the last ten years but the number of norfloxacin-resistant strains increased from 4% to 11% and ciprofloxacin-resistant strains increased from none to 9% in the last 10 years.

Akthar (1988), performed antimicrobial susceptibility studies on *C. jejuni* isolates from patients with diarrhoea, asymptomatic carriers and domestic animals. All isolates were sensitive to erythromycin, gentamicin, furazolidone and kanamycin and 7% of the isolates were resistant to tetracycline which was observed to be plasmid mediated.

The analysis of the above literature shows that antibiotic resistance is an unavoidable consequence of the use of antibiotics in human therapy and is observed in most countries at various levels depending on the usage. Examples of this are the unusually high levels of resistance to erythromycin reported in *Campylobacter* spp. from Thailand and Nigeria most probably due to its indiscriminate use and relatively lesser in other developed countries. However, it has been observed lately that resistance to an important class of antibiotics, the quinolones which was negligible or present at very low levels increased dramatically ever since it was introduced as a growth promoter in poultry. This combined with the increased use of antibiotics in human therapy as well as their use in the treatment of diseases and prophylaxis in food producing animals and the possibility of transfer of resistance genes in the environment, could have serious implications for the effective treatment of human diseases.

To combat this emerging situation it is important to minimise the use of antibiotics in animal feeds and give more emphasis on the achievement of management goals through increased sanitation and hygiene and better disease control methods in livestock rearing. Similarly in human medicine, use of antibiotics for the treatment of infectious diseases needs to be restricted to avoid inappropriate use. Where possible the choice of antibiotics should be based on identification and sensitivity of the offending bacterium or at least knowledge of the likely causative agent and its current sensitivity profile.

CHAPTER 3

3. ISOLATION AND IDENTIFICATION OF *CAMPYLOBACTER SPECIES* FROM FAECES OF CATTLE, SHEEP AND HUMANS.

3.1. INTRODUCTION

Campylobacter (previously termed *Vibrio*) spp. were known to cause abortion in sheep and cattle from the early 1900s (McFadyean and Stockman, 1913). It was only in the 1970s after a suitable isolation method was developed (DeKeyser *et al.*, 1972), that *C. jejuni* was recognised as an enteric pathogen in humans. At present, *Campylobacter* spp. especially *C. jejuni* and *C. coli* constitute the leading cause of gastrointestinal disease in humans in the developed world (McNicholas *et al.*, 1995; Pearson and Healing, 1992; Tauxe, 1992; Skirrow, 1991). *C. jejuni* also causes infertility and abortion in cattle, sheep (van Donkersgoed *et al.*, 1990; Varga *et al.*, 1986; Prescott and Munroe, 1982), and other animals (Prescott and Bruin-Mosch, 1981).

The major reservoirs of thermophilic campylobacter are believed to be the intestines of birds and other warm-blooded animals (Park *et al.*, 1991), including adult ruminants (Stern, 1992), in which, it is thought, they exist as commensals with the gut flora

A major cause of campylobacteriosis in humans is the consumption of raw or undercooked foods of animal origin (Harris *et al.*, 1986). Outbreaks of campylobacteriosis have been prominently associated with consumption of unpasteurised milk (Tauxe, 1992; Korlath *et al.*, 1985; Breiseman, 1984; Robinson and Jones, 1981; Porter and Reid, 1980). Dairy cows are frequently *Campylobacter* positive and appear to acquire the organism by the consumption of contaminated water (Humphrey and Becket, 1987). Some strains of *C. jejuni* can cause mastitis in dairy cows and such infections have led to milk-borne outbreaks (Hutchinson *et al.*, 1985).

More usually however, milk becomes infected with *Campylobacter* as a consequence of faecal contamination during milking (Humphrey and Becket, 1987).

Table 3.1 Reported carriage rates of *Campylobacter* species in adult cattle in various countries

COUNTRY	CARRIAGE RATE (%)	REFERENCE
Australia	59.3	Grau, 1988
Canada	22.34	van Donkersgoed *et al.*, 1990
Japan	46.7	Giacoboni *et al.*, 1993
Norway	0.8	Rosef *et al.*, 1983
Portugal	19.5	Cabrita *et al.*, 1992
United Kingdom	21	Bolton *et al.*, 1982
"	23.5	Manser and Dalzeil, 1985
"	89.4	Stanley *et al.*, 1998a

The *Campylobacter* colonisation of cattle is not only significant, because of its potential contamination of milk on the farm and the carcass at slaughter, but also with regards to the environmental and water contamination during disposal of abattoir effluents and slurries to land or run off from farms (Korhonen and Martikainen, 1991; Terzieva and McFeters, 1991; Bolton *et al.*, 1987; Carter *et al.*, 1987)

The present survey of adult cattle, calves and sheep was done to estimate the prevalence of thermophilic *Campylobacter* spp. in the Manawatu region of New Zealand and to better understand the importance of cattle and sheep in the epidemiology of these microorganisms.

3.2. MATERIALS AND METHODS

3.2.1. SAMPLING

To determine the prevalence of intestinal thermophilic *Campylobacter* in cattle and sheep three types of intestinal material were examined; rectal faeces, intestinal content from the large intestine and samples of fresh faeces collected on defecation.

A total of 300 samples from adult cattle and calves were collected for the survey. The samples from beef cattle were collected from an abattoir 25 km from Palmerston North. The samples from dairy cattle were collected at Massey University dairy farms, and the samples from the new-born calves were collected from both Massey farms and a private farm near Palmerston North.

One hundred and fifty eight sheep faecal samples and 58 samples of the sheep abattoir environment were collected from an abattoir about 35 km from Palmerston North. This work was done with the help of a summer studentship recipient Rachel Logan.

Sampling of Beef Cattle: Samples of the intestinal contents from 120 beef cattle were collected after evisceration by making an incision into the terminal part of the colon of slaughtered animals and collecting about 10g of contents into a sterile plastic container. All samples were transported to the laboratory on ice and were processed within 2 hours of collection.

Sampling from Dairy Cattle: Faecal samples from 80 dairy cattle from two farms were collected during milking in most instances directly into sterile plastic containers when the animals defaecated. The samples were transported on ice and processed immediately on arrival.

Sampling from Calves: Sterile cotton wool swabs were used to collect rectal contents from 100 calves at the farms. The heifer calves were 1 day old to 1 week old

whereas the bull calves were about 4 weeks old. Each swab was rotated within the rectum for approximately 30 seconds, ensuring that it was in contact with the mucosa. Rectal swabs were transported on ice in sterile tubes on ice and processed within 2 hr of collection.

Sampling from Sheep: Samples were collected from the rectum with the help of sterile swabs and then placed in transport medium. The environmental samples were collected by swabbing different sources and placed in the transport medium and transferred to the laboratory.

3.2.2. HUMAN CLINICAL ISOLATES

A total of 105 human clinical isolates were obtained form the Palmerston North Medical Laboratory. These isolates had been presumptively identified as *Campylobacter* spp. on the basis of their characteristic growth on campylobacter selective agar and Gram staining. The isolates were transported to the laboratory on the selective agar plates, purified by subculture and further biochemical tests were performed.

3.2.3. ISOLATION TECHNIQUES

An enrichment and selective plating method was used for the isolation of thermophilic *Campylobacter* spp. from the faecal samples. About 1g of the faeces or intestinal contents was inoculated into 10 ml of Preston enrichment broth (Nutrient broth No.2, Oxoid) supplemented with Campylobacter growth supplement (Oxoid), Preston selective supplement (Oxoid) and 5% defibrinated sheep blood) and incubated for 48 hr at 42°C in a microaerophilic atmosphere produced by using CampyPak Plus (BBL) envelopes in a GasPak (BBL) jar.

Preston broth was inoculated into campylobacter blood-free agar (CBF) with cefoperazone charcoal desoxycholate agar (CCDA) selective supplement (Oxoid) and the plates incubated for 48 hr at 42°C in a GasPak jar with CampyPak Plus (BBL, Cockeysville,USA) gas generating envelopes.

3.2.4. IDENTIFICATION OF CAMPYLOBACTER

3.2.4.1. PRESUMPTIVE IDENTIFICATION OF INTESTINAL THERMOPHILIC *CAMPYLOBACTER*

Typical colonies grown on campylobacter selective agar were examined by Gram staining and for motility by the hanging drop method, by taking a loop-full of culture on a glass slide with a drop of saline and putting a cover slip and watching under high magnification under a microscope.

Oxidase reaction: The oxidase test was performed by using Spot test oxidase reagents (Difco laboratories, Detroit, USA). Two or three drops of the reagent were put on the colonies to be tested directly on the plate. A positive reaction (presence of cytochrome oxidase) was indicated by the appearance of a deep purple colour within a few seconds. Negative reactions remained colourless or turned light pink or light purple after 30 seconds.

Catalase activity: The catalase activity was tested by a slide test. One drop of a 3.5% solution of hydrogen peroxide was added to a microscope slide, placed over a black background and a small amount of growth was transferred with a loop to the drop. The reaction was positive when effervescence and bubbles appeared within a few seconds (Skirrow and Benjamin 1980)

Preparation of pure cultures: Preparations of presumptive intestinal thermophilic *Campylobacter* were made by subculturing individual colonies from the selective media on to blood agar plates (Oxoid Blood agar base no 2 and 7% defibrinated sheep blood), which were incubated for 48 h at 42°C. These pure cultures provided the material for the subsequent tests.

3.2.4.2. CONFIRMATIVE IDENTIFICATION AND SPECIES DIFFERENTIATION OF THE THERMOPHILIC *CAMPYLOBACTER*

The presumptively identified *Campylobacter* were further tested to confirm their identity and to differentiate to species level by the following tests:

Hippurate Hydrolysis Test: The hippurate hydrolysis test was done by a rapid method using Bacto differentiation disc hippurate (Difco laboratories, Detroit, USA). It involved emulsifying a loop-ful of culture in a small tube containing 0.4 ml distilled water and a bacto-differentiation disc and incubation at 37°C for 2 h. Then 0.2 ml of SpotTest Ninhydrin reagent (Difco laboratories, Detroit, USA) was added. After gentle mixing and re-incubating for 10 minutes the reaction was read. A positive hippurate reaction was indicated by the formation of a deep purple blue colour. A light purple colour or no colour change indicated negative hippurate reactions.

Sensitivity to 30μg Nalidixic Acid and Cephalothin Discs: The sensitivity to 30μg nalidixic acid and cephalothin discs (Oxoid) was determined by a disc diffusion test. A loop-full of pure growth was taken from the blood agar plates and rotamixed in 2 ml sterile saline. A sterile cotton swab was then used to inoculate a blood agar plate and the discs impregnated. The plates were then incubated at 42°C for 48 h in a microaerophilic environment. The absence of a clear zone of inhibition around the discs was indicative of resistance.

Hydrogen Sulphide Production: The butt and slant of a triple sugar iron (TSI) agar in a tube were stab inoculated with a straight wire. A lead acetate indicator paper was suspended over the medium. The tube was capped and incubated at 42°C for 5 days under microaerophilic conditions. The appearance of black colour on the lead acetate indicator paper, or in the medium and the indicator, was recorded as positive.

Table: 3.2 Protocol used for the Identification of Thermophilic *Campylobacter* species

Campylobacter species	Grams Staining Cell form	Motility	Oxidase	Hippurate hydrolysis	H_2S Prod.	Sensit. Cephal -othin	Sensit. Nalidixic acid
C. jejuni	G-ve s-shape	+	+	+	_	R	S
C. coli	"	+	+	_	_	R	S
C. lari	"	+	+	_	_	R	R
C.hyointestinals	"	+	+	_	+	S	R

3.2.5. PRESERVATION OF CULTURES

Pure growth of cultures on a blood agar plate, resulting from the incubation of a thermophilic *Campylobacter* spp. at 42°C for 48 h, were harvested with a sterile cotton swab in 3 ml 15% glycerol broth. Approximately 2 ml of the suspension was transferred into sterile 2 ml Nunc cryo-tubes and stored at -70°C till further use.

3.3. RESULTS

3.3.1. RESULTS OF CAMPYLOBACTER ISOLATION FROM BEEF CATTLE

Intestinal contents from a total of 120 beef cattle slaughtered at the abattoir were cultured for thermophilic *Campylobacter* spp. over a period of 5 months. The cattle originated from farms in the Manawatu region.

Fifty (41.6%) of the samples were positive for *Campylobacter* spp. The predominant species recovered were *C. hyointestinalis* 26 (52%) followed by *C. jejuni* 9 (18%), *C. coli* 8 (16%), *and C. lari* 7 (14%). (Table 3.3).

3.3.2. RESULTS OF CAMPYLOBACTER ISOLATION FROM DAIRY CATTLE

Faecal samples from a total of 80 (30+50) cows from two Massey University dairy farms were screened for thermophilic *Campylobacter* spp. over a period of 3 months.

Campylobacter spp. were isolated from 43 (53.7%) faecal samples. *C. jejuni* constituted the largest number of isolations 25 (58.1%), followed by *C. hyointestinalis* 13 (30.2%) and *C. coli* 5 (11.6%). (Table 3.3).

Individual isolation rates from the two dairy farms were 70% (21/30) and 44% (22/50) respectively.

3.3.3. RESULTS OF CAMPYLOBACTER ISOLATION FROM HEIFER CALVES

Faecal samples from 50 heifer calves aged from 0 to 3 weeks from Massey University farms were screened for thermophilic *Campylobacter* spp.

Nineteen (38%) of the samples were positive for *Campylobacter* spp. The predominant species was *C. hyointestinalis* 15 (78.9%) followed by C. lari 2 (10.5%) and *C. jejuni* (5.2%) and *C. coli* 1 (5.2%). (Table 3.3)

New-born calves (1-3 days old) had an isolation rate of 20% (5/25) whereas 2-3 week old calves had an isolation rate of 56% (14/25).

Table 3.3 Showing the percentages of isolations of *Campylobacter* spp. from adult cattle, calves and sheep

	% Positive	% *C. jejuni*	% *C. coli*	% *C. hyointe-stinalis*	% *C. lari*
Beef Cattle (n=120)	41.6 (n=50)	18	16	52	14
Dairy Cows (n=80)	53.7 (n=43)	58.1	11.6	30.2	-
Heifer calves (n=50)	38 (n=19)	5.2	5.2	78.9	10.5
Bull Calves (n=50)	50 (n=25)	-	-	100	-
Sheep (n=158)	44.3 (n=70)	100	-	-	-

Table 3.4 Results of sheep abattoir environment sampling

DESCRIPTION	NO. OF SAMPLES	NO. OF POSITIVE
Gut Room:		
Aprons	1	1
Drain	2	2
Water tank	1	1
Casings tank	1	1
Floor	1	1
Chute	1	1
Basin	1	1
Steps	1	1
Bench top	1	0
Machinery		
Roller	1	1
Knife washer	1	0
SI puller	1	1
Hide puller rails	2	2
Drinking fountain	1	1
Floors, Walls etc:		
Paunch table	2	1
Aprons	6	1
Drip tray	1	1
Caecum spray screen	1	1
Gut puller floor	2	0
Wall by gut trays	1	1
Tail cutting area	2	0
Gut trays	2	0
Foot/apron wash area	2	2
Manual skinner	2	2
Water source	1	0
Floor by gut washer	1	1
Floor by stunner	3	1
Drain	2	1
Carcass post-perineum	6	2
Total	50	28

Table 3.5 Isolations of *Campylobacter* spp. during different months in cattle.

MONTH	NO. OF SAMPLES	NO. OF POSITIVES(%)
April	15	4 (27)
May	30	14 (47)
June	25	10 (40)
July	25	10 (40)
August	50	19 (38)
September	50	25 (50)
October	25	12 (48)
November	30	21 (70)
December	25	12 (48)
January	25	10 (40)

3.3.4. RESULTS OF CAMPYLOBACTER ISOLATION FROM BULL CALVES

Faecal samples from 50 bull calves from a local farm were screened for thermophilic *Campylobacter* spp.

Twenty five (50%) of the samples were positive for *Campylobacter* spp. and all the isolates were found to be *C. hyointestinalis.* (Table 3.3)

3.3.5. RESULTS OF CAMPYLOBACTER ISOLATION FROM SHEEP

Faecal samples from 158 sheep were screened for thermophilic *Campylobacter* spp.

Seventy (44.3%) of the samples were found positive for *Campylobacter* spp. All the isolates were typed as *C. jejuni* (Table 3.3).

3.5.6. RESULTS OF CAMPYLOBACTER ISOLATION FROM SHEEP ENVIRONMENTAL SAMPLES

Fifty samples from the environment of the sheep abattoir were taken and screened for *Campylobacter* spp.

Twenty eight (56%) were found positive for *Campylobacter* spp. All of them were typed as *C. jejuni* (Table 3.4).

3.3.7. RESULTS OF IDENTIFICATION OF CAMPYLOBACTER SPP. *FROM HUMANS*

A total of 105 isolates obtained from clinical samples and presumptively identified as *Campylobacter* spp. were procured from the Palmerston North Medical Laboratory.

The isolates, which were transported on the selective agar plates, were further purified on blood agar and species identification performed. Of the 105 isolates 100 were found to be *C. jejuni* and five *C. coli.*

3.4 DISCUSSION

The present study focussed on the association of thermophilic campylobacters with colonisation of the intestinal tract of cattle and sheep. Hence the incubation temperature of 42°C was employed, eliminating the possibility of isolating *C. fetus* and other species which do not grow at this temperature.

The isolation of thermophilic campylobacters from cattle of different ages and types revealed the presence of a range of *Campylobacter* spp. and a large variation in the isolation rates in different categories of animals tested. Dairy cows showed the highest overall rate of 53.7%. There was a noticeable difference between the two herds tested with one showing an isolation rate of 70% the other only 44%. A lower isolation rate of 38% was found in the heifer calves. Grau (1991) and Blaser *et al.* (1984), have shown that thermophilic *Campylobacter* are readily isolated from the intestinal tracts of apparently healthy ruminants and that the carriage rate varies significantly between individual herds and flocks and in different age groups.

In the present study faecal culture from 120 beef cattle at slaughter revealed 41.6% to be positive for *Campylobacter* spp. The predominant species identified were *C. hyointestinalis* (52%) *C. jejuni* (18%) and *C. coli* (16%). Similar results were found in an Australian study in which faecal samples from adult cattle at slaughter revealed a prevalence of 46.9% *C. hyointestinalis* and 12.5% *C. jejuni* (Grau, 1988). In a study in Japan (Giacoboni *et al.*, 1993), found an overall prevalence of 46.7% in adult cattle and the presence of a range of *Campylobacter* species including *C. jejuni, C. hyointestinalis, C. coli* and *C. lari.* However, the rate of isolation of *C. hyointestinalis* was lower than that found in the present and in the Australian studies (Grau, 1988). Also the rate of

isolation of *C. jejuni* was lower compared with that observed by other workers (Kursteiner *et al.*, 1985; Doyle and Roman, 1982;).

The isolation rate of *Campylobacter* spp. from dairy cattle was 53.7% in the present study. The predominant species was *C. jejuni* (58.1%) followed by *C. hyointestinalis* (30.2%) and *C. coli* (11.6%). Similar results have been obtained by a number of workers (Stanley *et al.*, 1998a; Cabrita *et al.*, 1992; Humphrey and Beckett, 1987).

The faecal samples collected from 50 heifer calves revealed 19 (38%) positive for *Campylobacter* spp. However it was seen that samples from new-born calves (1-3 days old) had an isolation rate of 20% compared to 56% in calves 2-3 weeks old. It seems that the animals acquire the bacteria at quite an early age when there is less resistance from the still developing gut microflora. The predominant species isolated was *C. hyointestinalis* (78.9%) followed by *C. lari* (10.5%), *C. jejuni* (5.2%) and *C. coli* (5.2%). In comparison Adesiyun *et al.* (1992), isolated *Campylobacter* from 20.5% of calves in Trinidad and *C. jejuni* accounted for 53.3% and *C. Coli* for 46.7% of the isolations.

The predominant species isolated from both the bull and heifer calves was *C. hyointestinalis*. In a study by Atabay and Cory (1998), *C. hyointestinalis* was isolated at a rate of 32%. The reason for the high incidence of the *C. hyointestinalis* in calves found in the present study is difficult to explain. Maybe the population of *Campylobacter* in the gut changes with the age of the animal as the normal gut microflora develops and perhaps in the older population there is a subsequent re-infection. Myers *et al.* (1984), reported the isolation of *C. hyointestinalis* in 20.5% of faecal samples from calves between 1-4 weeks of age, but did not detect it in any other groups. Also it was observed that the bull calves did not carry any *C. jejuni* as opposed to 5% of the heifer calves, this might be attributed to the rearing of bull calves separate from the adult dairy herds after birth. Dairy cows have been frequently found to be *Campylobacter* positive and appear to acquire the organism by the consumption of contaminated water (Humphrey and Becket, 1987) and this may be the main source of infection for calves.

Faecal samples from 158 slaughtered sheep in the present study revealed the presence of *Campylobacter* in 70 (44.3%) of the samples. All the isolates were characterised as *C. jejuni*. Two recent studies in UK have revealed carriage rates of 91.7% from the small intestines of lambs at slaughter (Stanley *et al.*, 1998b) and 88% of these isolates were *C. jejuni*. In the other study by Jones *et al.* (1999), to assess the shedding of campylobacter in grazing sheep at different times of the year showed a highest rate of 100% coinciding with lambing, weaning and movement on to a new pasture and the lowest (0%) occurred when the sheep were fed on hay and silage. *C. jejuni* was the predominant species (90%) recovered from the above study. Reported results from similar studies overseas revealed considerable variation, between 0- 94% (Adesiyun *et al.*, 1992; Green *et al.*, 1990; Turkson *et al.* 1988; Elegbe *et al.*, 1987; Sarkar *et al.*, 1984; Rosef *et al.*, 1983). As no other studies on large numbers of sheep have been carried out and reported before in New Zealand, it is assumed that the findings from this study are indicative of the true carriage rate. However it is noted that this rate is influenced by many factors, including age, sex, weather, season, geography, time and immune status of animals.

The occurrence of a *C. jejuni* in the environment of the abattoir and the equipment (56%) is understandable given the high prevalence of the organism in the sheep faeces. An overseas study (Bolton *et al.*, 1982) isolated *Campylobacter* spp. from the external surfaces of 70% of sheep carcasses, and 27-38% of implements e.g.: knives, cleavers, saws, cutting blocks, hooks, racks and scales. Another survey conducted in an abattoir in Calcutta found that 86.7% of floors and 50% of walls were contaminated. The author stated that the extensive contamination of the slaughterhouse represented a phenomenal risk to operating staff (Sarkar *et al.*, 1984). The difference between the isolation rate from sheep and the environment may reflect a build up of the organism, indicating a higher risk for meatworkers, especially in the highly contaminated areas.

The *Campylobacter* isolates from human clinical samples were identified as *C. jejuni* (100) and *C. coli* (5). The predominance of *C. jejuni* as a cause of human disease is well known throughout the world (Griffith and Park, 1990). Similar findings have been made in different countries where *C. jejuni* constitute more than 95% of the isolations from clinical cases and *C. coli* account for roughly 5% (Pearson and Healing, 1992).

The overall prevalence of thermophilic campylobacters in our study was observed to be on the higher side when compared to other studies in different countries (Table 3.1). This can be attributed to different factors. 1) Increased sensitivity and specificity of culture methods for campylobacter have shown to lead to a significant increase in the rate of isolations (McNicholas, 1995). The method of isolation used by us involved a selective enrichment step using Preston broth, which might have enhanced the isolation rate particularly by eliminating other competitive flora and also by reviving injured cells. Under certain environmental conditions for example, stationary phase of growth or exposure to atmospheric oxygen, campylobacters become round or coccoid in shape. This shape change has been associated with a transition from a viable culturable form to a viable but unculturable state (Rollins and Colwell, 1986), and this phenomenon might contribute to the differences in the isolation rates depending upon the cultural conditions employed.

2) Difference in the number and isolation rate between different countries might be due to differences in environmental and husbandry conditions of cattle (Giacoboni *et al.*, 1993). The conditions in which cattle are raised in New Zealand on open pastures are different from those found in many other parts of the world and might contribute to the differences in the isolation rates and the species isolated. Stern (1981), has pointed out that variations such as herd type, season, animal age, feeding regimen, crowding and geography can account for significant differences in isolation rates. *Campylobacter* are normal components of the intestinal flora of several wild rodents and wild birds. These reservoirs are believed to spread *Campylobacter* strains in nature. Exposure to their excrements or consumption of contaminated water constitutes the main routes of infection for food producing animals (Blaser *et al.*, 1983b).

The incidence of campylobacteriosis in humans in New Zealand in shown to follow a seasonal trend (Brieseman, 1990). It is reported to be highest in summer and lowest in winter (Ikram *et al.*, 1994). Meanger and Marshall (1989), found the highest prevalence of thermophilic campylobacter in dairy cattle in New Zealand to be in autumn (31%) followed by summer (24%) and the lowest (12%) in winter. This partially follows the

pattern for human clinical episodes so would be consistent with cattle being an important source of human infections.

Although we did not set out to determine the prevalence of *Campylobacter* spp. in different seasons, the sampling in the present study was done over a period of 10 months (April to January) thus incorporating a part of all seasons. Analysis of data reveals that there was no substantial difference in the isolation rates of thermophilic campylobacter recovered from the animals over different seasons although isolations made in the summer months show a slight increase (Table 3.5).

The high incidence of human infections in summer could be attributed to the general population undertaking more outdoor activities in this season and thus exposing themselves to contaminated stream waters or to drinking untreated water (Bohmer, 1997; Brieseman, 1987) and milk (Brieseman, 1984). A change in the food habits from well-cooked meats at home to eating barbecued and less thoroughly cooked meats (Ikram *et al.,* 1994), may be of more significance than any postulated increase in the excretion of the organism from the animal reservoirs.

Campylobacter outbreaks due to consumption of contaminated water have been documented in New Zealand (Bohmer, 1997; Stehr-Green *et al.,* 1991; Brieseman, 1987). It has been suggested that heavy rain may wash campylobacter organisms from the surrounding beef and sheep farms into rivers and facilitate seepage into springs and other drinking water sources.

The isolation of *C. hyointestinalis* and *C. lari* from cattle is an important finding of the present study. *C. hyointestinalis* was first isolated from pigs but is now being increasingly isolated from other animals especially cattle (Grau, 1988; Gebhart *et al.*, 1985; Myers *et al.,* 1984; Ursing *et al.,* 1984). *C. lari* was first described as a group of strains isolated from seagulls (Skirrow and Benjamin, 1980) and later from other birds and animals (Lindblom *et al.,* 1990). Both *C. hyointestinalis* and *C. lari* have been isolated from human clinical cases overseas and are now recognised as infrequent but

important human enteric pathogens (Borczysk *et al.*, 1987; Edmonds *et al.*, 1987; Tauxe *et al.*, 1985).

The occurrence of these species in cattle makes them potential pathogens for humans in this country. Since most medical laboratories in New Zealand, do not identify campylobacter to species level we do not have sufficient information on the involvement of the different species of campylobacter in the cases of human infection. If this collection of 105 isolates from Palmerston North is indicative of the national status then a small but significant proportion of cases of campylobacteriosis is due to *C. coli*.

A high percentage of people in New Zealand are involved in farming and occupations associated with handling animals and raw meat such as butchers, and staff at freezing works and supermarkets. There are thus many opportunities for infection with *Campylobacter* spp. (Brieseman, 1985). If the high prevalence of campylobacteriosis in the human population is a reflection of the high prevalence of infection in cattle and sheep found in this study then measures need to be implemented to break this cycle of infection. Such measures might include education about the potential sources of infection and the need to ensure safe water and milk supplies as well as the importance of properly cooking meats. There is therefore a need to determine whether or not other species such as deer are reservoirs and, if so, to implement appropriate control measures to prevent spread to the human population. While it is assumed at this stage that the species of campylobacter in farm animals are the same as those involved in human disease this is not always certain. There is also a need to characterise isolates more completely at the molecular level to determine whether or not the human clinical isolates are the same as the animal isolates.

CHAPTER 4

4. TYPING OF *CAMPYLOBACTER JEJUNI* ISOLATES FROM CATTLE, SHEEP AND HUMANS BY PULSED-FIELD GEL ELECTROPHORESIS.

4.1. INTRODUCTION

Campylobacter spp. represents a taxonomically heterogeneous group. The identification of these campylobacters can be difficult since strains have relatively fastidious growth requirements, are asacchrolytic and only a few biochemical tests give adequate discrimination (Goossens and Butzler, 1992).

Serotyping has been the most common typing method (Lior, 1984; Penner *et al.*, 1983), although biotyping and phagetyping have also been employed (Patton and Wachsmuth, 1992) to show the epidemiological association of isolates from patients or to trace the possible routes of transmission from animals to humans. However, strain discrimination using phenotypic typing methods may be significantly compromised due to poor reproducibility or inadequate discriminatory power (Arbeit, 1995).

A feature of the epidemiology of human campylobacteriosis is that most infections are sporadic cases with relatively few community outbreaks. The sources of the sporadic infections are rarely established with strains being widely distributed in both wild and domesticated birds and mammals, as well as in sewage and untreated water.

Pulsed-field gel electrophoresis (PFGE) of genomic DNA restricted with low frequency cutting endonucleases, has proved to be a valuable tool in the epidemiological investigations of a wide range of medically important bacteria (Tenover *et al.*, 1995), probably as a consequence of its ability to examine restriction polymorphisms along the entire bacterial chromosome.

This method has been found more distinguishing than serotyping because several genotypes are found within a serotype (Gibson *et al.*, 1995; Suzuki *et al.*, 1994). PFGE has been found to be the most discriminatory of several typing methods employed by various workers in characterising campylobacter isolates (Steele *et al.*, 1998; Shi *et al.*, 1996) and has considerable potential for tracing the possible routes of transmission of the campylobacters causing sporadic infections.

The aim of the present study was to type the *Campylobacter jejuni* isolates from cattle and sheep by PFGE using the restriction endonuclease *Sma* I and to generate a PFGE profile of these isolates to asses their relatedness and also compare them with *C. jejuni* isolates from human clinical cases in the Manawatu region. This exercise would give us an idea as to the similarities or differences between the *C. jejuni* strains prevalent in animals and humans and lead us to better understand the epidemiology of campylobacter infections in this region.

4.2. MATERIALS AND METHODS

4.2.1. BACTERIAL ISOLATES

C. jejuni isolates from humans, cattle and sheep were used for this study. The isolates were stored in glycerol broth at -70°C until used. They were revived by thawing, plating on blood agar plates and incubating in microaerophilic environment at 42°C for 24 h.

4.2.2. DNA PREPARATION

Intact genomic DNA was prepared by a modification of the methods described by Barret *et al.* (1994) and Olsen *et al.* (1994).

Single colonies were selected from purity blood agar plates and grown in trypticase soya broth at 42°C for 48 h. One millilitre aliquot of each broth culture was pipetted into a sterile micro-centrifuge tube and centrifuged at 10,000 rpm for 4 min. The supernatant was removed, the pellet resuspended in 1.5 ml ice-cold Pett IV buffer (1M NaCl, 10mM

Tris HCl pH 8.0, 10mM EDTA pH 8.0) and the suspension centrifuged at 10,000 rpm for a further 4 minutes. The supernatant was removed and the pellet resuspended in 50 µl of cold Pett IV buffer and kept on ice until required. Eighty µl of molten, 1%, pulsed-field certified (PFC) agarose (10 mg ml^{-1} agarose in Pett IV buffer) was added to the suspension and mixed carefully by pipetting to avoid air bubbles. The resultant agarose/buffer suspension was transferred quickly into a plug mould and left to solidify on ice for 20 min. Then, the solid plugs were removed into fresh micro-centrifuge tubes containing 1 ml Urea-ESP buffer (6M Urea, 50mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1% sodium lauryl sarcosine, 0.2% sodium deoxycholate 0.5 mg ml^{-1} Proteinase K) and incubated overnight in a waterbath at 56°C. After the incubation the plugs were washed 8 times in ice-cold TE pH 8.0, for at least 30 min each wash and stored in TE pH 8.0 at 4°C until required.

4.2.3. RESTRICTION ENDONUCLEASE DIGESTION OF PLUG-INCORPORATED DNA

The restriction endonuclease enzyme *Sma* I was selected based on reports by other workers (Salama *et al.*, 1992; Yan *et al.*, 1991) about its cutting ability in *Campylobacter* spp. and after preliminary trials with a few other restriction enzymes. An optimum concentration of the enzyme and other parameters were determined by initial experimentation (data not presented).

Approximately one quarter of each plug was cut off using a sterile scalpel blade, placed in 100 µl of 1x restriction buffer (12 µl 10x New England Biolabs Buffer 4, 1 µl 100 mg ml^{-1} Bovine Serum Albumin (BSA), 87 µl sterile distilled water) and equilibrated for at least 45 min on ice. About 20 plugs were processed at one time and a cocktail of the restriction enzyme was made up, depending on the number of plugs being digested, and aliquotted accordingly. The restriction buffer was decanted and replaced with 80 µl cutting buffer (8 µl 10x restriction buffer, 0.8µl 100mg ml^{-1} BSA, 20 U restriction endonuclease, distilled water). Plugs and buffers were equilibrated for 45 min before incubation overnight in a waterbath at 25° C.

4.2.4. PULSED-FIELD GEL ELECTROPHORESIS OF DIGESTED DNA

A contour-clamped homogenous electric field (CHEF) apparatus, including variable speed pump, gel chamber, power module and mini chiller (CHEF Mapper, Bio- Rad Laboratories, Richmond, California, USA) was the system used for PFGE. A gel running protocol was optimised for maximum resolution of the digested DNA fragments (Table 4.1).

After completion of digestion, plug slices were loaded into the wells of a 1% PFC agarose gel (800 mg in 80 ml 0.5x TBE buffer), which had been equilibrated in the electrophoresis chamber with the buffer (0.5x TBE) circulating, for at least 2 h. A molecular weight marker was also loaded in each gel.

After electrophoresis, gels were stained in fresh aqueous ethidium bromide (80 µl of 10mg ml^{-1} stock solution in 800 ml-distilled water) for 1 h, then destained overnight. Gels were examined under UV light and photographed.

Table 4.1: Gel Running Parameters

PARAMETER	
Agaraose Type / Concentration	1% Pulsed Field Grade Agarose
Buffer Type / Concentration	0.5X TBE
Voltage gradient	6.0 V cm^{-1}
Buffer temperature	14° C
Pulse angle	120°
Initial switch time	1 sec
Final switch time	28 sec
Run time	24 h

4.2.5. INTERPRETATION OF PULSED-FIELD PROFILES

The gels were analysed visually for differences in the banding patterns. The first isolate examined from each source was arbitrarily designated as Type 1 and all other isolates were compared with this "type strain". Bands were identified numerically from the highest molecular weight downwards, with positions fixed in relation to Type 1. Bands were also described by their sizes in kilobases relative to one of the molecular weight markers. Isolates were subsequently classified into Types and Pulsotypes on the basis of their band differences to the Type 1 strain. Following the guidelines published by Tenover et al. (1995) on the interpretation of chromosomal DNA restriction patterns produced by PFGE, isolates were assigned to new Types when they differed by four or more bands from the Type 1 strain, and to pulsotypes when they differed by 1-3 bands from the Type 1 strain.

4.3. RESULTS

4.3.1. RESULTS OF PFGE OF C. JEJUNI STRAINS ISOLATED FROM CATTLE

Genomic DNA from 35 *C. jejuni* isolates from cattle was characterised by PFGE after digestion with the restriction enzyme *Sma* I. The resolution of *Sma* I digested DNA yielded between 5 and 9 fragments ranging in size from 40 - 480 kbp. The 35 isolates were classified into 11 different Types (Type 1 to 11) having a difference in 4 or more bands (Fig 4.1; Table 4.2). Type 1 was subdivided into 3 pulsotypes each, having up to three band differences from the principal strain (Table 4.2).

Types 1_C, and 2 were found in both beef and dairy animals. Whereas Types 1, 1_B, 3 and 5 were unique to beef cattle and Types 4, 6, 7, 8, 9, 10, and 11, to dairy cattle.

Table 4.2 Types and Pulsotypes of *Campylobacter jejuni* among 35 cattle isolates

TYPES	NUMBER OF ISOLATES (%)
Type 1 (Pulsotype 1_A)	1 (3)
Pulsotype 1_B	1 (3)
Pulsotype 1_C	5 (14)
Type 2	12 (34)
Type 3	1 (3)
Type 4	6 (17)
Type 5	1 (3)
Type 6	1 (3)
Type 7	1 (3)
Type 8	2 (6)
Type 9	1 (3)
Type 10	1 (3)
Type 11	2 (6)

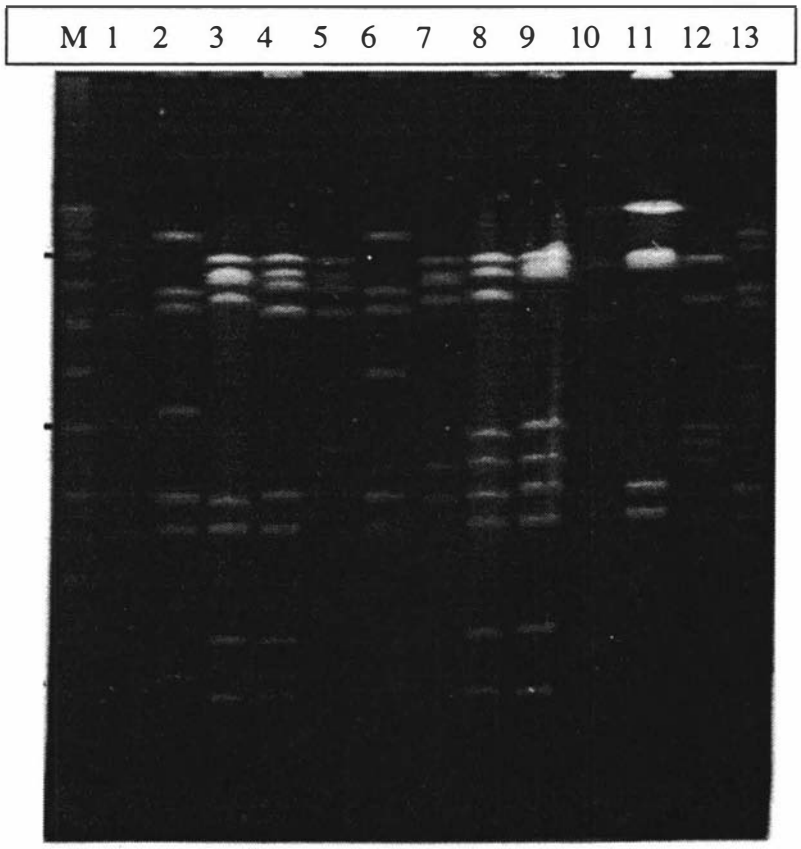
Table 4.3 Types and Pulsotypes of *Campylobacter jejuni* among 50 Sheep isolates

TYPES	NUMBER OF STRAINS (%)
Type 1 (Pulsotype 1_A)	2 (4)
Pulsotype 1_B	10 (20)
Pulsotype 1_C	5 (10)
Pulsotype 1_D	4 (8)
Pulsotype 1_E	5 (10)
Type 2	1 (2)
Type 3	2 (4)
Type 4	5 (10)
Type 5	3 (6)
Type 6	4 (8)
Type 7	3 (6)
Type 8	1 (2)
Type 9	1 (2)
Type 10	1 (2)
Type 11	1 (2)
Type 12	2 (4)

Table 4.4 Types and Pulsotypes of *Campylobacter jejuni* among 50 human isolates

TYPES	NUMBER OF ISOLATES (%)
Type 1 (Pulsotype $1_{A)}$	9 (18)
Pulsotype 1_B	11 (22)
Pulsotype 1_C	2 (4)
Pulsotype 1_D	3 (6)
Pulsotype 1_E	2 (4)
Pulsotype 1_F	2 (4)
Type 2	1 (2)
Type 3	9 (18)
Type 4	3 (6)
Type 5	2 (4)
Type 6	1 (2)
Type 7	1 (2)
Type 8	1 (2)
Type 9	1(2)
Type 10	1 (2)
Type 11	1 (2)

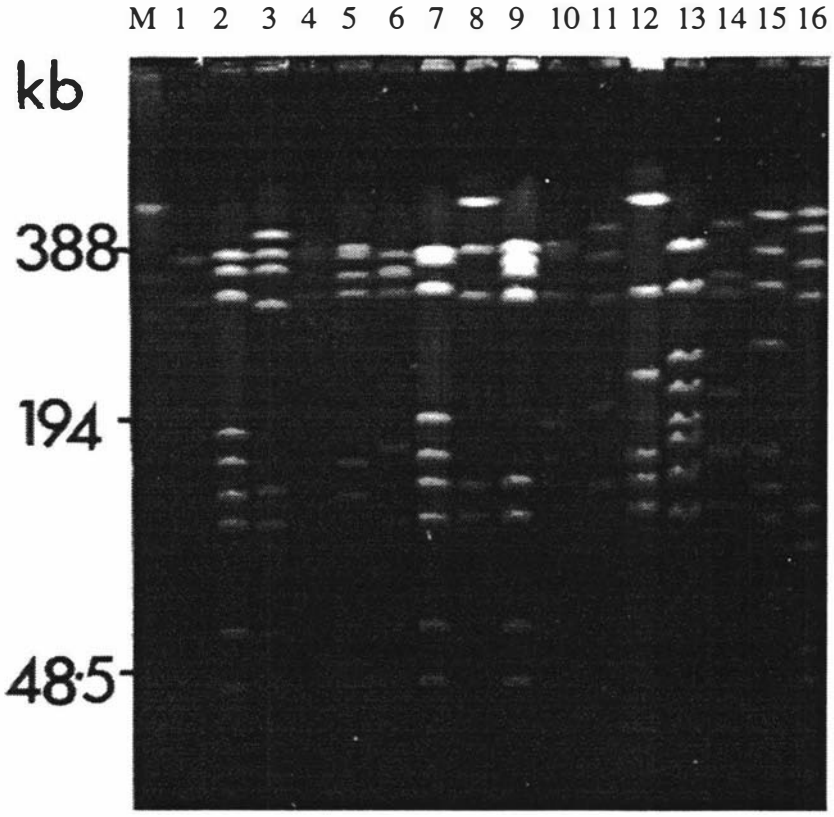
Figure 4.1 Representative PFGE profiles *C. jejuni* cattle isolates



M: Molecular size marker (Lambda ladder)

Lanes: 1-Type 1(Pulsotype 1), **2-**Type 2, **3-** Pulsotype 1_B, **4-** Pulsotype 1_C, **5-** Type 3, **6-**Type 4, **7-** Type 5, **8-** Type 6, **9-** Type 7, **10-** Type 8, **11-** Type 9, **12-** Type 10 **13-** Type 11.

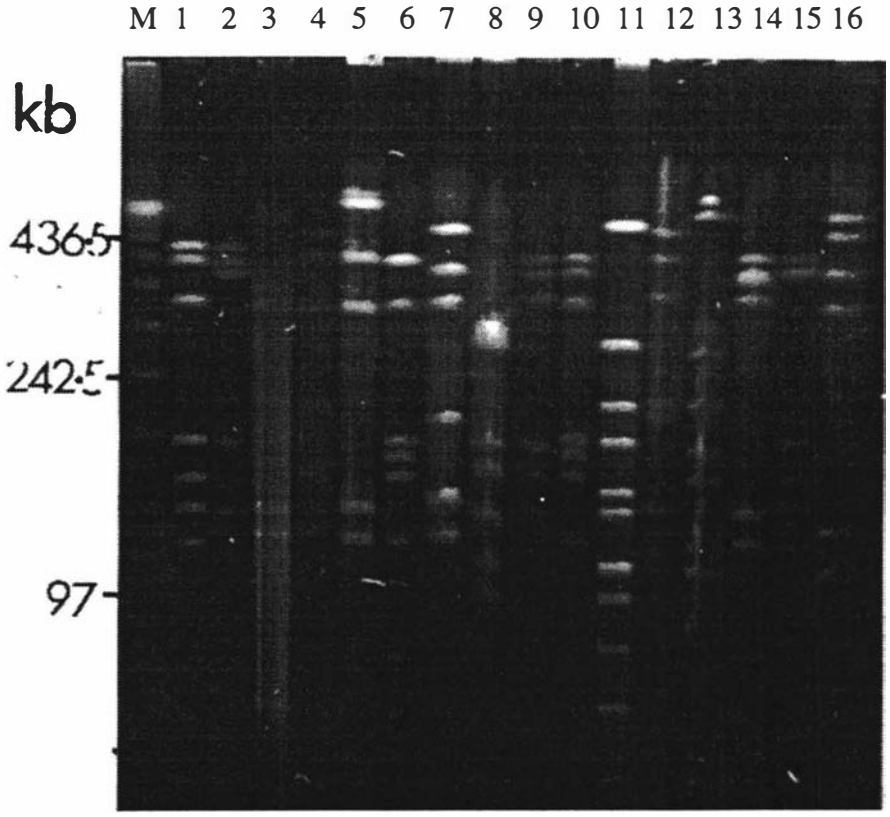
FIGURE 4.2 Representative PFGE profiles of *C. jejuni* sheep isolates



M: Molecular size marker (Lambda ladder)

Lanes : 1- Type 1 (Pulsotype 1_A), **2-** Pulsotype 1_B, **3-** Type 2, **4-** Type 3, **5-** Type 4, **6-** Pulsotype 1_C, **7-** Pulsotype 1_D, **8-** Type 5, **9-** Type 6, **10-** Pulsotype 1_E, **11-** Type 7, **12-** Type 8, **13-** Type 9, **14-** Type 10, **15-** Type 11, **16-** Type 12.

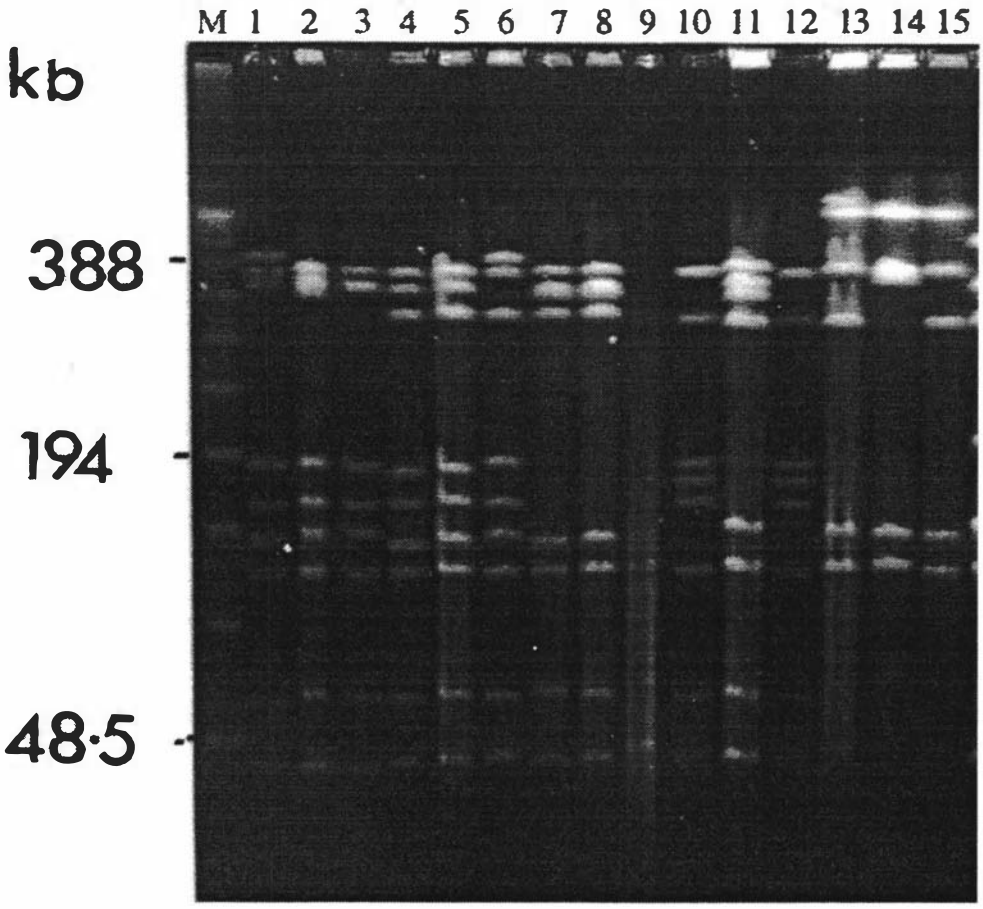
FIGURE 4.3 Representative PFGE profiles of *C. jejuni* human isolates



M: Molecular size marker (Lambda ladder)

Lanes: 1- Type 1(Pulsotype 1), **2-** Pulsotype 1_B, **3-** Type 2, **4-** Type 3, **5-** Type 4, **6-** Pulsotype 1_c, **7-** Type 5, **8-** Type 6, **9-** Pulsotype 1_D, **10-** Pulsotype 1_E, **11-** Type 7, **12-** Type 8, **13-** Type 9, **14-** Type 10, **15-** Pulsotype 1_F, **16-** Type 11

Figure 4.4 Common PFGE profiles in human, cattle and sheep isolates of *C. jejuni*



M: Molecular size marker (Lambda ladder)

Lanes 1 and 2 Human and cattle isolates- pulsotypes

Lanes 2 and 3 cattle and human isolates - indistinguishable

Lanes 4 and 5 human and cattle - indistinguishable

Lanes 5 and 6 cattle and human - pulsotypes

Lanes 7 and 8 human and cattle - indistinguishable

Lanes 10 and 12 human and sheep- indistinguishable

Lanes 13 and 15 human and sheep- indistinguishable

Lanes 14 and 15 cattle and sheep - pulsotypes

4.3.2. RESULTS OF PFGE OF C. JEJUNI STRAINS ISOLATED FROM SHEEP

Genomic DNA from a total of 50 *C. jejuni* isolates from sheep were digested with the restriction endonuclease *Sma* I and subjected to PFGE. The analysis of digested DNA yielded six to nine fragments ranging in size from 40- 480 kbp. The isolates were grouped in to 12 different Types (Type 1 to 12) based on a difference of four or more bands (Fig 4.2; table 4.3). Type 1 was further subdivided into five pulsotypes having up to 3 band difference from the principal type (Fig 4.2; Table 4.3).

4.3.3. RESULTS OF PFGE OF C. JEJUNI STRAINS ISLOATED FROM HUMANS

Genomic DNA from 50 *C. jejuni* isolates from humans was digested with the restriction endonuclease enzyme *Sma* I and resolved by pulsed-field gel electrophoresis. The strains could be grouped into 11 different Types (Type 1 to 11) based on a difference of 4 or more bands (Fig 4.3 ; Table 4.4). Type 1 (Pulsotype 1_A) was further subdivided into five pulsotypes (Pulsotypes 1_B to 1_F) having up to 3-band difference from the principal strain (Fig 4.3; Table 4.4).

4.3.4. COMPARISON OF THE PFGE PROFILE OF C. JEJUNI ISOLATES FROM ANIMAL AND HUMAN SOURCES

The PFGE profiles of the cattle, sheep and human isolates were compared visually to determine their relatedness to each other.

A total of six cattle and human *C. jejuni* isolates were found to have identical PFGE patterns. Another 25 isolates were found to differ by less than three bands from each other and were classified as pulsotypes and considered to be closely related (Fig 4.4).

Among the sheep and human isolates there were two groups consisting of a total of ten isolates, which had identical patterns to each other (Fig 4.4).

The comparison of cattle and sheep PFGE profile revealed three sheep isolates to be pulsotypes of a cattle isolate.

4.4. DISCUSSION

PFGE has been used for typing various organisms (Wrights *et al.*, 1998; Feizabadi *et al.*, 1997; Hosaka *et al.*, 1997; Harsono *et al.*, 1993). Digestion of chromosomal DNA with a restriction endonuclease that has infrequent DNA recognition sites results in fewer but larger fragments and greatly simplifies analysis compared to restriction endonuclease analysis (REA) and standard electrophoresis (Kakoyiannis *et al.*, 1988).

The aim of the present study was to characterise the large number of *C. jejuni* strains isolated from cattle and sheep at meat works and farms in the Manawatu region and to compare them with *C. jejuni* strains isolated from clinical cases in humans at the Palmerston North Public Hospital.

Gibson *et al.* (1995) reported the first use of PFGE in an epidemiological investigation of *C. jejuni*. The results indicated that this technique was most discriminatory of other typing methods used namely ribotyping and phage-typing and was capable of discriminating not only between strains of different serotypes but also between strains within a single serotype. The present study confirmed that PFGE of chromosomal DNA was a useful technique in separating large molecular weight fragments with high resolution into simple restriction profiles, which could be easily compared.

The restriction enzyme *Sma* I provided the best discrimination among the enzymes tested in the preliminary trial runs, with the most easily interpreted patterns consisting of 6-10 fragments between approximately 40 kbp to 500 kbp. This was subsequently used in the restriction analysis of all the isolates.

Some workers have suggested that the use of two restriction enzymes might give better discrimination (Gibson *et al.*, 1995) however, Steele *et al.* (1998) compared restriction analysis using *Sma* I and *Sal* I and came to the conclusion that the discriminatory index of PFGE with either of the enzymes was excellent and could be enhanced only slightly by combining the two enzyme groups.

The technique was found to be robust with good reproducibility as demonstrated by the consistent patterns obtained when a number of isolates where run several times during the investigation (data not presented).

In this study the PFGE analysis of isolates of *C. jejuni* from cattle, sheep and humans with the *Sma* I restriction endonuclease typically resolved 5-10 bands ranging in size from 40 kbp to 480 kbp. Gibson et al. (1997) made similar observations in an epidemiological investigation of *C. jejuni* in the UK.

The phenotypically identical *C. jejuni* cattle isolates were characterised into 11 different types, which reflects the high degree of discrimination obtained by PFGE and the diversity of the organism within a limited geographic area. However, the majority of the isolates (more than 70%) fell under three predominant types (Type 1, 2 and 4) showing the occurrence of a few dominant clonal types in most of the animals. The other eight types were found to be represented by only one or two strains each.

In a similar study On *et al.* (1998), separated 34 Danish strains of *C. jejuni* from cattle, poultry, humans and water and assigned them to one of six *Sma* I profile groups. Two groups of indistinguishable isolates contained randomly isolated strains from the human cattle and poultry isolates providing evidence of identical clones infecting humans and animals.

Some strains having identical PFGE profiles were common to beef and dairy cattle, showing the widespread presence and dissemination of the same clonal types in the region, probably originating from a common ancestry.

Types 1_C, and 2 were not only found most predominant but were found in both beef and dairy animals which belonged to different farms from different locations. There were also Types which were either unique to beef cattle (Types 1, 1_B, 3 and 5) or to dairy cattle (Types 4, 6, 7, 8, 9, 10, 11,).

The sheep isolates were characterised into 12 different types based on a difference of four or more bands in their PFGE patterns. There was only one major type (Type 1/Pulsotype 1_A) with its four pulsotypes (1_B, 1_C, 1_D & 1_E) which encompassed more than 52% of the isolates. But unlike the cattle strains the sheep isolates were found to be more varied with the other Types (2 to 12) being represented by two to ten percent of the isolates each.

The human isolates more or less followed a distribution similar to the sheep isolates and were categorised into 11 Types based on their band differences. Type 1 and its five pulsotypes (1_B to 1_F) were found to be the most predominant types with about 58 % of the isolates falling under this group. This could suggest a common origin or source of infection, which is represented by infecting *C. jejuni* strains showing more genetic homogeneity.

Hanninen *et al.* (1998b), identified a total of 69 PFGE types among 176 *C. jejuni* isolates from Finnish patients. Five predominant types were found to comprise 40% of the isolates. In investigations of small outbreaks, identical PFGE patterns were demonstrated, indicating a common source of infection.

The comparison of the animal strains with the human isolates provided quite illuminating data with a large number of cattle strains having identical patterns to the human isolates implying a clonal relationship between them.

Similarly the common PFGE patterns and closely related pulsotypes found between the sheep and human isolates strongly suggest that the sheep isolates are the same clone as the human isolates. The occurrence of only one cattle isolate which had three pulsotypes in the sheep isolates suggests that the cattle and sheep isolates are more

distantly related and probably have diverged very early from each other in their evolution.

Most cases of campylobacteriosis in humans occur sporadically with the principal route of infection believed to be foodborne (Skirrow, 1994; Tauxe, 1992). Our results provide substantial genotypic evidence for a link between sporadic human infection and two food animal sources; cattle and sheep in the region.

PFGE has been used successfully in earlier studies to establish links between food animal sources and human disease. Lorenz *et al.* (1998), used *Sma* I PFGE profiles to characterise chicken and human isolates and was able to detect similar types between the two sources. Similarly in another study Hanninen *et al.*, (1998b), found identical PFGE patterns in human and poultry isolates. However four of the ten most common human PFGE types were not found among chicken isolates, which suggests that in addition to poultry other sources like cattle and sheep might contribute to human infections as has been demonstrated by the occurrence of closely related strains between the cattle, sheep and human isolates in the present study.

Although poultry are traditionally regarded as the principal source of such infections (Shane, 1992) cases linked to bovine and ovine sources have been described previously (Dilworth *et al.*, 1988; Orr *et al.*, 1995). Also the data presented in the present study indicate the need for further investigation concerning the infectious potential of other foodstuffs such as beef and mutton as a source of human infections or as a reservoir for *C. jejuni* strains pathogenic to humans.

Laboratory and epidemiological methods used previously to establish chains of transmission in foodborne infections have been limited to serotyping and classical epidemiological case studies, all of which are not sufficiently sensitive to distinguish individual strains (Patton *et al.*, 1991; Arbeit, 1995). In addition, a substantial number of isolates have been found to be untypable by Lior and Penner serotyping and phage-typing (Patton and Wacshsmuth, 1992).

The ability to rapidly identify certain clones of known pathogenicity may thus be more relevant to public health protection than simply detecting the presence of *C. jejuni*. The application of PFGE has been shown to provide precise information that can be used to accept or reject epidemiological associations to a high degree of confidence (Smith and Cantor, 1987; Smith *et al.*, 1988).

The present study has endeavoured to classify the cattle, sheep and human strains in the region and identify the important types prevalent in the region and to enhance our understanding of the epidemiology of campylobacter infections in the area. The close relationship found between certain animal Types with the human isolates has stressed the importance of monitoring the bovine and ovine sources also as an important source of human campylobacter infections.

The use of PFGE in typing *Campylobacter* spp. isolated from human and animal sources and maintenance of a national database should offer an excellent means of epidemiological monitoring of the disease situation and make comparisons between the human and animal strains possible. This would ultimately help in better understanding of the infectious potential of various animal species and offer a means of control of human infections.

CHAPTER 5

5. ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF *CAMPYLOBACTER JEJUNI* ISOLATED FROM CATTLE, SHEEP, AND HUMANS

5.1. INTRODUCTION

Campylobacter jejuni is recognised as one of the most important causes of acute diarrhoeal disease in humans throughout the world (Taylor and Blaser, 1991). Affected persons usually recover without antimicrobial therapy but in some patients with prolonged illness therapy may be indicated. In these circumstances erythromycin or fluroquinolones are often recommended and tetracyclines used as alternatives (Blaser, 1997; Blaser, 1990; Goodman *et al.*, 1990).

An alarming trend has been the increase in resistance in *Campylobacter* spp. isolated from humans to antibiotics which are used in the treatment of human infections (Hoge *et al.*, 1998; Velazquez *et al.*, 1995). Resistant bacteria can diminish the effectiveness of antibiotics and demand the use of more expensive or less safe alternatives.

This has been attributed to the use of antibiotics in food producing animals as growth promoters, in prophylaxis and treatment with antimicrobial agents that are important in human therapy (Tollefson *et al.*, 1998; Reina *et al.*, 1992). It has been reported that worldwide growth promotants account for 42% of the mass of veterinary pharmaceuticals used (Gold and Moellering, 1996).

In addition, it has been demonstrated that the flow of resistance genes in the environment from animals to man is possible (Johnson *et al.* 1994; Nijsten *et al.*, 1994; Linton 1986; Scoli *et al.* 1980).

In New Zealand, the incidence of campylobacter infections in humans is amongst the highest in the world and it is the most commonly diagnosed cause of acute diarrhoeal disease (McNicholas *et al.*, 1995). In a recent study, low level resistance to erythromycin, ciprofloxacin and doxcycline was detected in *Campylobacter* isolates from Auckland (Dowling *et al.*, 1998) However, very little information is available on the prevalence of antimicrobial resistance in other areas of New Zealand in animal and human isolates.

The aim of this study was to determine the antimicrobial profile of *C. jejuni* isolated from humans, cattle and sheep to six antibiotics commonly used in the treatment of campylobacter infections in humans.

5.2. MATERIALS AND METHODS

5.2.1. BACTERIAL STRAINS

One hundred *C. jejuni* human isolates from human patients with diarrhoea were procured from the Palmerston North Medical Laboratory. The 35 cattle and 100 sheep isolates were isolated from faecal samples collected at local meat-works and farms from apparently healthy animals. All the isolates were stored frozen in glycerol broth at -70°C and revived by culturing on blood agar. The control organism was *E. coli* NCTC 10418.

5.2.2. ANTIBIOTIC SUSCEPTIBILITY TESTING

Susceptibility testing was performed by the controlled disc diffusion method (Hunter, 1994) on Muller-Hinton agar plates supplemented with 5% sheep blood. A well-isolated colony was selected from a blood agar plate and inoculated into 3 ml of sterile

saline and briefly rotamixed. A suspension for the control *E. coli* was similarly prepared. A sterile swab was used to apply the control and the test suspension spirally on the agar medium using a rotary plater (Pbi International). The control was spread from the area of the disc to the outside of the plate and the test sample from the centre of the plate to the area of the discs.

The six antibiotic discs (Oxoid) of erythromycin (15 μg), tetracycline (10 μg), enrofloxacin (10 μg), chloramphenicol (10 μg), gentamicin (10 μg) and neomycin (10 μg) were applied on the agar between the test sample and control using a disc dispenser (Oxoid). The plate was allowed to dry for 30 minutes and then incubated in an anaerobic jar with a Campy-Pak (BBL) gas envelope at 42°C. The plates were read after 48 h and the diameter of the zones of inhibition around the antibiotic discs recorded.

5.2.3. MINIMAL INHIBITORY CONCENTRATION (MIC)

The MICs of the resistant isolates was determined by an agar dilution method (Mendez *et al.*, 1980). Isolates were grown in blood agar plates containing 1 μg/ml of tetracycline. Growth was suspended in 0.1 M phosphate buffer saline (PBS) to 0.5 McFarland standard. The suspension was diluted 1:10 in PBS and inoculated into Muller-Hinton agar plates with doubling concentration of tetracycline ranging from 2 to 128 μg/ml in duplicates. The plates were incubated at 42°C and observed after 24 h and 48 h for growth. The highest concentration in which growth was absent was taken as the MIC of that strain.

5.3. RESULTS

5.3.1. RESULTS OF ANTIMICROBIAL SUSCEPTIBILITIES OF HUMAN, CATTLE AND SHEEP ISOLATES

All the 100 human isolates were found sensitive to erythromycin, enrofloxacin, gentamicin, chloramphenicol and neomycin. Two isolates were found resistant to tetracycline.

All 35 *C. jejuni* isolates from cattle as well as the 100 sheep *C. jejuni* isolates were found sensitive to all the six antibiotics tested.

5.3.2. RESULTS OF DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATIONS (MICs) OF THE RESISTANT ISOLATES

The minimal inhibitory concentrations of the tetracycline resistant isolates of *C. jejuni* were determined by the agar dilution method. MICs of the two resistant isolates were found to be $\geq$ 128 μg/ml. Tetracycline susceptible isolates demonstrated MICs of $\leq$ 2 μg/ml.

The two resistant isolates were in the process of testing sub-cultured 3 times after the first determination of resistance and still found resistant.

5.4. DISCUSSION

In modern breeding of farm animals, practised in most countries, large amounts of antibiotics are used for a variety of reasons, including growth promotion, disease prophylaxis, and therapy and there is an increasing concern that such widespread use of antibiotics is contributing to the emergence of resistant bacteria that are contaminating the food supply (WHO Report, 1997). However, this is not at all the New Zealand situation except for intensive industries such as pigs and poultry. Many of the 40 million sheep in New Zealand would never see antibiotics.

Infections with quinolone-resistant strains of *Campylobacter* organisms are now found with increasing frequency in clinical settings, following a trend of food borne pathogens that have become resistant to antibiotics and have caused an increase in morbidity and mortality among humans (Tee *et al.*, 1995; Alder-Mosca *et al.*, 1991; Endtz *et al.*, 1991a)

In the United States, Smith *et al.* (1999) found that resistance to nalidixic acid increased approximately eight-fold from 1.3% in 1992 to 10.2% in 1998. All isolates that were resistant to nalidixic acid were also resistant to ciprofloxacin also 2% were resistant to erythromycin and 61% resistant to tetracycline.

Data from other countries provide evidence that the use of fluoroquinolones in poultry production has a primary role in increasing resistance to quinolones among *C. jejuni* isolates from humans. In the Netherlands, an increase in ciprofloxacin resistant human isolates from 0% through 1985 to 11% in 1989 paralleled the increase in ciprofloxacin-resistant isolates from retail poultry products (Endtz *et al.*, 1991a).

Likewise in Spain, an increase in the prevalence of ciprofloxacin-resistant human isolates from between 0% - 3% in 1989 to 30% - 50% in 1991 coincided with the introduction of enrofloxacin for veterinary use in 1990 (Reina *et al.*, 1992; Sanchez *et al.*, 1994).

In the present study the antimicrobial susceptibility of *C. jejuni* isolates to six antibiotics was examined by the disc diffusion method. It was simple to perform and results were easily interpreted. Standard methods for susceptibility testing and interpretation of resistance in *Campylobacter* spp. are lacking and different workers have employed various methods. However, the disc diffusion method has been widely applied with good results for campylobacter testing (Gaudreau and Gilbert, 1997, Huysmans and Turnidge, 1997; Koenraad *et al.*, 1995b; Vanhoof *et al.*, 1984). If there is a need to obtain quantitative data regarding the resistant isolates, then minimal inhibitory concentrations could be determined by an agar dilution method.

All the 35 cattle and 100 sheep isolates were found to be susceptible to all the six antibiotics used including enrofloxacin. This is contrary to findings in many countries where resistance to antibiotics is being increasingly detected in animal isolates particularly against the quinolones.

Smith *et al.* (1999) found 20 % of *Campylobacter* isolates from poultry to be resistant to ciprofloxacin and Jacobs-Reitsma (1997) found 29 % to be resistant to quinolones. This may be due to the extensive systems in which these animals are raised in New Zealand with minimal to no use of antibiotics.

The likely times when antibiotics are used in these animals would be to treat sick animals. Antibiotic resistant bacteria are more frequently isolated from pigs and poultry than from extensively grazed cattle or sheep indicating the greater use of antimicrobial agents in intensively housed species for growth promotion (Threlfall, 1993). A study of *Campylobacter* isolates from pigs and poultry in New Zealand may give very different results and should be conducted in a suitable range of isolates.

In our study only two human isolates (2%) of *C. jejuni* were found resistant to tetracycline. No resistance to the other five antibiotics was detected in human, cattle or sheep isolates. The findings agree with a recent study done in Auckland which reported two of 202 human isolates to be resistant to tetracycline (Dowling *et al.*, 1998).

Overseas research shows that tetracycline resistance is more common in human isolates than among isolates from animals. In studies by Aarestrup *et al.* (1997) 11 % of the human isolates as compared to 2 % of the animal isolates were resistant to tetracycline. Huysmans and Turnidge (1997) in Australia studied 100 clinical isolates of thermophilic *Campylobacter* species from humans and did not detect resistance to erythromycin, gentamicin or chloramphenicol but nine strains were found resistant to tetracycline.

The absence of resistance to important antibiotics like erythromycin and enrofloxacin seen in the present study speaks well for the country although low level resistance was seen in human isolates for erythromycin (1.5%) and ciprofloxacin (2.5%) in the Auckland study (Dowling *et al.*, 1998). This is still very much at a lower level when compared to the extent of resistance observed overseas.

Levels as high as 84% in Thailand (Hoge *et al.*, 1998) to 34% in Spain (Velazquez *et al.*, 1995) and 18.6% in Sweden (Sjogren *et al.*, 1997) to fluoroquinolones have been reported. Similarly rates as high as 79% resistance to erythromycin have been reported in Nigeria (Coker and Adefeso, 1994) to 0.6 % in Japan (Tadano *et al.*, 1996). It is however advisable to monitor this situation so that appropriate action could be instituted should resistant strains be detected in the future.

Further studies on the antibiotic susceptibility patterns of *C. jejuni* from intensively farmed species in New Zealand such as poultry and pigs are needed to better

understand the situation in this country with regard the prevalence of resistance, to come to definite conclusions about the extent of the problem if any.

CHAPTER 6

6. DEVELOPMENT OF A POLYMERASE CHAIN REACTION (PCR) FOR IDENTIFICATION OF *CAMPYLOBACTER JEJUNI* FROM MEAT SAMPLES.

1. INTRODUCTION

The demanding growth requirements of *Campylobacter* spp. make their microbiological detection and identification a time consuming and onerous task. Discrimination among closely related thermophilic campylobacters is by biochemical and serological tests and is labour intensive. The detection and identification of these organisms by PCR is now being recognised as a reliable alternative to the traditional tests (Harmon *et al.*, 1997).

The use of PCR to screen samples of retail poultry and red meats as a rapid method of analysis of samples from the supermarkets in addition to the culture method was evaluated in the present study. Therefore the initial task of developing and optimising the PCR was undertaken.

6.2. MATERIALS AND METHODS:

6.2.1. OLIGONUCLEOTIDE PRIMER SELECTION

The objective of the development of the PCR was to identify *C. jejuni,* the principal *Campylobacter* spp. responsible for the overwhelming majority of infections in humans. After perusal of available literature it was decided to select and use primers directed against the hippuricase gene previously described by Linton *et al.*, (1997), which is unique and highly conserved in *C. jejuni* (Slater and Owen, 1997; Hani and Chan, 1995).

The primer pair was as follows:

PRIMER AH 1: 5'- GAA GAG GGT TTG GGT GGT G -3'

PRIMER AH 2: 5'- AGC TAG CTT CGC ATA ATA ACT TG -3'

The primers were synthesised by Life technologies, Auckland, New Zealand and were reconstituted in sterile distilled water to give 200 µM solution of each primer. Primers were stored in aliquots of 50 µl at -20°C.

6.2.2. DNA EXTRACTION FROM BACTERIA

Preparation of genomic DNA from *C. jejuni* was carried out by three different methods for use as templates for the PCR. The bacteria were grown from the frozen cultures on blood agar for 48 h at 42°C and broth cultures made by growing in Preston broth for 18 h at 42°C.

6.2.2.1. DNA extraction based on proteinase-K digestion and phenol-chloroform purification

One millilitre of the broth culture was taken in a microfuge tube and centrifuged for 10 min at 2000 rpm and the supernatant discarded and the pellet washed two times with TE buffer. To the pellet was added 500 µl of TE buffer and 100 µl (10 mg/ml) of Lysosyme mixed well and the tube was incubated at 37°C in a waterbath for 30 min and 100 µl of 10% sodium dodecyl sulphate (SDS) added and incubated for a further 30 min. Thereafter 5 µl (10 mg/ml) Proteinase K (Boehringer Mannheim, Germany) was added to the tube and incubated again at 56°C for 1 h.

After the incubation an equal volume of 25:24:1 phenol:chlorofom:isoamyl alcohol solution was added to the tube, mixed well by vortexing and centrifuged at 12000 rpm for 10 min. The upper phase of this solution, which contained the DNA, was transferred to a fresh tube and the extraction process repeated. Then to the solution was added an

equal volume of chloroform, mixed well and centrifuged at 12000 rpm for 10 min and the upper phase transferred to a new tube.

The DNA in the solution was precipitated by adding an equal volume of absolute ethanol and 1/10 volume of 3 M sodium acetate and left for 1 h at - 80°C and then centrifuged at 12000 rpm for 20 min. The pellet was washed with 70 % ethanol and vacuum dried and resuspended in 200 µl of TE and the DNA concentration measure directly by the GeneQuant (Pharmacia Biotech, Cambridge, England) DNA calculator and 1-5 µl used in the PCR or stored at -20°C for subsequent use.

6.2.2.2. DNA extraction by boiling method

The DNA was extracted by whole cell lysis brought about by boiling the bacteria. 1 ml of the broth culture was taken in a microfuge tube and centrifuged at 8000 rpm for 5 min. Supernatant was discarded and the pellet washed twice in 1 ml TE buffer. A 200 µl of the suspension was taken in a tube and boiled for 10 min and immediately cooled on ice and 5 µl of this was used as a template in the PCR reaction.

6.2.2.3. DNA extraction using QIAGEN DNA kit

The DNA was extracted using QIAamp (QIAGEN, Germany) rapid DNA extraction kits using the manufacturers protocol. The procedure in brief was as follows: About 1ml of the enrichment culture was pipetted out into a 1.5 ml microfuge tube and centrifuged for 5 min at 7500 rpm. The supernatant was discarded and to the pellet was added 180 µl of buffer ATL supplied in the kit and 20 µl of proteinase K stock solution (20 mg/ml) and mixed by vortexing and incubated at 55°C for 1 h. After the incubation 200 µl of buffer AL supplied with the kit was added and mixed thoroughly and incubated at 70°C for 10 min in a water bath. Then 210 µl of ethanol (96-100%) was added to the sample and vortexed. This mixture was applied carefully to QIAamp spin column with a collection tube and centrifuged at 8000 rpm for 1 min. The filtrate was collected in the collection tube and was discarded along with the collection tube. The spin column was washed twice with 500 µl of buffer AW supplied with the kit at 8000 rpm for 1 min and each time the filtrate was collected in a clean collection tube and discarded. After the wash

steps the DNA was eluted twice by applying 200 µl of buffer AE preheated to 70°C to the spin column and centrifuging at 8000 rpm for 1 min. The DNA was eluted into a microfuge tube and the concentration determined directly by the GeneQuant DNA calculator and used as template in the PCR or stored in the same buffer at -20°C till further use.

6.2.3. PCR REACTION COMPONENTS

6.6.3.1. Taq DNA polymerase system

During optimisation of the PCR the reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM dNTPs, and 2.5 U of Taq DNA polymerase (Boehringer Mannheim, Germany) in a total volume of 25 µl.

6.2.3.2. HotStarTaq™ DNA polymerase system.

The HotStarTaq DNA polymerase system (QIAGEN, Germany) is a modified form of Taq DNA polymerase, which is supplied in the inactive form and is activated by heating to 95°C for 15 min just before the thermal cycling begins. It was used with a 10x PCR buffer (Supplied with the polymerase) and 0.2 µM dNTPs (Boehringer Mannheim, Germany).

6.2.4. OPTIMISATION PROCEDURES

6.2.4.1. Magnesium concentration

Magnesium concentrations in the reaction were increased by the addition of sterile $MgCl_2$ (25 mM, Boehringer Mannheim, Germany). The following concentrations were tested: 1.5, 2.0, 2.5 and 3.0, mM

6.2.4.2. Denaturation, annealing and extension times and temperature

A standard temperature of 94°C was used for denaturation. Initially, a temperature of 55°C was chosen for annealing, however, this was raised to 60°C and finally to 65°C to eliminate non-specific priming during PCR. Primer extension was performed at a

standard 72°C. The times of denaturation, annealing and extension were kept at 1 min each.

6.2.4.3. Number of amplification cycles

The number of cycles can affect the yield of amplification product so 25, 30 and 35 cycles were performed and evaluated.

6.2.5. STANDARD PCR REACTION COMPONENTS AND CONDITIONS

The components of the PCR reaction were assembled in 25 µl volumes containing the following -

Addition order	Quantity (µl)	Final Concentration
Distilled water	13.8-17.8	-
10x reaction buffer	2.5	1x
$MgCl_2$	1.0	3.0 mM
dNTP's	0.5	200 µM each
Primer AH 1	1.0	1.0 µl
Primer AH 2	1.0	1.0 µl
Hotstar DNA polymerase	0.2	1 U
Template	1-5	1ng/µl

Master mixes of all reagents were made and aliquots placed in 0.2 ml PCR reaction tubes to avoid reagent losses. Tubes were kept on ice until template was added. The reactions were performed using sterile procedures and following contamination-free guidelines. Separate rooms, equipment, reagents and disposables were used for making up reagent mixtures, adding template, running the PCR and analysis of PCR products. The addition of template was conducted in a class II bio-safety cabinet (Gelman Science, Australia).

Amplifications were performed in 0.2 ml PCR tubes in a programmable DNA thermal cycler (Perkin-Elmer Cetus Instruments, Connecticut, USA). An initial temperature of 95°C for 15 min was used to activate the Hotstar Taq polymerase followed by 35 cycles of denaturation, annealing and extension as follows -

PCR steps	**Temperature**	**Time**
Denaturation	94°C	1 min
Annealing	65°C	1 min
Extension	72°C	1 min

After completion of the amplification cycles the tubes were cooled to 4°C to stop the reaction.

6.2.6. SPECIFICITY AND SENSITIVITY OF THE PCR

The in vitro detection limit or sensitivity of the PCR (defined as the lowest quantity of template detectable in the assay) was assessed using DNA extracted from *C. jejuni* reference strain NCTC 11351. Starting from a known quantity of DNA, estimated by GeneQuant DNA Calculator (Pharmacia Biotech, UK), ten-fold dilutions were made from approximately 1 ng to 1 fg and amplified under standard PCR cycling conditions.

The specificity of the PCR (defined as the ability of the PCR to amplify only DNA from *C. jejuni*) was assessed using bacterial genomic DNA as a template in a standard PCR. Species of bacteria used in the present study and their source are presented in Table 6.1

Table 6.1 Bacterial species and sources of isolates used in the assessment of the PCR specificity and sensitivity

BACTERIAL STRAINS	SOURCE
1. *Salmonella brandenburg*	Reference and clinical isolates obtained from the Veterinary Microbiology Laboratory, Massey University, Palmerston North.
2. *Escherichia coli O157:H7*	
3. *Escherichia coli NCTC 10418*	
4. *Yersinia enterocolitica*	
5. *Yersinia pseudotuberculosis*	
6. *Streptococcus agalactiae*	
7. *Staphylococcus aureus*	
8. *Brucella ovis*	
9. *Campylobacter jejuni* NCTC 11351	
10. *Campylobacter coli* NCTC 11366	
11. *Campylobacter lari*	Isolated in the present study
12. *Campylobacter hyointestinalis*	

6.2.7. GENERATION AND LABELLING OF PROBE

The primers AH 1 and AH 2 were employed to produce a 735 bp fragment that served as a template of a probe, which was used to confirm the identity of the PCR products. The template of the probe was generated by PCR using the standard PCR conditions. After amplification, 15 µl aliquots of amplification mixture were electrophoresed in 2% agarose gel in TBE with molecular size markers (ΦX174 RF DNA/*Hae* III Fragments). The 735 bp band of ethidium bromide-stained DNA was visualised on a 312 nm UV transilluminator, carefully excised from the gel and transferred to an Eppendorf tube. The PCR product was purified using a CONCERT™ gel extraction system (Life Technologies) according to the manufacturers instructions. After elution in TE the concentration of the purified probe was estimated directly by GeneQuant DNA calculator (Pharmacia Biotech, Cambridge, UK).

The purified probe was labelled using a DIG High Prime DNA labelling Kit (Boehringer Mannheim). One µg of purified probe was suspended in 16 µl of sterile distilled water in a reaction vial and heated in a boiling water bath for 10 min to denature the DNA. The probe was then rapidly cooled on ice and 4 µl DIG-High Prime added and mixed and centrifuged briefly. The mixture was incubated for 20 h at 37°C and the reaction stopped by heating to 65°C for 10 min.

The efficiency of the DIG labelling was quantified by comparison with DIG labelled control DNA supplied with the kit.

6.2.8. VALIDATION AND IDENTITY OF 735-bp PCR PRODUCT

After purification of the 735-bp PCR product using CONCERT™ rapid PCR purification system the sequencing of the amplicons was performed by the Massey University DNA analysis service.

6.2.9. PCR PRODUCT ANALYSIS

After amplification, the analysis consisted of electrophoresis of PCR products in 1.5% agarose gel and then the identity of the amplicons generated by the AH 1 and AH 2 primers confirmed by hybridisation with the DIG-labelled probe.

Electrophoresis of PCR products

Aliquots (10µl) of each amplification mixture were mixed with 2 µl of loading dye and were electrophoresed in 1.5% Agarose gels in 1%TBE at 100 volts for 1 hr. Molecular size markers (ΦX174 RF DNA/*Hae* III fragments, GIBCO BRL, USA) were run concurrently. The ethidium bromide stained DNA bands were visualised on a 312 nm UV transilluminator, and photographed using Polaroid 667 film.

Dot blot hybridisation

DNA-DNA hybridisation was performed to confirm the identity of the amplicons. Amplification mixtures in each PCR tube were heated for 4 min at 94°C, immediately transferred on ice, and then 1 µl aliquots of each reaction mixture deposited manually onto a Hybond-N membrane (Amersham, UK). After DNA cross-linking by ultraviolet light irradiation for 3.5 min, the membrane was hybridised for 2 h at 65°C in 10 ml of Rapid-hyb buffer (Amersham, UK) containing 5 ng of the DIG-labelled 735 bp probe per 1 ml of the buffer. After hybridisation, the membrane was washed under stringent conditions: once in 2 X SSC, 0.1% SDS at room temperature for 10 min, followed by one wash in 1 X SSC, 0.1% SDS at 65°C for 10 min and finally in 0.7 X SSC, 0.1% SDS at 65°C for 15 min.

After post-hybridisation washes, the membrane was equilibrated at room temperature for 1 min in 40 ml of washing buffer (10 mM maleic acid, 15 mM NaCl; pH 7.5, 0.3% (v/v) Tween® 20) and blocked for 60 min at room temperature in 40 ml of blocking solution (10 mM NaCl; pH 7.5 1% (w/v) blocking reagent). The blot was then incubated at room temperature for 30 min in freshly prepared antibody solution (40 ml blocking solution to which 4 µl anti-digoxigenin antibody was added), washed twice (15 min per wash) in washing buffer, equilibrated for 2 min in 40 ml of detection buffer (10

mM Tris-HCl, 10 mM NaCl pH 9.5). After equilibration, the membrane was incubated at room temperature for 2 h in 40 ml of freshly prepared colour substrate solution containing detection buffer to which 4.5 µl of NBT solution (75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformide) and 3.5 µl of BCIP solution (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformide) was added per 1 ml of the buffer. The colour was developed in the dark and was stopped by washing the membranes with 100 ml of TE for 10 min.

6.3. RESULTS

6.3.1. DNA EXTRACTION

DNA extracted by the phenol chloroform method resulted in a good amount of pure DNA and the process required about 3 h to complete and the use in the amplification reaction gave a good product. (Fig 6.1)

The DNA extraction using the QIAamp kit gave consistently good amounts of highly pure DNA and the whole process took less than 90 min to complete and produced a strong signal (product) on the gel (Fig 6.1).

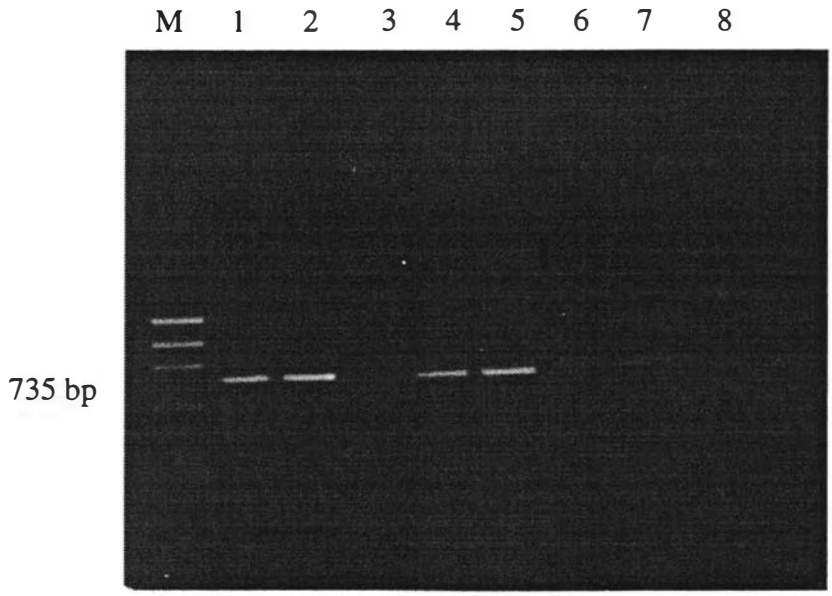
The use of crude DNA extracted by boiling did not give good amplification of the product (Fig 6.1).

6.3.2. POLYMERASE SYSTEMS

Two polymerase systems were evaluated. The Taq polymerase system (Bohreinger Manheim) when used for amplifying DNA extracted from the enrichment medium in the PCR gave a large number of non specific amplifications (Fig 6.2A).

The use of Hotstar Taq polymerase (QIAGEN) system was found to specifically amplify the target region and the non specific reactions were eliminated (Fig 6.2B).

Figure 6.1 1.5% Agarose gel with PCR products amplified by the AH 1 and AH2 primers using DNA extracted by different methods from *C. jejuni*.



M: Molecular marker ∅X 174 RF DNA / *Hae* III fragments

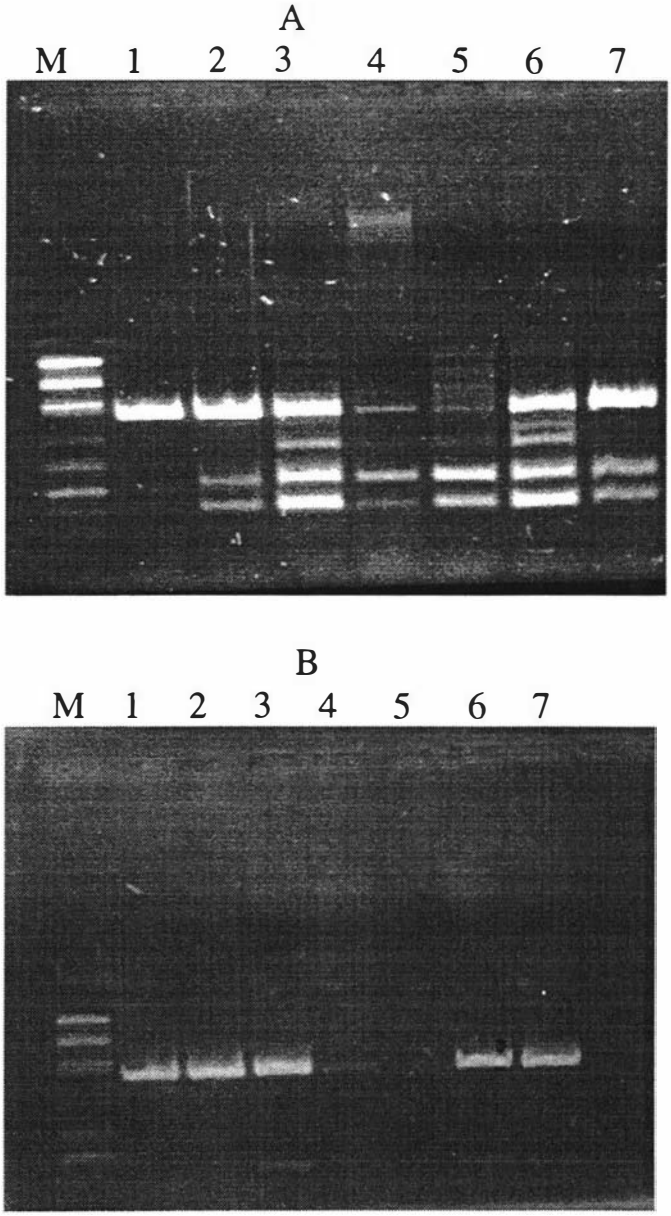
Lane 1 and **2:** 735 bp product amplified from Phenol chloroform extracted DNA

Lane 3 Negative reagent control

Lane 4 and **5:** 735 bp product amplified from QIAGEN Kit extracted DNA

Lane 6 and **7:** Faint bands visible from DNA extracted by boiling method.

Figure 6.2 1.5 % Agarose gels showing PCR products using the standard Taq Polymerase system and HotStarTaq polymerase system.

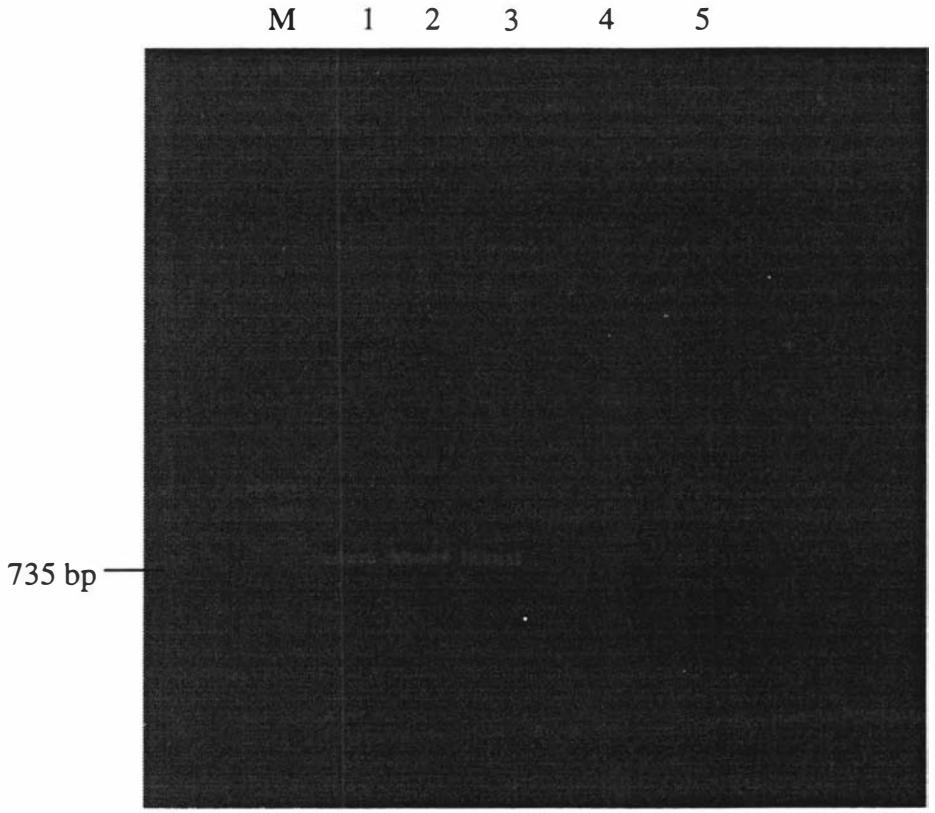


M: Molecular marker ØX 174 RF DNA / *Hae* III fragments

Fig A: Amplification of non specific products when Taq polymerase system is used for amplification of DNA extracted from mince incoculated with *C. jejuni* in lanes 1,2,3,6 and 7 and lanes 4 and 5 containing uninoculated mince samples as negative controls.

Fig B: The same with HotStar Taq polymerase shows the elimination of non specific product amplification.

Figure 6.4 Sensitivity of the PCR using purified DNA



M: Molecular marker ∅X 174 RF DNA/ Hae III fragments

Lane 1: 1ng of *C. jejuni* DNA

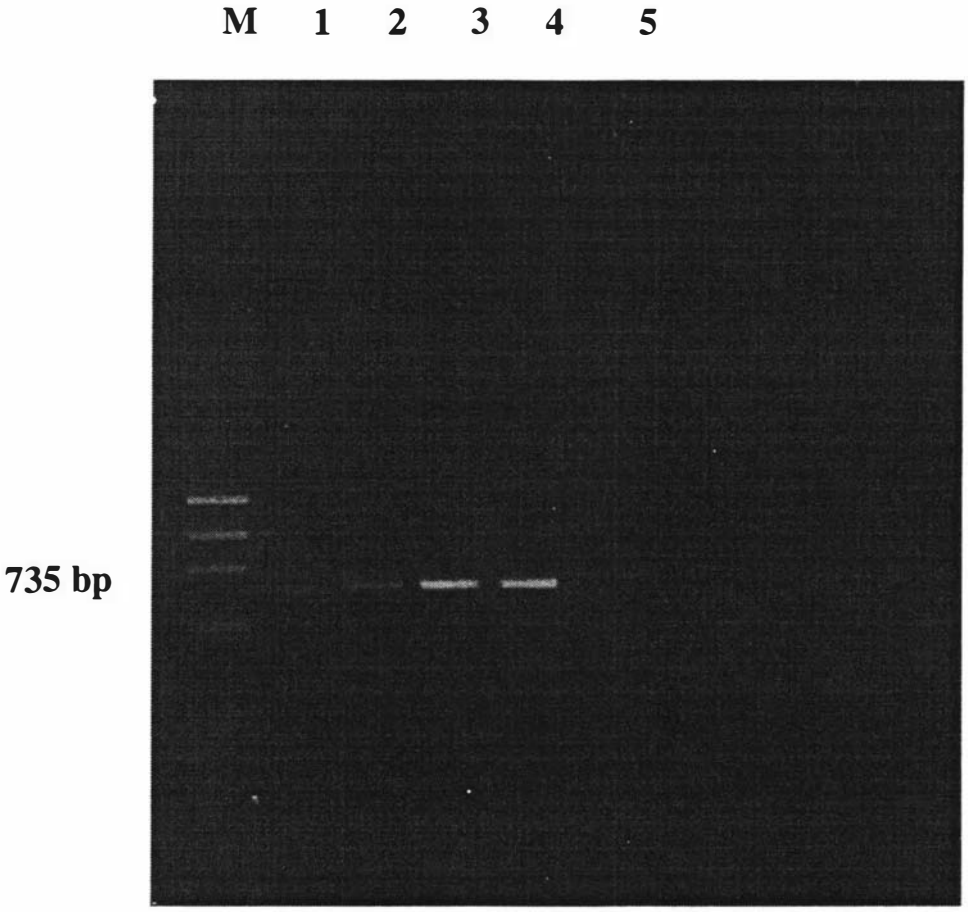
Lane 2: 1pg of *C. jejuni* DNA

Lane 3: 100 fg of *C. jejuni* DNA

Lane 4: 1 fg of *C. jejuni* DNA

Lane 5: reagent negative control.

Figure 6.3 Optimisation of magnesium concentration



M : Molecular weight marker-∅X 174 RF DNA/ *Hae* III fragments

Lane 1: *C. jejuni* + 1.5 mM Mg (commercial buffer)

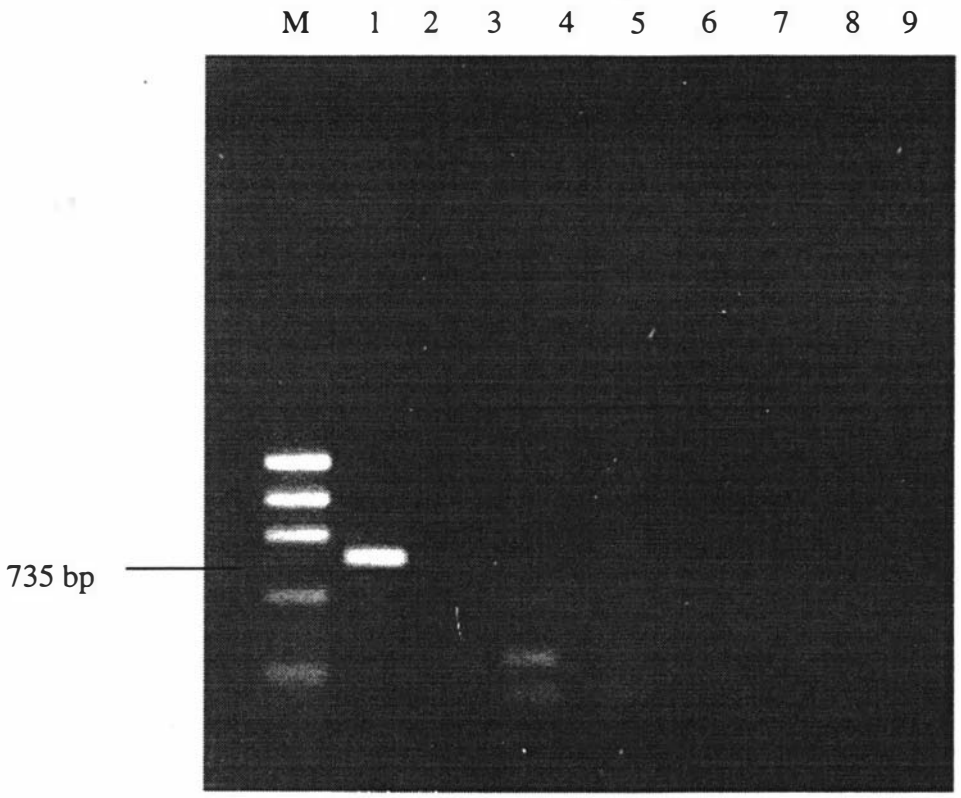
Lane 2 : *C. jejuni* +2.0 mM Mg

Lane 3 : *C. jejuni* + 2.5 mM Mg

Lane 4 : *C. jejuni* + 3.0 mM Mg

Lane 5 : reagent negative control

Figure 6.5 Specificity of the PCR



M: Molecular size marker ∅X 174 RF DNA/ *Hae* III fragments
Lane 1: *C. jejuni* NCTC 11351
Lane 2: *C. coli* NCTC 11361
Lane 3: *C. lari*
Lane 4: *C. hyointestinalis*
Lane 5: *E. coli*
Lane 6: *Salmonella brandenburg*
Lane 7: *Yersinia enterocolitica*
Lane 8: *Brucella ovis*
Lane 9: Reagent negative control

6.3.3. OPTIMISATION OF PCR

A range of magnesium concentrations starting from 1.5, 2.0, 2.5 and 3.0 μM were evaluated. A good amplification was seen at a concentration of 3.0 μM (Fig 6.3) and was considered to be optimum for the PCR reaction.

A denaturation temperature of 94°C, annealing temperature of 65°C and extension temperature of 72°C with a time of 1 min each were found optimum and used in the standard PCR reaction.

Amplification using 35 cycles was seen to produce the strongest signal on the gel and used in the standard assay.

6.3.4. SENSITIVITY AND SPECIFICITY OF PCR

The sensitivity of detection or the lowest amount of DNA that could be detected in the standard PCR was seen to be 100 fg (Fig 6.4).

DNA from no organisms (Table 6.1) except *C. jejuni* was amplified using the primers AH 1 and AH 2 (6.5)

6.3.5. VALIDATION AND IDENTITY OF PCR PRODUCT

The analysis of the sequence of the 735 bp product using the BLAST GenBank database program confirmed the identity of the PCR product generated by the AH 1 and AH 2 primers.

6.4 DISCUSSION

Detection of *Campylobacter* spp. in foods takes 5-6 days and involves selective cultural enrichment followed by isolation from selective agar (Skirrow, 1977; Humphrey 1986) and finally identification by a range of biochemical and serological tests.

These limitations emphasise the need for a rapid reliable and sensitive technique for the detection of *Campylobacter* in foods. The PCR has been used for the detection and typing of *Campylobacter* (Birkenhead *et al.,* 1993), but its use in the food industry has been limited by the inhibition of Taq polymerase by components from food (Giesendorf *et al.,* 1992).

Therefore in the development of the PCR for the detection of *C. jejuni* in meat, it was decided to employ a short enrichment before extracting the DNA so as to increase the number of target organisms as well as remove the target bacteria from the food matrix and thereby resolve any likely problems with inhibition of PCR reaction from the substance in the meat.

The hippuricase gene sequence was selected as the primer as it was unique to *C. jejuni.* Slater and Owen (1997) have shown by restriction fragment length polymorphism analysis that the hippuricase gene of *C. jejuni* is highly conserved.

It was observed in the study that the PCR products were consistent and of the correct size indicating that the amplified sequence is conserved among different strains of *C. jejuni.*

The sensitivity of the PCR assay was seen to be in femtogram quantities of genomic DNA. This corresponds to fewer than four bacterial cells, on the basis of a 1,700 kb genome in *Campylobacter* spp. (Chang and Taylor, 1990).

The assay did not generate a detectable PCR product with DNA from *C. fetus, C. lari, C. coli, C. hyointestinalis, C. upsaliensis* or from other Gram-negative bacteria likely to be encountered in the same clinical environment as *C. jejuni* and can therefore be considered to be highly specific for the identification of *C. jejuni.*

A standard PCR buffer is suitable for most PCR assays but better results were obtained with higher $MgCl_2$ concentrations (final concentration 3.0mM) than are generally found in commercially prepared buffers (1.5mM). The high Mg^{2+} concentration can affect primer annealing, strand disassociation temperature, product specificity and enzyme activity and fidelity. When the Mg^{2+} concentration increased to 3.0 mM the products of the size amplified were clearly visible.

The use of chromosomal templates extracted by boiling whole cells has the advantage of speed and convenience but is dependant on the inoculum size as too much inoculum (>50,000 cells) can inhibit the PCR reaction (Nachamkin *et al.,* 1996). It was seen that compared to the other two methods of DNA extraction it produced a very faint signal which could suggest a decrease in sensitivity of the PCR when it is applied to crude DNA extracts and is in agreement with other studies (Wards *et al.,* 1995; Fenwick, 1997). Possible explanations include insufficient extraction of DNA from *C. jejuni*, the presence of PCR inhibitors, or a combination of both of these.

The use of HotStarTaq™ DNA polymerase system in the PCR assay had a very beneficial effect in eliminating the non specific amplifications seen when standard Taq polymerase was used in the assays with DNA extracted from enrichment broth with meat mince present. HotStarTaq™ is a modified form of Taq DNA polymerase and is supplied in an inactive state and can be activated by 15 min incubation at 95°C just before the thermal cycling begins. Since it is inactive during PCR setup and prior to the initial denaturation step non-specifically annealed primers are not extended, which reduces amplification of nonspecific products, background, and primer-dimer formation in each PCR cycle.

The objective of the present study was to design a PCR assay for the detection of *C. jejuni* in meat samples. This was successful and the application of the PCR assay to retail meat samples is presented in Chapter 7.

CHAPTER 7

7. SURVEY OF RETAIL MEATS FOR THE PRESENCE OF *CAMPYLOBACTER JEJUNI* BY CULTURE AND POLYMERASE CHAIN REACTION (PCR) METHODS.

7.1. INTRODUCTION

The microbiological detection and identification of *Campylobacter* spp. by conventional methods is a time consuming and delicate process due to the demanding growth requirements of campylobacters. Discrimination among closely related thermophilic campylobacters by biochemical and serological tests is cumbersome. Also the possibilities for reliable species differentiation is very limited due to the fastidious and asaccharolytic nature of the organism. Differentiation between species is sometimes based on a single test for example *C. jejuni* is differentiated from *C. coli* by a positive hippurate hydrolysis reaction.

Genotype-based identification systems like the polymerase chain reaction (PCR) offer a reliable means of identification of organisms and can cut down on the amount of time involved. This methodology allows the amplification of a specific gene fragment by *in vitro* enzymatic amplification of the target DNA. In the past few years this approach has been evaluated by various researchers as a means of identification of *Campylobacter* in faeces (Linton *et al.,* 1997; Oyofo *et al.,* 1997; Rasmussen *et al.,* 1996), water (Jackson *et al.,* 1996; Hernandez *et al.,* 1995), sewage (Koenraad *et al.,* 1995a), dairy products (Docherty *et al.,* 1996; Wegmuller *et al.,* 1993) and poultry (Ng *et al.,* 1997; Docherty *et al.,* 1996; Manzano *et al.,* 1995).

Constraints commonly encountered in the PCR assays were those due to genomic anomalies which affect the size of the PCR product, result in a false negative test or fail

to differentiate species with the primers used. Sometimes the efficacy of the assays is affected by agents present in faecal and tissue samples with purification of bacterial DNA required for optimum performance. Specialised materials and equipment may be required when radiolabelled probes are used for detection of PCR products. However, the advantages of specific and rapid detection outweigh these problems.

This study was undertaken to estimate the prevalence of *C. jejuni* in retail samples of red meat and poultry mince in local supermarkets by culture and enrichment PCR. It is possible that red meats and poultry are a major source of campylobacter infections especially so since the high isolation rates of *Campylobacter* spp. from cattle and sheep faeces in the Manawatu region in the earlier studies. The use of PCR and culture simultaneously permitted a comparison between the two methods and enabled an evaluation of the PCR with an initial enrichment step as a rapid means for identification of *C. jejuni* in meat samples.

7.2. MATERIALS AND METHODS

7.2.1. COLLECTION OF SAMPLES

Sampling was carried out over a six-week period and retail packs of beef mince (n=25), lamb mince (n=25) and poultry mince (n-50), were bought from five supermarkets in Palmerston North. The mince packs were transported on ice in insulated boxes, and processed immediately upon arrival at the laboratory.

7.2.2. PROCESSING OF SAMPLES

About 10 g of each sample were homogenised in 40 ml of peptone water in a Colwarth stomacher 400 (A.J. Seward, London, UK) for 5 minutes. One ml of the contents of the stomacher bag was inoculated into 9 ml Preston broth (Oxoid) and incubated microaerobically at 42°C. After about 18 h, a 1ml portion of the broth was pipetted out

to be used for DNA extraction and the remainder of the broth was reincubated for another 30 h at 42°C. After the 48 h incubation a loop-full of the Preston broth was streaked onto a Cefoperazone charcoal deoxycholate agar (CCDA) plate. The plates were incubated for 48 h at 42°C in a microaerophilic atmosphere. Colonies showing typical morphology were picked up and purified by inoculating onto blood agar plates for a further 48 h and identified biochemically.

7.2.3. PHENOTYPIC IDENTIFICATION OF ISOLATES

Colonies grown on CCDA plates were presumptively identified as *Campylobacter* spp. as described in Chapter 3 (3.2.3) according to their colony form, Gram stain, cell form, motility and oxidase reaction. The species identification was carried out as described in Chapter 3 (3.2.4) by the following tests: hippurate hydrolysis, H_2S production (triple sugar iron agar) and sensitivity to 30 μg of nalidixic acid and cephalothin discs.

7.2.4. EXTRACTION OF DNA FOR PCR

The DNA was extracted using QIAamp (QIAGEN, Germany) rapid DNA extraction kits according to the manufacturers protocol. The procedure as given in chapter 6 (6.2.2.3) was followed.

7.2.5. MEASUREMENT OF DNA CONCENTRATION

The DNA concentration was measured directly by the GeneQuant DNA Calculator (Pharmacia Biotech, Cambridge, England).

7.2.6. POLYMERASE CHAIN REACTION

The Polymerase chain reaction was conducted on the samples using a Perkin Elmer thermocycler. Hot start Taq polymerase and buffer and magnesium chloride buffer was obtained from QIAgen; dNTPs were procured from Boehringer Mannheim, primers were synthesised by Life Technologies, Auckland, NZ and were reconstituted in sterile distilled water to give a 200 µM solution of each primer. Primers were stored in aliquots of 50 µl at -20°C.

Primer 1: 5'- GAA GAG GGT TTG GGT GGT G -3'
(Length 19 bases, GC content 57%)

Primer 2: 5'- AGC TAG CTT CGC ATA ATA ACT TG -3'
(Length 23 bases, GC content 39%)

PCR reactions were carried out in 25 µl volumes as described in chapter 6 with 35 cycles of amplification.

7.2.7. GEL ELECTROPHORESIS OF PCR PRODUCTS

Aliquots (10µl) of each amplification mixture were mixed with 2 µl of loading dye and were electrophoresed in 1.5% Agarose gels in 1%TBE at 100 volts for 1 hr. Molecular size markers (ΦX174 RF DNA/*Hae* III fragments, GIBCO BRL, USA) were run concurrently. The ethidium bromide stained DNA bands were visualised on a 312 nm UV transilluminator, and photographed using Polaroid 667 film.

7.2.8. DOT BLOT HYBRIDISATION

DNA-DNA hybridisation was performed to confirm the identity of the amplicons. Amplification mixtures in each PCR tube were heated for 4 min at 94°C, immediately transferred on ice, and then 1 µl aliquots of each reaction mixture deposited manually onto a Hybond-N membrane (Amersham, UK). The same procedure given in chapter 6 (6.2.9) was followed.

7.3. RESULTS

7.3.1. RESULTS OF THE SCREENING OF POULTRY SAMPLES

Selective enrichment and isolation for *Campylobacter* spp. revealed 22 (44%) of 50 samples processed positive for *Campylobacter* spp. They were identified phenotypically as 20 *C. jejuni* and 2 *C. coli* (Table 7.1).

7.3.2. RESULTS OF THE SCREENING OF BEEF SAMPLES

The 25 beef mince samples examined revealed 4 (16%) positive for *Campylobacter* spp. All the 4 isolates were typed as *C. jejuni.*

7.3.3. RESULTS OF THE SCREENING OF LAMB SAMPLES

Three lamb mince samples (12%) out of the 25 sampled were found positive for *Campylobacter* spp. All of them were typed as *C. jejuni.*

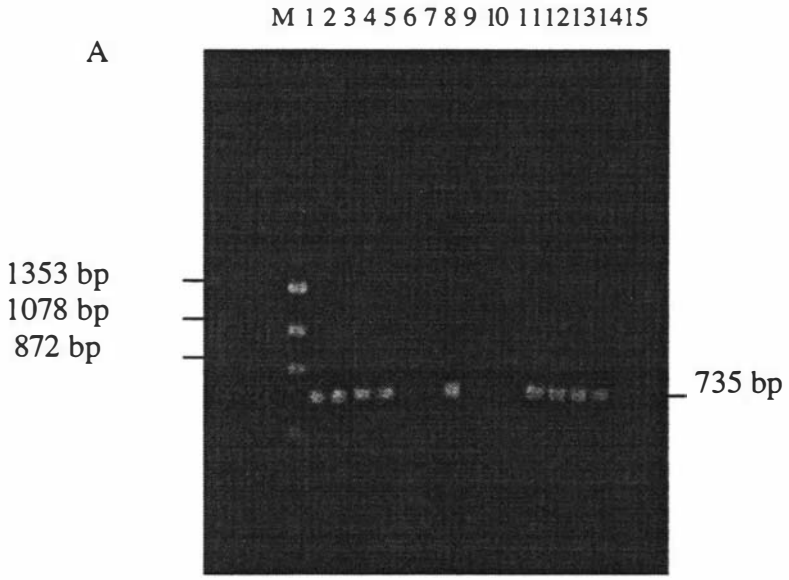
7.3.4. RESULTS OF THE SCREENING BY PCR

The screening of the chicken, lamb and beef mince samples after selective enrichment and PCR with primers directed against the hippuricase gene present in *C. jejuni* amplified an approximately 735 bp product in 22 chicken mince, 4 beef mince and 3 lamb mince samples as seen on the agarose gel after electrophoresis (Figs 7.1. 7.2 and 7.3). The products were confirmed by dot blot hybridisation. The isolates that yielded positive results in the PCR and dot blots were those that were phenotypically identified as *C. jejuni* plus the two positive samples from chicken mince that were phenotypically identified as *C. coli.*

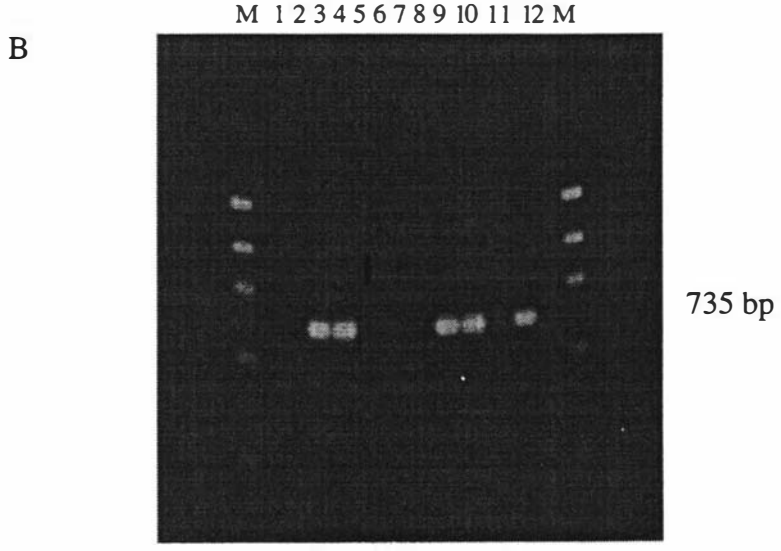
Table 7.1 Results of isolation of *Campylobacter jejuni* from different meat samples by culture and PCR

MINCE SAMPLE	CULTURE ISOLATION POSITIVE (%)	PCR POSITIVE (%)
Poultry (n=50)	22 (44)	22 (44)
Lamb (n=25)	4 (16)	4 (16)
Beef (n=25)	3 (12)	3 (12)

Figure 7.1 PCR of Poultry mince samples

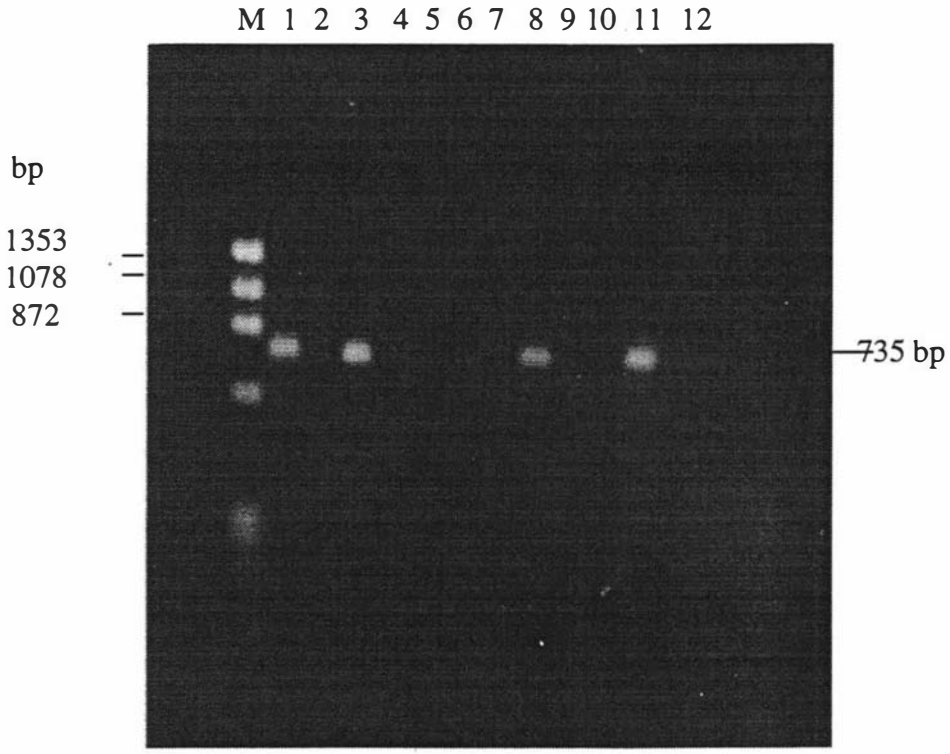


M: Molecular size marker ØX 174 RF DNA/ Hae III fragments
Lanes 1, 2, 3, 4, 7, 11, 12 & 13: Poultry Mince samples showing a 735 bp product
Lanes 5, 6, 8, 9 & 10: poultry mince sample DNA showing negative
Lane 14: positive control *C. jejuni* NCTC 11351 DNA
Lane 15: reagent negative control



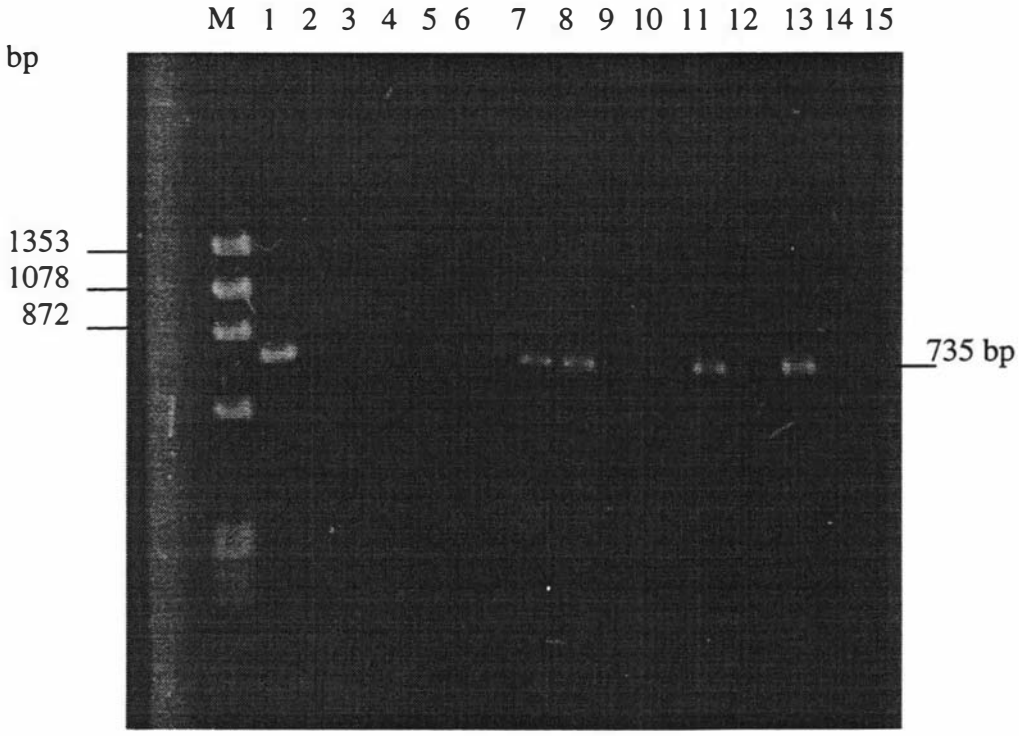
M: molecular size marker ØX 174 RF DNA/Hae III fragments
Lanes 2, 3, 7 & 8: DNA Poultry mince samples showing a 735 bp product
Lanes 1, 4, 5, 6, 9 & 10: Poultry mince samples DNA showing no product
Lane 11: positive control *C. jejuni* NCTC 11351 DNA
Lane 12: reagent negative control

Figure 7.2 PCR of Lamb mince samples



M: molecular size marker ∅X 174 RF DNA Hae III fragments
Lanes 1, 3 & 8: Lamb mince samples DNA showing a 735 bp product.
Lane 11: positive control *C. jejuni* NCTC 11351 DNA
Lane 12: reagent negative control.

Figure 7.3 PCR of beef mince samples



M: molecular size marker ∅X 174 RF DNA/ Hae III fragments
Lanes 7, 8, 11 & 13: Beef mince samples DNA showing a 735 bp product
Lanes 2, 3, 4, 5, 6, 9, 10, 12 & 14: Beef mince samples showing negative
Lane 1: positive control *C. jejuni* NCTC 11351 DNA
Lane 15: reagent negative control.

7.4. DISCUSSION

It has been postulated that *C. jejuni,* a commensal in the intestinal tract of poultry and warm blooded animals may find its way into meat as a faecal contaminant during processing (Doyle, 1990). Food is recognised as the most significant vehicle of human infections (Stern and Kazmi, 1989). The examination of mince meat samples from chicken, beef and lamb in the present study revealed the presence of *C. jejuni* in 44% of the chicken, 16% of the beef and 12% of the lamb samples making all three types of meat potential sources of transmission of campylobacter to consumers.

Studies of campylobacteriosis both in New Zealand and overseas (Eberhart-Phillips *et al.,* 1997) have implicated chicken (Stern and Line, 1992) beef (Lammerding *et al.,* 1988) and lamb (Varnam and Evans 1991) with chicken being considered the most common source.

Investigations of retail meats in other countries have found the occurrence of *C. jejuni* in retail meats more or less comparable to the levels of contamination seen in our study. Fukushima *et al.* (1987) in Japan found 50% of chicken and 1.7% of beef samples positive for *C. jejuni* and more recently Ono and Yamamoto (1999), found 45.8% of retail poultry positive but failed to detect the organisms in beef. In Canada Lammerding *et al.,* (1988) found 5% of raw ground beef and 40% of veal specimens to be positive for *C. jejuni.* However Madden *et al.* (1998) in Northern Ireland could not detect *C. jejuni* in red meats but 38% of retail packs of chicken were found positive. In Australia, sampling of sheep carcasses and frozen bulk packs revealed 1.3% of 885 samples positive for *Campylobacter* spp. (Vanderlinde *et al.,* 1999) and in the U.K. Little et al. (1999) found 28% of raw meat products positive for *Campylobacter* spp.

The levels of contamination in the beef and lamb mince samples were found to be higher than those found in other studies probably because our study used mince meat for isolation, which is usually prepared from trimmings from a variety of sources and is handled extensively. It has also been observed that one heavily contaminated piece of

meat is sufficient to contaminate the entire batch as it passes through the grinder (Jay, 1992).

In our study the mince samples were procured from supermarkets which had their own butchery section where the mince was made and packed. Therefore the possibility of cross contamination of the beef and lamb mince from the chicken meat during the mincing process cannot be ruled out.

There was concurrence between the two methods used, culture and PCR identification in detection of *Campylobacter* spp. Linton et al. (1997) and Giesendorf et al., (1992) observed similar findings of the concurrence of culture isolation and PCR with regards to identification of *C. jejuni.*

Phenotypic discrimination of the *Campylobacter* isolates revealed two of the isolates as *C. coli*, on the basis of a negative hippurate hydrolysis test. However PCR identified these isolates as *C. jejuni.*

The PCR primers used in the present study for the hippuricase gene, which is present only in *C. jejuni* strains (Hani and Chan, 1995) are capable of hybridising to genomic DNA of phenotypically hippurate hydrolysis negative strains (Linton *et al.,* 1997). There is the likelihood that two isolates identified as *C. coli* on the basis of negative hippurate hydrolysis reaction could have been atypical *C. jejuni* (Nicholson and Patton, 1993). Thus the PCR is potentially more reliable in detecting all isolates of *C. jejuni.* It is also possible that other surveys which have relied on isolation and identification may have under-estimated the true prevalence and called some isolates *C. coli* when in fact they were hippuricase-negative strains of *C. jejuni.*

Although the culture isolation method using Preston enrichment broth and Campylobacter selective agar with CCDA selective supplement was found an effective and efficient technique for the isolation of *Campylobacter* spp. from meat, the process is labour intensive and time consuming. The isolation and identification process taking about 6 days to complete. The detection of *C. jejuni* from meat samples utilising the

enrichment step and PCR could be completed in less than 24 h without compromising the accuracy and reliability of the results. The enrichment-PCR identification using HotstarTaq polymerase system as used in the present study has the potential to offer an excellent means of rapidly identifying the organism from meat samples.

A short selective enrichment step in the present study was used to overcome likely problems of low numbers for detection, growth of background flora and inhibition of the PCR reaction due to substances in the food. The use of pre-enrichment has been shown to be effective in detection of *Salmonella* from chicken mince (Mahon *et al.,* 1994) and *Campylobacter* from faeces (Giesendorf *et al.,* 1992) by PCR.

The infective dose for humans of *C. jejuni* is very small. It has been estimated that as few as 500 cells of *C. jejuni* can cause human illness (Black *et al.,* 1988; Robinson, 1981). This means that even a very small number of *Campylobacter* cells in the meat may be a potential health hazard, so a sensitive detection method is required.

The high percentage of *C. jejuni* contamination found in the retail meats may help to explain the high incidence of enteritis in humans caused by *Campylobacter* spp. in New Zealand (Ikram *et al.,* 1994; Lane and Baker 1993). If contamination of the end product is a reflection of infection in live animals, then control of *Campylobacter* contamination on the farm, may reduce the contamination of carcass, poultry and red meat products at the retail level (Kapperud *et al.,* 1992).

Several measures have been advocated for control of *Campylobacter* at the farm level. These include adoption of strict hygiene measures including thorough cleaning and disinfecting procedures especially at poultry farms and monitoring of the environment of the farms for *Campylobacter* spp. (van de Giessen *et al.,* 1998). Another program finding increased acceptance in certain countries is the specific pathogen free (SPF) breeding programs to supply animals free of enteropathogens like *Campylobacter* (Kuiper and Martens, 1994). However, the big challenge is then to maintain freedom from infection during the growing phase on farms.

At the abattoir level, the slaughtering process presents many opportunities for contamination of the carcass by intestinal material. Practices such as 'rodding' and 'bunging', which involve sealing the oesophagus and anus in cattle and sheep, can greatly reduce the risk of contamination during carcass dressing process. Hanging and chilling of the carcass after slaughter can cause the surface to dry out and result in reduction of the number of *Campylobacter* present due to the sensitivity of the organism to desiccation. Radical approaches such as irradiation may also serve to eliminate the organisms (Lambert and Maxey, 1984).

The meat industry has now generally adopted quality assurance systems and is implementing the Hazard Analysis Critical Control Point (HACCP) concept. There is a need for rapid microbiological monitoring systems, which can be adapted to the technology and logistics of specific production processes whereby contribution of critical phases in the production chain towards end product contamination can be estimated in order to take the necessary intervention or corrective steps. Traditional microbiological methods are highly unlikely to meet these requirements. A PCR-based identification of *Campylobacters* would offer a definite advantage.

At the retail level, in butcheries and supermarkets, the equipment associated with grinding of meat should be thoroughly cleaned to prevent successive build up of microbial numbers and also cleaned between grinding different meats to prevent cross-contamination. Cooked and raw meat should be kept separately with staff maintaining adequate hygiene requirements.

Finally, consumers carry an equal responsibility in ensuring that the meat they eat is free from *Campylobacter* by eating thoroughly cooked meats and taking care to avoid cross contamination between cooked and raw meats.

CHAPTER 8

8. GENERAL DISCUSSION / SUMMARY

The emergence of *Campylobacter* spp. as the leading cause of gastrointestinal infections in humans worldwide has been swift and dramatic. They are recognised as the principal agents of bacterial intestinal infections in many countries surpassing the traditional organisms *Salmonellae* (Tauxe, 1992). In New Zealand over the last two decades *Campylobacter* has been the chief cause of human gastrointestinal infections and the most commonly notified disease (McNicholas *et al.,* 1995). Since 1993 notifications of campylobacteriosis have consistently exceeded 200 cases per 100 000 population per annum (Withington and Chambers, 1997).

Campylobacter infections vary from a mild attack of diarrhoea lasting 24 hours to a severe illness lasting more than a week characterised by persistent colicky abdominal pain, blood-stained faeces and general malaise (Butzler and Oosterom, 1991; Healing *et al.,* 1992). Serious ongoing sequelae include reactive arthritis and Guillain-Barre' syndrome (GBS). The former is estimated to complicate campylobacter infection in approximately 1-4% of cases and is associated with considerable ongoing morbidity (Fan and Yu, 1993; Highton and Priest, 1996). GBS is a rare but potentially fatal neurological condition usually requiring extensive periods in hospital and prolonged rehabilitation (Mishu and Blaser, 1993). Some 20-30% of cases of GBS have been associated with campylobacter infection (Rees *et al.,* 1995).

Studies both overseas and in New Zealand have identified consumption of undercooked chicken as a major risk factor for human infections (Eberhart-Phillips *et al.,* 1996; Ikram *et al.,* 1994). However, the potential of cattle and sheep as a contributor to human infection has attracted little attention. The major reservoirs of thermophilic *Campylobacter* are believed to be the intestines of birds and warm-blooded animals (Park *et al.,* 1991), including ruminants (Stern, 1992). The intestinal colonisation of cattle and sheep with campylobacters is significant not only for the contamination of

milk on the farm and the carcass at slaughter but also with regards to environmental and water contamination during disposal of abattoir effluents and slurries to land or run off from farms (Terzieva and McFeters, 1991). This is of direct concern in the New Zealand situation.

The present study was envisaged to explore the possible role of cattle and sheep in the epidemiology of human campylobacter infections in the Manawatu region with the overall objective to determine whether or not cattle and sheep constitute a source of *Campylobacter* for humans in New Zealand. To achieve this goal the work was devised into several parts, each represented by a chapter in the thesis. The primary work involved the isolation of *Campylobacter* spp. from faeces of cattle and sheep. This was undertaken chiefly from animals presented at abattoirs in the region, and also from some dairy and beef farms.

The results of this study indicated an overall prevalence of 45% for *Campylobacter* spp. in the faeces of cattle and sheep which was quite unexpected and contrasted with the limited information already published about the prevalence of *Campylobacter* in cattle and sheep in New Zealand (Meanger and Marshall, 1989). This previous study done to estimate the seasonal prevalence of thermophilic *Campylobacter* found a rate of isolation of only 12% in winter to 31% in autumn. The faecal sampling of cattle was done from both young and adult groups of dairy cattle and from beef cattle sent for slaughter from different farms in the Manawatu region with a view to get a representative prevalence rate of *Campylobacter* spp. in different groups of cattle populations in the region. The rate of *Campylobacter* isolations from the different groups was found to vary between the two different dairy herds, between the different age groups of calves and also between the bull and heifer calves. It is difficult to speculate why this differences might occur probably factors such as access to clean water, contact with other animals and wild birds might play a role in dissemination of the organism. Control programs could probably look at these factors and the possibility of rearing calves away from adult cattle.

A range of thermophillic *Campylobacter* species were identified from the cattle with *C. jejuni* and *C. hyointestinalis* predominating followed by *C. coli* and *C. lari.* A similar

observation was made in an Australian study which estimated the prevalence of *Campylobacter* in adult cattle (Grau, 1988). However, other studies have reported a lower isolation rate of *C. hyointestinalis* in Japan (Giacoboni *et al.,* 1993) and the U.K. (Doyle and Roman, 1982). The difference in the number and isolation rate between different countries might be due to variations such as herd type, season, animal age, feeding regimen, crowding and geography (Stern, 1981). The above features of sheep and cattle farming seen in New Zealand contrast with those overseas and may account for the higher prevalence found in this present study.

The occurrence of *C. hyointestinalis* and *C. lari* in the faeces of cattle in the present study recognises the importance of these species in the epidemiology of human infections here in New Zealand. These species which were originally isolated from pigs and birds are being increasingly isolated from other animals especially cattle (Lindblom *et al.,* 1990; Grau, 1988) and are now recognised as infrequent but important human enteric pathogens (Borczyk *et al.,* 1987; Edmonds *et al.,* 1987). It is difficult to say whether these constitute an emerging disease or just that they were present in the past but not isolated or identified due to unavailability of proper selective media or use of some antibiotics in the media which suppressed their growth.

The occurrence of an isolation rate of 44% *Campylobacter* from sheep faecal samples with all isolates being typed as *C. jejuni* was significant as no other studies on large numbers of sheep have been carried out and reported before in New Zealand. *C. jejuni* was the only species isolated from the sheep samples and this suggests that there is potentially an important role for sheep in the epidemiology of human infections. The likely routes of infection in addition to contamination of meat and surface waters could be through the direct contact of infected animals with people engaged in farming.

If there is a considerable build up of *C. jejuni* in the abattoir environment and equipment then this could have serious implications for the safety of the operating staff (Sarkar *et al.,* 1984), and make the contamination of meat a genuine possibility.

The occurrence of a range of thermophilic *Campylobacter* species in animals makes it important that *Campylobacter* isolated in the medical laboratories be typed to the

species level to ascertain the involvement of different species in causing human infections. The current trend is to presumptively identify campylobacter based on growth on selective media and confirmation of typical morphology. However, of the 105 isolates supplied from the medical laboratories for this study all were *C. jejuni* except for five isolates, which were *C. coli.* Therefore, even if the correct species of *Campylobacter* is determined this may not indicate the animal species of the source. There is thus a need for a subtyping scheme of sufficient discriminatory ability to allow epidemiological tracing of human and animal isolates.

A previous New Zealand study by Meanger and Marshall (1989), to study the seasonal influence on the isolation of thermophilic *Campylobacter* spp. from cattle demonstrated the highest prevalence in autumn followed by summer and winter. However data from this present study showed that the isolation rates were more or less the same over different seasons with only a slight increase in the summer months. This increase in summer seems to coincide with the pattern of human clinical episodes observed which increase in the summer months (Brieseman, 1990). This would suggest that cattle may be an important source of human infection. However, the vehicle of infection may not always be cattle meat but rather contaminated water, which may be encountered more frequently during the summer months due to more outdoor activities.

The next phase of the study involved collection from the medical laboratory at the Palmerston North public hospital of presumptively identified *Campylobacter* isolates from diarroheal cases and these were then typed to species level in our laboratory. The majority of the isolates were found to be *C. jejuni* (95%) and the rest *C. coli.* Our findings are consistent with those observed elsewhere which implicate *C. jejuni* as the principal campylobacter species isolated from clinical cases (Pearson and Healing, 1992). Therefore for clinical management of human infections, presumptive identification of isolates is probably sufficient but this is not so for epidemiological studies.

Thereafter *C. jejuni* isolates from cattle, sheep and human sources were typed at the molecular level by pulsed field gel electrophoresis (PFGE) employing the restriction endonuclease *Sma* I to determine the relationship between each other and from the

different isolation sources. The technique was found to be reproducible and allowed good discrimination between the isolates.

The PFGE profiles of the *C. jejuni* isolates revealed a large variation both between and within source species, with each source exhibiting between 13 to 16 different genotypic profiles. However, a majority of the isolates of each species were found to have a few profiles, which were more predominant suggesting the presence of a few dominant clones. There were profiles which were indistinguishable and pulsotypes (clonally related) to each other, between the human and cattle, and human and sheep groups as well as a few which showed a close relationship between cattle and sheep isolates.

The appearance of closely related isolates as observed by PFGE typing suggests that cattle and sheep offer alternative sources of *Campylobacter* infections in humans in New Zealand. This is more so because of the mainly pastoral lifestyle and high number of beef and sheep farms found in this country and the large number of people involved in farming activities. If the rates of isolation of *Campylobacter* in cattle and sheep in the Manawatu region are taken as a representative of the whole of New Zealand then these animal species could potentially be very important sources of human infections.

The potential for *Campylobacter* contamination of meat from the faeces of cattle and sheep during the slaughtering process from carriers among cattle and sheep is ever present. To investigate the presence of these microorganisms at the retail level closest to the consumer chain, samples of beef and lamb mince as well as poultry mince were examined for the presence of *Campylobacter* spp.

As the cultural isolation of *Campylobacter* is time consuming and difficult due to the relatively fastidious nature of the organism, a short enrichment and PCR based method of identification was developed to examine the meat samples for the presence of *C. jejuni.*

A survey of retail meat from six local supermarkets, revealed that both lamb and beef mince in addition to chicken mince were contaminated with *C. jejuni,* reflective of the occurrence of this organism in sheep and cattle faeces observed in our study. The

enrichment-aided PCR detection offered a sensitive, rapid and highly specific identification method for detection of *C. jejuni* from meat samples

The presence of a large collection of *C. jejuni* isolates from animal and human sources coupled with the burning issue of increasing occurrence of resistance in *Campylobacter* spp. to antibiotics, which has been attributed to the indiscriminate use of antimicrobial agents as growth promoters in animals, lead us to investigate this aspect with respect to our isolates. Six antibiotics, which are commonly used in the treatment of human infections, were chosen to test by a modified disc diffusion method.

The results indicated that resistance to antibiotics in the animal and human isolates was notably absent except for two human isolates, which showed resistance to tetracycline. This finding, though contrary to the findings in many countries where resistance to antibiotics is being increasingly detected in animal isolates (Jacobs-Reitsma, 1997), may be due to the extensive systems in which these animals are raised in New Zealand with minimal to no use of antibiotics. However, it is important to monitor this situation in the future to take advantage of this fortunate status by encouraging responsible use of antibiotics in animals and humans.

Significant practices designed to reduce the emergence of resistant bacteria in animals include emphasis on the preventive strategies such as quality assurance programmes, appropriate husbandry and hygiene, routine health monitoring and immunisation. Use of therapeutic antimicrobials only when it is known or suspected that an infectious agent is present which will be susceptible to therapy and taking care to use narrow spectrum antimicrobials and only for as long as necessary and as short as possible.

On the basis of the results obtained and the inferences made from them, the following summary of this thesis is presented.

1) There is a considerable presence of *Campylobacter* spp. in the faeces of cattle and sheep that has the potential to contaminate milk on the farm, carcasses at slaughter and surface waters and pass into the food supply.

2) PFGE typing of the *C. jejuni* from the human and animal sources showed that there are profiles which are indistinguishable between isolates from the same sources as well as between the cattle and human and sheep and human sources which suggest a close relationship between them. There is thus a good possibility that cattle and sheep derived *C. jejuni* contribute to human infections.

3) The presence of *C. jejuni* in the beef, lamb and chicken mince samples lends credibility to the hypothesis that faecal contamination of carcasses at slaughter by *Campylobacter* from the cattle and sheep is a strong possibility and this has serious public health implications.

4) The enrichment-based PCR method of identification of *C. jejuni* from meat is a viable, rapid, sensitive and highly specific method for detection of *C. jejuni* and could offer an alternative to cultural isolation.

5) The antimicrobial susceptibility studies of the *C. jejuni* isolates from the human and animal sources show that the resistance to the commonly used antibiotics for treatment of human infections is infrequent but vigilance is required to monitor and maintain this favoured status.

The study emphasised the need for affective monitoring of the prevalence of *Campylobacter* spp. in the animal populations in New Zealand for the containment of human infections. The methods used in this study can be effectively utilised to achieve these goals.

Molecular typing using PFGE looks a promising tool which can be utilised along with the newer computer software programs available now to type the *Campylobacter* organisms isolated from various sources and maintain a national database which can be utilised for developing epidemiological strategies to combat this disease in humans.

PCR aided identification of *C. jejuni* in meats offered a rapid alternative to culture and could be further evaluated with the other *Campylobacter* spp. to estimate the extent of their presence in food given their substantial presence in the animal populations.

Lastly, the need for a co-ordinated surveillance for the presence of antimicrobial resistance cannot be over emphasised. If the present favourable status is to be maintained constant efforts are required to monitor the situation in cattle and sheep and study the extent of the problem in poultry and pigs and take effective action including passing appropriate legislation's to limit the use of antimicrobials for growth promotion in animals.

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APPENDIX

A. MEDIA

Preston Enrichment Broth

Composition per litre:

Nutrient broth No.2 (Oxoid) 25 g

Distilled water 925 ml

Dissolve in distilled water, sterilise and cool to 50°C.

Add 50 ml of sterile defibrinated sheep blood.

Add 1 vial of reconstituted Preston campylobacyter supplement (Oxoid S 117E)

Add 1 vial of reconstituted campylobacter growth supplement (Oxoid SR 084E)

Aseptically dispense in 10 ml amounts into sterile bijoux bottles.

***Campylobacter* Blood-Free Selective Agar Base**

Campylobacter blood free selective agar base (Oxoid CM 739)

Typical formula	(g/L)
Nutrient broth No 2	25.0
Bacteriological charcoal	4.0
Casein hydrolysate	3.0
Sodium desoxycholate	1.0
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar	12.0

22.75 g is dissolved in 500ml of distilled water and autoclaved.

Cooled to 50°C and aseptically added 1 vial of CCDA selective supplement SR 155 (Oxoid)

Blood Agar No 2 (Oxoid)

	g/litre
Protease peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0

Suspend 40 g in 1 litre distilled water and autoclave.

Cool to 50°C and add 7% defibrinated sheep blood.

Muller-Hinton Agar

	g/lt
Beef dehydrated infusion form	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Suspend 38 g in 1 litre distilled water. Bring to boil to dissolve.

Sterilise by autoclaving.

Trypticase Soya Broth (Difco)

	g/l
Bacto Tryptone	17.0
Bacto Soytone	3.0
Bacto Dextrose	2.5
Sodium chloride	5.0
Dipotassium phosphate	2.5

Suspend 30 g in 1 litre distilled water and sterilise by autoclaving.

Muller-Hinton Broth

Composition	g/l
Beef, dehydrated infusion form	300.0
Casein hydrolysate	17.5
Starch	15.0

pH 7.4

Dissolve 21 g in 1 litre distilled water and sterilise by autoclaving.

15% Glycerol Broth

Nutrient broth No 2 (Oxoid) 25 g/lt

15 % v/v glycerol

Triple Sugar Iron Agar (Oxoid)

	g/l
Lab lemco powder	3.0
Yeast extract	3.0
Peptone	20.0
NaCl	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	q.s
Agar	12.0

pH 7.4

Suspend 65 g in 1 lt of distilled water. Bring to dissolve and mix well and distribute and autoclave. Allow the medium to set in sloped form with a butt 1 inch deep.

B. BUFFERS

PETT IV BUFFER

1 M NaCl
10 mM TRIS.Cl pH 8.0
10 mM di-Na^+ EDTA pH 8.0

UREA -ESP BUFFER

6 M Urea
50 mM Tris pH 8.0
50 mM di-Na^+ EDTA pH 8.0
Just prior to use add
Sodium lauroyl sarcosinase to 1%
Sodium desoxycholate to 0.2%
Proteinase-K to 0.5 mg/ ml

5 X TBE BUFFER (Tris-Borate-EDTA)

1 M TRIS base
1 M HBO_3
2 mM di-Na^+ EDTA pH8.0

10/1 T.E. BUFFER pH 8.0

10 mM TRIS.Cl pH 8.0
1 mM di-Na^+ EDTA pH 8.0