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**SPARROWS, FLIES, AND RODENTS AS RESERVOIRS OF
CAMPYLOBACTER SPP. ON A DAIRY FARM**

**A thesis presented in partial fulfilment of the requirements for the
degree of Master of Veterinary Science in Veterinary Public Health
and Meat Hygiene**

**At Massey University, Palmerston North,
New Zealand.**

BIJAY ADHIKARI

2003

ERRATA

- Page v, line 13: This study investigated (rather than investigates)
Page v, line 20: a sample size of 52 was taken
Page V, line 23: collected (rather than calculated)
Page xi, 1.1 & 3.2: *Campylobacter* in italics
Page 16, line 18: Table 2 should be Table 1.2
Page 17, Table 1.2: *C. jejuni* subsp *doylei* should be hippurate V
C. nitrofigillis should be catalase +, nitrate + and hippurate -
C. upsaliensis should be catalase W
Page 28, line 7 & 8: detection (rather than isolation)
Page 31, line 5: contaminated (rather than contamination)
Page 34, line 16: calves (rather than cattle)
Page 39, line 9: remove since from the sentence beginning "Since sheep and goats..."
Page 49, line 4: 40-fold (rather than 40 times)
Page 56, line 7: remove "(Fig 2.8)"
Page 61, line 5: A 30µg nalidixic acid (NA30) and a 30µg cephalothin (C30) antibiotic disc
Page 67, last line: remove "and Table 3.2"
Page 73, line 7: *Campylobacter jejuni* isolates were then classified into patterns A to V on the basis of a one or more band difference.
Page 80, line 1: shows (rather than showing)
Page 80, line 3: Letters (rather than Alphabetes...)
Page 81, line 1: *C. jejuni* in italics
Page 82, Figure 3.7: proportion (rather than percentage)
Page 85, line 1: there was a presence of *Campylobacter jejuni* in some milking cows at Massey No. 4 dairy farm over a 24 month period.
Page 88, line 13: restriction pattern X and XIV (Table 3.6 and Fig. 3.8)
Page 89 and 90 revision of conclusions as below:

Campylobacter jejuni has been isolated from most animal species worldwide. Despite its importance as a human and animal pathogen, relatively little is understood of the mechanisms of *C. jejuni*-associated disease in animals and humans.

This study suggested that dairy cows, rodents, sparrows and flies could be potential reservoirs of *Campylobacter* on a dairy farm. The PFGE analysis of *C. jejuni* isolates from the dairy farm showed a high degree of diversity of the organisms within a limited geographical area. Isolates with common restriction patterns (identical clones) infecting cattle, sparrows, flies and rodents suggested a common source of infection.

The high prevalence of asymptomatic carriage of *C. jejuni* found in cows could be sufficient to maintain infections within the dairy farm ecology via environmental contamination. The number of campylobacters shed by cattle defaecating 25 kg of fresh faeces per animal per day (Matsuzaki, 1975) would exceed that shed by sparrows or rodents, and as such cattle would be expected to constitute a more significant source of environmental contamination. To determine the most likely and significant routes of transmission, further studies of the epidemiology of *Campylobacter* in the farm ecology are needed.

This thesis is dedicated to my beloved parents
Shree Chiranjibi Adhikari
&
Tulasa D. Adhikari

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ABSTRACT

The reported numbers of human *Campylobacter jejuni* infections have increased considerably in many countries during the last few years. In New Zealand, the current annual incidence rate (302.5 cases/100 000) of human campylobacteriosis is higher than that of any other notifiable disease, and surpasses the incidence of campylobacteriosis reported by other developed countries. Although *Campylobacter jejuni* has been isolated from poultry at high prevalence rates worldwide, poultry are probably not the only important source of human campylobacteriosis as it is well documented that many other animal species (sheep, pigs, cattle and free-living birds and mammals) can be carriers of zoonotic campylobacters. The high incidence of the disease in people could be related to the consumption of poorly cooked meat, drinking contaminated water, overseas travel and animal contact.

This study investigates the potential role of free-living animals (sparrows, rodents and flies) as potential reservoirs of *Campylobacter* spp. and was carried out at Massey University No. 4 dairy farm. We isolated *Campylobacter* from the faeces of cattle and from other samples, and used pulsed-field gel electrophoresis (PFGE) typing of the organisms to determine the similarity between isolates. This study also includes a comparison of the prevalence and genetic diversity of *Campylobacter* isolated from sparrow populations on the farm and from an urban environment.

Based on the results of a previous study on the same farm, sample size of 52 were taken for the dairy cows in order to obtain results at the 90% confidence level within 10% accuracy. Faecal samples from 53 farm sparrows, 65 rodents and 56 flies were collected and examined for the presence of thermophilic *Campylobacter* spp. Faecal samples were also collected from 53 urban sparrows from "The Square" in the central urban area of Palmerston North city about 7 km from the dairy farm. A convenient number of samples of five of grass silage and two from each of water, worker's boots and aprons were collected with the aim to determine the presence of campylobacters in these samples.

All samples were collected between the 5th April 2002 and 25th May 2002. Random samples of rectal contents from 52 Friesian dairy cows were collected during milking time. Rodents were trapped in the feed storage premises approximately 15m from the milking shed using standard spring loaded, baited traps. Flies were captured around the milking shed using standard fly-traps. Bird samples were collected from an 8×10 feet tarpaulin placed on the ground under a tree where sparrows were roosting about 50m from the milking shed. Feed was provided to attract the birds. The same method was used to collect sparrow droppings in the urban area about 7 km from the farm.

Campylobacter jejuni was the only *Campylobacter* species isolated from the 290 samples collected at the dairy farm and from sparrows in the urban area. The highest isolation rate was found in dairy cows (54%), followed by urban sparrows (40%), farm sparrows (38%), rodents (11%) and flies (9%). Other samples from the farm environment such as grass silage, water, worker's apron and boots were also found to be positive for *C. jejuni*. Most of the rodents caught during the study period were mice. The high isolation rate in this study of *Campylobacter* from dairy cows (54%) and sparrows (40%) supports the notation that these species are important reservoirs of infection. Overall the results of the present and previous study show that at least some dairy cows from the same farm can be asymptomatic carriers (intermittent or persistent) of *Campylobacter jejuni* for at least 24 months.

Molecular characterisation of genomic DNA from 61 *C. jejuni* isolates from farm and urban sources obtained during the study was performed by PFGE after digestion with the enzyme *Sma* I. Of the 22 restriction patterns obtained seven were common to more than one source. The PFGE typing yielded seven, six, nine, six and three restriction patterns from dairy cows, farm sparrows, urban sparrows, rodents and flies respectively. PFGE analysis of the *C. jejuni* isolates shows a high degree of diversity of the organisms within a limited geographical area. But the finding of some common restriction patterns provides evidence of identical clones infecting cattle, sparrows, flies and rodents.

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LIST OF ABBREVIATIONS

BHI	Brain heart infusion
BRENDA	Bacterial restriction endonuclease DNA analysis
BS	Butzler selective
BU	Butzler
Campy-BAP	Campy brucella agar
CBFS	<i>Campylobacter</i> blood-free selective
CCD	Charcoal-cefazolin-sodium deoxycholate
CS	Charcoal-based selective
CVA	Campylobacter-cefoperazone-vancomycin-amphotericin
EDTA	Ethylenediamine tetra-acetic acid
GB	Guillain-Barré
Kb	Kilobase
Mb	Megabase
MBU	Modified Butzler
mCCD	modified charcoal-cefoperazone-deoxycholate
mCCDA	modified charcoal-cefoperazone-deoxycholate agar
MF	Miller-Fisher
MPN	Most probable number
MQ	Milli – Q
NARTC	Nalidixic-acid-resistant thermophilic <i>Campylobacter</i>
OD	Optical density
PR	Preston
REA	Restriction endonuclease analysis
rpm	Revolutions per minute
SK	Skirrow
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
WHO	World Health Organisation

CHAPTER ONE: LITERATURE REVIEW

1.1 General introduction

Campylobacteriosis is a major public health and economic burden in developed as well as developing countries. It is the most commonly reported cause of foodborne enteritis in people worldwide (Skirrow, 1990; Butzler and Oosterom 1991).

Campylobacter jejuni is the most common species causing campylobacteriosis in people. *C. jejuni* are microaerophilic, thermophilic rods, growing best at 42°C and low oxygen concentration. These characteristics are adaptations for growth in its normal habitat - the intestine of warm-blooded birds and mammals (Owen and Gibson, 1994). Food for human consumption can become contaminated from intestinal contents during processing of poultry and other food animals. *Campylobacter jejuni* grows poorly on properly refrigerated foods but survives refrigeration and will grow if contaminated foods are left out at room temperature. *Campylobacter* are sensitive to heat and other common disinfection procedures. Pasteurisation of milk, adequate cooking of meat including poultry, and chlorination of water will destroy the organism (Steele *et al.*, 1997).

Diarrhoea, which often is bloody, is the most consistent and prominent manifestation of campylobacteriosis in people. Other typical symptoms of *C. jejuni* infection include fever, nausea, vomiting, abdominal pain, headache, and muscle pain. The majority of cases are mild and self-limiting and do not require hospitalisation. However, Ang *et al.* (2001) reported that *C. jejuni* infection can be severe and life threatening. Death is more common when other conditions (e.g., cancer, liver disease, and immuno-deficiencies) are present. Children under the age of five and young adults aged 15-29 are the age groups most frequently affected (Ang *et al.*, 2001). The incubation period is typically two to five days, but may as long as 10 days after ingestion. The illness usually lasts no more than one week but severe cases may persist for up to three weeks.

Despite its importance as a human pathogen, relatively little is understood of the mechanisms of extra-intestinal *C. jejuni* associated diseases. The Guillain-Barré syndrome (GB), which affects the peripheral nerves of the body, occasionally develops following campylobacteriosis. Although rare, it is the most common cause of acute, generalized paralysis in the western world (Ang *et al.*, 2001). Beginning several weeks after the diarrhoeal illness in a small minority of *Campylobacter* victims. The GB-syndrome occurs when the immune system makes antibodies against lipopolysaccharide components of the *Campylobacter* cell. These antibodies attack components of the body's nerve cells that chemically similar to the bacterial components (Ang *et al.*, 2001). Although full recovery is common, some victims may be left with severe neurological disorders. It is estimated that approximately one in every 1000 reported campylobacteriosis cases leads to the development of the GB-syndrome. Up to 40% of the reported GB syndrome cases occurred in the USA, are associated with campylobacteriosis (Rees *et al.*, 1995).

Miller-Fisher Syndrome (MF) is another related neurological syndrome that can follow campylobacteriosis. In patients suffering from the MF syndrome, the nerves of the head are affected more than the nerves of the body (Rees *et al.*, 1995).

Reiter's syndrome may be associated with *Campylobacter* infection. This is a chronic, reactive arthritis that most commonly affects large, weight-bearing joints such as the knees, and the lower back. It is a complication that is strongly associated with a particular genetic make-up; persons having the human lymphocyte antigen B27 being most susceptible (Rees *et al.*, 1995).

The economic losses associated with the *Campylobacter* infections have attracted increasing attention in developed countries, in particular in the USA, Canada and Europe including the UK. In the USA, costs (both medical and as a consequence of lost productivity) related to the acute effects of food borne campylobacteriosis have been estimated at \$0.6–1.0 billion annually (Buzby, Allos and Roberts, 1997). Further analyses of *Campylobacter*-associated GB syndrome in the USA have indicated that

these cases may incur an additional \$0.2 – 1.8 billion annually (Buzby, Allos and Roberts, 1997). The same authors reported that because only a small proportion of patients with diarrhoea seek medical attention and faecal testing is performed for only a small number of cases, the true incidence and cost of campylobacteriosis is probably significantly underestimated.

The direct cost of campylobacteriosis to the New Zealand community is estimated to be \$4.48 million per annum (Withington and Chambers 1996), with true costs possibly in the order of \$40 million per annum when underreporting and loss of productivity is considered. This figure includes 58% of cost due to time off work with the remainder attributed to medical tests, treatments, and consultations for campylobacteriosis and the GB syndrome. The impact may be greater in the developing countries for which very little data are available to determine the actual cost. However, because of the increasing incidence, expanding spectrum of infections, potential of HIV-related deaths due to *Campylobacter*, and the availability of the complete genome sequence of *C. jejuni* NCTC 11168, interest in campylobacteriosis research and control in developing countries is growing (Coker *et al.*, 2002).

Campylobacteriosis is one of the most frequently reported diseases in infants in developing countries, due to consumption of contaminated food or water (Oberhelman and Taylor, 2000). The scenario for campylobacteriosis in developing countries is different when compared to developed countries. Many developed countries have well documented records for health related diseases. Frost (2001) reported that the disparity between developed and developing countries may be because *Campylobacter* in developing countries is not pathogenic in patients over 6 months of age. However, one community-based longitudinal study provided evidence that infection could be pathogenic beyond the first 6 months of life in developing countries (Rao *et al.*, 2001).

Most estimates of the incidence of human campylobacteriosis in developing countries are from laboratory-based surveillance of pathogens responsible for diarrhoea. *Campylobacter* isolation rates in developing countries range from 5 to 20%

(Oberhelman and Tayloy, 2000). Table 1.1 shows the *Campylobacter* isolation rates for some countries according to World Health Organisation (WHO).

Table 1.1. Isolation rates of *Campylobacter* spp from diarrhoea specimens from children under five years of age in selected developing countries (Coker *et al.*, 2002).

WHO region and country	Isolation rate (%)	Reference
Africa		
Algeria	17.7	Megraud <i>et al.</i> (1990)
Cameroon	7.7	Koulla-Shiro, Loe and Ekoe (1995)
Ethiopia	13.8	Gedlu and Aseffa (1996)
Nigeria	16.5	Coker and Adefeso (1994)
Tanzania	18.0	Lindblom <i>et al.</i> (1995)
Zimbabwe	9.3	Simango and Nyahanana (1997)
Americas		
Brazil	9.9	Mangia <i>et al.</i> (1993)
Guatemala	12.1	Ramiro <i>et al.</i> (1994)
Eastern Mediterranean		
Egypt	9.0	Rao <i>et al.</i> (2001)
Jordan	5.5	Nawas and Abo-Shehada, (1991)
Southeast Asia		
Bangladesh	17.4	Albert <i>et al.</i> (1999)

Some *Campylobacter* strains are highly infective. The infective dose of *C. jejuni* ranges from 500 to 10,000 cells, depending on the strain, damage to cells from environmental stresses, and the susceptibility of the host (Blaser *et al.*, 1986; Nachamkin, Blaser and Tompkins, 1992). Only the mesophilic *C. fetus* is normally invasive. Thermophilic

Campylobacter species with an optimum temperature for growth of 42°C such as *C. jejuni* are occasionally invasive. Such infections are manifested as meningitis, pneumonia, miscarriage, and a severe form of the Guillain-Barré Syndrome (Blaser *et al.*, 1986; Nachamkin, Blaser and Tompkins, 1992).

The epidemiology of *Campylobacter* is complex due to its ubiquitous nature. Poultry appear to be the main reservoir of *Campylobacter jejuni*. Up to 100% of chickens, turkeys, waterfowl and other wild birds have been shown to harbour this organism (Blaser and Reller, 1981).

Campylobacter jejuni was not recognized as a cause of human food-borne illness prior to 1975. Now, the bacterial organism is known to be the most common cause of food-borne illness in the U.S.A. The incidence of reported cases of *Campylobacter* gastroenteritis in the USA is approximately 5-6 cases per 100,000 populations (Lehner *et al.*, 2000). Although *Campylobacter* does not commonly cause death, according to data from the Economic Research Service (USDA), Buzby, Roberts and Allos (1997) reported that up to 500 human death occurs annually in the USA as a result of *Campylobacter* infection.

Frost, Kramer and Gillanders (1999) reported that the majority of human *Campylobacter* isolates in England and Wales were *C. jejuni* (90%), with most of the remainder being *C. coli*. Since 1981, *C. jejuni* is the most commonly identified human enteric pathogen in England and Wales (Pebody, Ryan and Wall, 1997; Anon_a, 2001; Anon_b, 2000). Approximately 44,000 and 58,000 laboratory cases of human *Campylobacter* infections were found during 1995 and 1998 respectively (Evans and Sayers, 2000). In New Zealand the figure is 302.5 cases per 100,000 populations, one of the highest rates in the world (Anon_b, 2002). In the Netherlands, about 10% of all patients with acute diarrhoea have *Campylobacter* infections (Severin, 1978). In Australia there are around 10,000 cases of *Campylobacter* infections each year (Stafford, Tenkate and McCall, 1996). The *Campylobacter* infections in Norway increased by 24% from 2331 cases in 2000 to 2890 cases in 2001 (Anon_c, 2002).

Campylobacter jejuni is commonly found in the gastrointestinal tract of healthy cattle, pigs, chickens, turkeys, ducks, and geese. Direct transmission of organisms from animals can lead to zoonotic infection of people. Pets that may carry *Campylobacter* include birds, cats, dogs, hamsters, and turtles. The organism has also occasionally been isolated from streams, lakes and ponds (Fang, Araujo and Guerrant, 1991).

Wild birds, rats, dogs and flies could be an important reservoirs in relation to disease outbreak and the high rate of carriage of *C. jejuni* in these species is well established (Rosef and Kapperud, 1983; Cabitra *et al.*, 1992; Pitkala *et al.* 1992; Gulosano *et al.*, 1993).

Although the epidemiology of human campylobacteriosis is not clearly understood, there is strong circumstantial evidence to implicate flies, rodents and wild birds in the direct or indirect transmission of *C. jejuni* to people (Rosef and Kapperud, 1983).

1.2 Historical review

2.1.1 Taxonomy

The taxonomy of campylobacters has changed since strains of the species *Campylobacter fetus* associated with ovine and bovine abortion were first recognised as 'vibrios' or spirilla by McFadyean and Stockman, (1913). Isolation of vibrio like organisms by Smith (1918) from aborted bovine fetuses supported the observations of McFadyean and Stockman (1913). Subsequently these organisms were characterised and named *Vibrio fetus* on the basis of comma-shaped cells predominating over spirilloid ones, particularly in young cultures (Smith and Taylor, 1919).

Since then, microaerophilic vibrios have been linked with a range of diseases of animals and human beings. Jones and Little (1931) isolated vibrio-like organisms from cases of winter dysentery in adult cattle and calves. To be confirmed as the cause of winter dysentery in cattle and calves, Jones and Little (1931) recovered the vibrios from the

jejunum of the dysenteric animals after experimentally challenging healthy cattle by feeding a pure culture of the organism. It was considered that the jejunum was the first site in the intestinal tract to be infected with these organisms and they named the organism *Vibrio jejuni*.

Doyle (1944) who isolated vibrios from pigs with swine dysentery and recovered the same organisms from the colon of pigs after experimental challenge, subsequently named the organism *Vibrio coli* (Doyle, 1948). Similar organisms were then detected by various researchers in blood cultures from humans with gastroenteritis (Levy, 1946; King, 1957; King, 1962).

Human cases of acute gastroenteritis infection due to microaerophilic vibrios were first reported by Levy (1946) in Illinois penal institutions in the USA. “Vibrio-like” organisms were observed in 31 of 73 stool specimens collected from inmates with acute gastroenteritis. A local dairy supplying skim milk to the institutions suspected that raw milk was the sources of the outbreaks, but proper isolation of organisms were unsuccessful because the suspected organisms were lost during subculture.

Hofstad (1956) was the first investigator to associate vibrios with hepatitis in poultry, which is characterised by low mortality and high morbidity with affected birds producing fewer eggs than healthy birds. In 1958, Peckham supported the findings of earlier researchers by reproducing hepatitis in healthy chicken after challenging them with a *vibrio* that was originally isolated from the livers of diseased birds. The disease was designated “avian vibrionic hepatitis”.

King (1957) compared the characteristics of two distinct groups of microaerophilic vibrios isolated from different sources, including blood cultures from infected people. The characteristics of the second group of vibrios were very similar to those of *V. fetus* except that their optimum temperature for growth was much higher than that of the isolates in the first groups.

Florent (1959) isolated strains of *Vibrio fetus* subsp *intestinalis*, primarily from the intestinal tract of sheep, cattle, swine, birds and humans. These strains had also been isolated intermittently from the genital tracts, the viscera and the blood. All strains were found to be the cause of sporadic abortion in cows and sheep (Akkermans, Terpstra and Van Waveren, 1956; Florent, 1959).

Vibrio fetus has a significantly different DNA base-pair ratio from that of the classical species of *vibrio* (Smibert, 1969). It is phenotypically different from other species of *Vibrio*, including the inability to ferment or oxidise carbohydrates and a requirement for microaerophilic conditions for growth. Most *Vibrio* can ferment carbohydrates and are facultative anaerobes (Smibert, 1969 and Smibert, 1978). The genus *Campylobacter* first proposed by Sebald and Veron (1963) included two species, *Campylobacter fetus* and *Campylobacter bubulis* (now *C. sputorum*) on the basis of characteristics exhibited. These taxa were formerly classified as *Vibrio* spp until 1963 and the authors concluded that *V. fetus* should be removed from the genus *Vibrio* and be re-classified as *Campylobacter*, with *C. fetus* being the type species of the genus.

Veron and Chatelain (1973) used biochemical, serological and DNA composition analysis and thereby established that the previously proposed genus is *Campylobacter*.

The development and increasingly widespread use of selective media for the isolation of *Campylobacter* from stool samples in the 1970s led to recognition of *Campylobacter* as a common cause of human gastroenteritis. By the end of 1980s, *Campylobacter* spp were reported to be one of the most common causes of diarrhoea worldwide (Allos, 2001).

Since the pioneer work by Smith and Taylor describing *V. fetus*, other researchers have reported and identified other species and subspecies of microaerophilic, curved bacteria included in the genus *Campylobacter* (Veron and Chatelain, 1973). Butzler *et al.* (1973) isolated *Campylobacter* from majority of human cases of enteritis with high prevalence. By this time *C. fetus* was already recognized as an important animal pathogen as a case

of abortion and infectious infertility, ensuring the increased attention of both human clinicians and veterinarians.

Woese (1987) described the potential role of the 16s rRNA gene for determining phylogenetic relationships among all living organisms. The 16s rRNA gene sequence identified several distinct clades within *Campylobacter* (Paster and Deuhirst, 1988). Of these *Campylobacter pylori* and *C. mustelae* were re-classified into a new genus *Helicobacter* to reconcile major differences from *Campylobacter* spp (Goodwin *et al.*, 1989).

Pasteur and Dewhirst (1988) found *C. cryaerophilia* and *C. nitrofigilis* as closely related organisms but distinct from other *Campylobacter* and *Helicobacter*. Much of the complex and problematic nomenclature of these organisms was finally resolved in an extensive, polyphasic, taxonomic study of the entire *Campylobacter* complex by exploring hybridisation positions between DNAs from more than 70 strains of *Campylobacter* spp. and related taxa and an immunotyping analysis of 130 antigens versus 34 antisera of campylobacters and related taxa (Vandamme and DeLay, 1991). They found that all of the named campylobacters and related taxa belonged to the same phylogenetic group named rRNA superfamily VI which is a group distinct from the gram-negative bacteria designated to the five other other rRNA superfamily. The authors proposed that the revised genus *Campylobacter* be limited to include *Campylobacter fetus*, *C. hyointestinalis*, *C. concisus*, *C. mucosalis*, *C. sputorum*, *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*. Subsequently, the authors transferred *Wolinella curva* and *W. recta* to the genus *Campylobacter* as *Campylobacter curvus* comb. nov. and *C. rectus* comb. nov., respectively. *Campylobacter cinaedi* and *C. fennelliae* were relocated as *Helicobacter cinaedi* comb. nov. and *H. fennelliae* comb. nov., respectively.

Currently, the genus *Campylobacter* contains 16 species and six subspecies (On, 2001). *Campylobacter jejuni* subsp *jejuni*, *C. jejuni* subsp *doylei*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus*, which are the most commonly isolated species from human and

animal patients with diarrhoea (Vandamme *et al.*, 1997) appear to be closely related with respect to morphological and genetical characteristics.

The taxonomic classification of *Campylobacter* that remains still unclear. Engberg *et al.* (2000) isolated *C. concisus* from oral cavity of humans and several reports suggested this strain was associated with diarrhoea showing a frequency equal to that of *C. jejuni*. Some strains of *C. concisus* from diarrhoea shows 46% related to the type strain in DNA-DNA hybridisation (Vandamme *et al.*, 1989). The presence of *C. concisus* in faeces of children with and without diarrhoea might be coincidental (Van Etterick *et al.*, 1996) or opportunistic (Engberg *et al.*, 2000). Strains of *Campylobacter lari* appeared to be host diverse. *Campylobacter lari* has been isolated from laridis of sea gulls and human cases of enteritis (Benjamin *et al.*, 1983; Tauxe *et al.*, 1985). The taxonomic statuses of these different variants have yet to be properly classified with biochemical, genetic and protein analysis.

2.1.2 Morphology

The word *Campylobacter* derived from the Greek word "campily" meaning curved and "bacter" meaning rod was proposed by Sebald and Veron (1963). Campylobacters are Gram-negative and non-spore forming bacilli or rods. Members of the family Campylobacteraceae are curved, s-shaped or spiral rod cells that are 0.2 to 0.5 μm wide and 0.5 to 8 μm long (Penner, 1988). Cells in old cultures or those subjected to environmental stresses display spherical or coccoid morphology but in young cultures cells may be comma, spiral, "S" or "gull-wing" shaped. Campylobacters are typically motile with a characteristic corkscrew-like motion, by means of a single, polar, unsheathed flagellum at one or both ends of the cell (Griffiths and Park, 1990). Cells of some species are non-motile (*C. gracilis*) or have multiple flagella e.g. *C. showae* (Nachamkin and Blaser, 2000).

1.3 Microbiology of *Campylobacter*

1.3.1 Isolation

Isolation of *Campylobacter* from faecal and environmental samples requires special selective media (i.e. *Campylobacter* selective media) and atmospheric conditions because of their microaerophilic nature and the many contaminating organisms often encountered. Various methods for isolating campylobacters have been established and include direct plating of specimens on suitable culture media as well as enrichment and pre-enrichment before sub-culture onto selective media. Enrichment broths used for the isolation and recovery of *Campylobacter* spp. include Campy-thio, Preston enrichment broth, *Campylobacter* enrichment broth and Bolton's broth.

The choice of isolation technique depends on availability of selective media, the nature of samples and facilities available in the laboratory. Primary isolation of organisms from specimens can be done on selective media. For a selective medium to be effective, it must efficiently propagate the target organisms and suppress the competing microorganisms. Examples include blood base media such as the Skirrow medium (SK) (Skirrow, 1977), *Campylobacter*-cefoperazone-vancomycin-amphotericin medium (CVA) (Reller, Mirret and Reimer, 1983), Preston medium (PR) (Bolton and Robertson, 1982); and Butzler selective medium (BS) (Goosens, De Boeck and Butzler, 1983); and blood-free media such as modified charcoal-cefoperazone-deoxycholate agar (mCCDA) (Hutchinson and Bolton, 1983) and charcoal-based selective medium (CS) (Karmali *et al.*, 1986). The development of such selective media provide better opportunity for isolation of campylobacters from faeces and other samples, and these techniques are widely used today (Griffiths and Park, 1990).

A number of studies comparing the efficiency of different isolation media for campylobacters have been carried out. Patton *et al.* (1981) found a higher isolation rate of campylobacters using modified Butzler medium (modification in BU media by increasing the concentration of colistin from 10 U/ml to 40 U/ml) as compared to BU and SK medium. Modified Butzler (MBU), and SK media were found the best media

combination for detecting campylobacters, which nearly detected 98% of the isolates (Patton *et al.* 1981; Rubsamen, 1986).

The ability to isolate campylobacters and the range of species isolated could be affected by the choice of growth medium and the isolation techniques. Madden, Moran and Scates (2000) found that direct plating of rectal swab samples on mCCDA (modified by the substitution of cefoperazone for cefazolin and blood for charcoal in the original CCD medium) was most efficient while for rectal contents prior enrichment in mCCD broth was best. In the same study it was reported that ileal content samples from pigs enriched in PR broth before plating on mCCDA enhanced isolation of three more *Campylobacter* species, which were not isolated from the same samples using mCCD broth.

Maximum number of campylobacters isolation from the faecal samples depends on the use of selective media. Bolton *et al.* (1983) carried out a comparative study on Skirrow's, Butzler's, Blaser's, Campy Brucella agar (Campy-BAP) and PR media for the isolation of *Campylobacter* spp from human, animal and environmental specimens. Butzler's medium provided the lowest isolation rate (68%) followed by Campy-BAP (69%), Blaser's (76%) and Preston medium (84%), which revealed the most selective and highest isolation rate. The authors also found that enrichment with PR broth produced a higher isolation rate than direct plating onto the PR medium. In contrast, Gonzalez and Abuxapqui (1989) found the BS medium better for *Campylobacter* isolation from chicken intestinal samples, compared to the SK medium.

Research by Ling (1994) showed that Skirrow's selective yolk medium with culture enrichment provided isolation of *C. jejuni* from various domestic birds. In contrast, Magdalenic (1992) preferred the use of PR medium to SK medium for the isolation of *C. jejuni* from raw milk.

Zanetti *et al.* (1996) observed no substantial difference between BU, CCD and PR medium, although CCD medium showed slightly better results than the BU and PR

media. The variable that had the most influence on the rate of isolation was the incubation temperature. A higher number of strains were obtained at 42 °C compared to 37 °C irrespective of medium used.

In contrast, Bolton *et al.* (1983) and Bracewell *et al.* (1985) found that the incorporation of an enrichment stage was superior to direct plating for human, animal and environmental specimens. Although enrichment procedures may enhance isolation rates, Turnbull and Rose (1982) found that of the eight samples of raw meat positive on direct plating and all were negative by enrichment procedures.

Samples of rectal content could be inoculated directly onto a small area of the surface of a selective agar. Ono, Masaki and Tokumaru (1995) compared charcoal-cefazolin-sodium deoxycholate agar (CCD) with BU agar for selectivity of campylobacters from faecal samples from cattle and pigs. They found 31.3% cattle infected with campylobacters positive by the direct plating technique on CCD agar as compared with 16.5% on BU agar. Similarly, positive rates of direct plating of pig rectal samples onto CCD agar and Butzler agar were 93.2 % and 83.5% respectively. The same authors suggested that the CCD medium which does not contain blood is easy to handle and superior to BU agar for isolation of *Campylobacter*.

Shih (2000) evaluated the efficacy of three selective media (Peterz's charcoal cefoperazone deoxycholate agar, Campy-Cefex agar, and CS medium) and found no differences among these selective media for isolation of campylobacters.

mCCD medium is recommended for the selective isolation of *C. jejuni*, *C. coli* and nalidixic-acid-resistant thermophilic *Campylobacter* (NARTC) from faecal specimens. This medium is considered more sensitive and selective for the isolation of *Campylobacter* because the addition of cefoperazone results in increased inhibition of contaminants and enhances the growth and aero-tolerance of *Campylobacter* spp. (Kwiatek, Wojton and Stern, 1990). The same authors recommended the mCCD

medium for the isolation of campylobacters in poultry carcasses in preference to Campy-BAP medium.

Koenraad *et al.* (1995) estimated *Campylobacter* populations by the most probable number (MPN) method using several combinations of enrichment and isolation media for isolating and enumerating *Campylobacter* in sewage. No significant difference in efficacy was revealed between the tested broths (PR broth, and CCD broth), and selective media such as Columbia agar base, *Campylobacter* blood-free selective medium-modified CCDA-Preston, *Campylobacter* blood-free selective medium-modified CCDA improved, and *Campylobacter* agar base Karmali.

Direct plating of samples is always better than MPN technique for detection and enumeration of *C. jejuni* in refrigerated chicken meat according to Beuchat (1985). In a recent comparative study of two MPN methods (using different selective enrichment broths and plating media) and the direct plating technique for enumeration of *Campylobacter* from freshly processed broiler chicken carcass, Line *et al.* (2001) found no significant difference between results obtained from the direct plating of carcass rinse samples on Campy-cefex agar and the MPN procedures. It is therefore suggested that the direct plating method offers a simpler, less expensive and more rapid alternative to MPN procedures for estimating *Campylobacter* populations associated with freshly processed broiler carcasses.

For the isolation of *Campylobacter* spp from cattle faeces contaminated with fungal species, modified-semisolid blood-free selective motility medium supplemented with amphotericin-B to inhibit the growth of fungi was found most effective (Ono *et al.*, 1996). Several enrichment media and direct isolation of *Campylobacter* have been studied. Monfort, Stills and Bech-Nielsen (1989) reported the direct plating is a reliable method for the isolation of *C. jejuni* and an enrichment step is not necessary to maximize its isolation from faecal specimens from dogs.

Peterz (1991) found no difference between the PR agar and *Campylobacter* blood-free selective (CBFS) medium in the recovery of *Campylobacter jejuni* from food. All samples were enriched in PR broth. However, the specificity of CBFS medium was better than that of PR agar. There was a slightly better growth of campylobacters, and competing organisms were more inhibited on CBFS as compared to PR agar.

Several enrichment media for better isolation of *Campylobacter jejuni* and *Campylobacter coli* from stool samples and foodstuff have been proposed during the last two decades. The semi-solid motility test medium (Chan and MacKenzie, 1982), enrichment medium (Bolton and Robertson, 1982) and *Campylobacter* enrichment broth medium have increased isolation rate by 6%, 21% and 46.3% respectively.

Gun-Munro *et al.* (1987) isolated four more strains of *C. jejuni* from human samples and 19 more strains of *C. jejuni* or *C. coli* from animal specimens on CS medium, which could not be isolated with SK medium. Suppression of normal faecal flora was also greater on charcoal-based selective medium. Byrne *et al.* (2001) found that recovery of *C. upsaliensis* from human faeces on cefoperazone amphotericin teicoplanin agar (CAT) was superior to that on mCCDA at lower concentrations of organisms (103 CFU/ml).

Campylobacter jejuni can survive 2-4 weeks under moist, reduced-oxygen conditions at 4 °C, often outlasting the shelf life of the product (except in raw milk products). They can also survive 2-5 months at -20 °C, but only a few days at room temperature (Castillo and Escartin, 1994). Environmental stresses, such as exposure to air, drying, low pH, heating, freezing, and prolonged storage, damage cells can hinder recovery of campylobacters to a greater degree than for most other bacteria. Older and stressed organisms gradually become coccoidal and increasingly difficult to culture (Nachamkin, Blaser and Tompkins, 1992). Oxygen quenching agents in media such as haemin and charcoal as well as a microaerophilic atmosphere, and pre-enrichment can significantly improve recovery of organisms (Tran and Yin, 1997). George *et al.* (1978) demonstrated that an atmospheric level of oxygen to be toxic to *C. jejuni*. However, the

holding atmosphere did not appear to be associated with a difference in isolation rates (Monfort, Stills and Bech-Nielsen (1989).

1.3.2 Identification:

Identification of species or subspecies of *Campylobacter* is performed by using phenotypic method, serological method, general genetic techniques and molecular genetic techniques. Presumptive identification of *Campylobacter* spp. is made by traditional morphological and biochemical methods, such as Gram stain, oxidase and catalase reactions, hydrogen sulfide production, hippurate hydrolysis, and susceptibility to cephalothin and nalidixic acid (Butzler, 1984; Morris and Patton, 1985).

Serological identification methods based on passive haemagglutination and colony identification based on latex slide agglutination have been described (Penner and Hennessy, 1980; Hodinka and Gilligan, 1988). In contrast, the Accuprobe *Campylobacter* culture identification test offers a rapid, non-subjective analysis of a bacterial colony for the presence of specific ribosomal RNA sequences that are unique to *Campylobacter jejuni*, *C. coli*, and *C. lari*, the three *Campylobacter* species most frequently isolated from humans (Tauxe *et al.*, 1988).

The identification of *Campylobacter* can be performed using biochemical tests (Table 2), such as oxidase, catalase, nitrate reduction, and hippurate hydrolysis (John *et al.*, 1994).

Table 1.2. Differential characteristics of the species of the genus *Campylobacter*

Organism	Susceptibility to			Other test			
	42 °C	Nalidixic	Cephalothin	Oxidase	Catalase	Nitrate	Hippurate
<i>Campylobacter cinaedi</i>	+	S	R	NR	NR	+	NR
<i>C. coli</i>	+	S	D	+	+	+	-
<i>C. concisus</i>	+	R	R	+	-	+	-
<i>C. cryaerophila</i>	D	D	D	NR	NR	+	NR
<i>C. fennelliae</i>	-	S	S	NR	NR	-	NR
<i>C. fetus</i> subsp <i>fetus</i>	D	R	S	+	-	+	-
<i>C. fetus</i> subsp <i>venerealis</i>	-	R	S	+	+	+	-
<i>C. hyointestinalis</i> subsp <i>hyointestinalis</i>	+	R	S	+	+	+	-
<i>C. jejuni</i> subsp <i>jejuni</i>	+	S	R	+	+	+	+
<i>C. jejuni</i> subsp <i>doylei</i>	W	S	S	+	V	-	NR
<i>C. lari</i>	+	R	R	+	+	+	-
<i>C. mucosalis</i>	+	D	S	+	-	+	-
<i>C. nitrofigillis</i>	-	S	S	NR	NR	NR	NR
<i>C. sputorum</i> biovar <i>sputorum</i>	+	D	S	+	-	+	-
<i>C. sputorum</i> biovar <i>bubulis</i>	+	D	S	+	+	+	-
<i>C. sputorum</i> biovar <i>fecalis</i>	+	R	S	+	-	+	-
<i>C. upsaliensis</i>	+	S	S	NR	NR	+	NR

+, Positive reaction or growth; -, negative reaction or inability to grow; D, contradictory report, V, 11-89% of strains are positive, W, weak reaction, S&R- susceptibility or resistance to 30 µg antibiotics., NR – Not reported. Source John *et al.* (1994).

1.3.2.1 Phenotypic methods

1.3.2.1.1 Biotyping

Phenotypic tests that assess the ability of a microorganism to produce or utilize biochemical substrates, replicate on media containing potentially inhibitory compounds or under potentially adverse environmental conditions are collectively known as biotyping. A number of biotyping schemes developed for the differentiation of campylobacters during the last two decades by Skirrow and Benjamin (1980), Lior (1984) and Patton and Wachsmuth (1992).

Biotyping has proved an effective method to identify type and sub-type of *Campylobacter* species and widely used scheme in epidemiological investigations (Lior, 1994). Biotyping and genotyping test could confirm the intestinal origin of *C. jejuni* present in the genital tract and on eggshells of poultry. Biotyping method proved that strains of *C. jejuni* isolated from human patients were similar to those isolated from the genital tract and eggshells of poultry, with implications for the epidemiology of human campylobacteriosis (Modugno *et al.*, 2000).

Hutchinson *et al.* (1987) suggested that the need to use at least two typing methods when studying epidemiologically related strains and further suggested that the most useful method of investigation could be a combination of a serotyping scheme and an extended biotyping scheme. This process could provide additional epidemiological evidence by further differentiating the serogroups by species and biotypes (Lior, 1984; Raji *et al.*, 2000).

The Preston biotyping scheme could be used in conjunction with the Penner system, because it was able to sub-type strains, and to identify isolates which could not be biotyped using the Penner scheme alone (Smith, Coker and Olukoyo, 1997).

Minimum criteria for the characterisation of species or subspecies of *Campylobacter* have been described (Ursing, Lior and Owen, 1994). These criteria include hippurate hydrolysis, catalase test, oxidase test, acid production, nitrate test, indoxyl acetate

hydrolysis, hydrogen sulfide gas production, and urea hydrolysis. Hippurate hydrolysis and catalase test are discussed in this section.

1.3.2.1.1.1 Hippurate hydrolysis test

The hippurate hydrolysis test developed by Ederer and Hwang (1975), is one of the phenotypic tests that discriminate *C. jejuni*, *C. coli* and other thermotolerant *Campylobacter*. The principle of the hippurate hydrolysis test was to produce glycine and sodium benzoate upon the hydrolysis of sodium hippurate by the enzyme hippuricase. Ninhydrin reagent is used to detect the glycine end product. The formation of a purple colour in a specified time frame is considered positive for glycine.

1.3.2.1.1.2 Catalase test

The catalase test involves addition of hydrogen peroxide to a culture sample or an agar slant. If the bacteria in question produce catalase, this will convert the hydrogen peroxide to water and oxygen gas. The evolution of gas causes bubbles within a few seconds is indicates as a positive test (Skirrow and Benjamin, 1980).

1.3.2.1.2 Phage typing

The phage typing scheme for *Campylobacter jejuni* and *C. coli* developed by Grajewski and co-workers in 1985 make use of a set of 14 lytic bacteriophages isolated from faecal samples of chickens (Wareing, Bolton and Hutchinson, 1996). Lior *et al.* (1982) introduced a phage-typing scheme, which included an additional 11 new bacteriophages. The Preston phage typing scheme proposed by Salama, Bolton and Hutchinson (1990) is now used routinely as an addition to serotyping for the epidemiological typing of *C. jejuni* and *C. coli* in England and Wales. This scheme utilised six phages of the Grajewski scheme with an addition of 10 new phages isolated in the United Kingdom. (Frost, 2001).

Phage typing provides essential data for epidemiological investigations by ascertaining if two or more strains are derived from a single parent organism and is usually carried out to determine whether two isolates from different sources represent the same strain or distinctly different ones. In some circumstances, phage typing is now being employed as an extension to serotyping (Newell *et al.*, 2000).

Phage typing of *Campylobacter jejuni* has been adopted by many researchers worldwide because the typing reagents was inexpensive and could be prepared locally, without the need for specialist procedures and equipment (Bolton and Owen, 1996). This procedure was a reasonably simple and reproducible means of typing *Campylobacter* isolates and is recommended for use with other typing methods such as serotyping or biotyping (Patton and Wachsmuth, 1992). Excellent results have been obtained when the three typing schemes serotyping, biotyping and phage typing are combined. But from a practical stand point, the combination of all these methods may be too time consuming and costly for most laboratories (Patton *et al.*, 1991).

1.3.2.1.3 Serotyping

Serotyping is used in most laboratories worldwide for the identification of strains of campylobacters and to provide a better understanding of both the source of infection and route of transmission (Frost *et al.*, 1998). For surveillance on a broad scale, serotyping could be the most practical approach of subspecies typing within the species *C. jejuni* and *C. coli* (Patton *et al.*, 1991; Owen and Gibson, 1994).

The Penner serotyping scheme developed in Canada by Penner and Hennessy (1980) recognizes soluble heat-stable antigens by passive haemagglutination (PHA). More recently, an adaptation of this scheme based on the detection of heat-stable antigens by absorbed antisera and utilizing whole cell agglutination has improved discrimination within both *C. jejuni* and *C. coli* (Frost *et al.*, 1998).

The Lior serotyping scheme based on slide agglutination of live bacteria with whole cell antisera absorbed with homologous heated, and heterologous, unheated cross-reactive antigens recognises about 130 serotypes of *Campylobacter* including *C. jejuni*, *C. coli* and *C. lari* (Lior *et al.*, 1982).

The main drawbacks of the serotyping scheme devised by Penner have been identified. Penner introduced PHA as the detection system in an attempt to eliminate non-specific agglutination reactions. However, reproducibility problems could occur as a result of variation in the source, age, concentration, and condition of the erythrocytes (Owen and Gibson, 1994). Also the Penner scheme used unabsorbed antisera, and a significant proportion of isolates agglutinated with more than one antiserum.

1.3.2.2 General genetic techniques

1.3.2.2.1 Plasmid analysis

Bacterial isolates might be discriminated from one another by the presence or absence of extra-chromosomal, self-replicating double-stranded DNA molecules known as plasmids. 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).

Plasmid typing is qualitative monitoring for the presence of plasmids of certain sizes, as determined by agarose gel electrophoresis, relative to a size marker (Ansary and Radu, 1992). Several methods of plasmid DNA extraction have been applied to the isolation of plasmids from *Campylobacter* spp (Patton *et al.*, 1991; Ansary and Radu, 1992). The Birnboim and Doly method is commonly used method for plasmid DNA analysis from *C. jejuni* and *C. coli*. (Birnboim and Doly, 1979). The presence of plasmid DNA was determined by loading an aliquot of the plasmid DNA suspension into an agarose gel. Gel concentrations vary from 0.66 to 0.85% (low percentage agarose gels resolve larger fragments of DNA more accurately than high percentage agarose gel). Samples are subjected to electrophoresis. Subsequently agarose gels were stained with ethidium

bromide and placed under a UV transilluminator to visualise the plasmid bands. Records of plasmid profiles are made by photography or digital image analysis.

1.3.2.2.2 Restriction endonuclease analysis or bacterial restriction endonuclease

DNA analysis

Isolation and analysis of bacterial restriction endonuclease groups produced from bacterial cells by using high frequency cutting enzymes referred to as restriction endonuclease analysis (REA) or bacterial restriction endonuclease DNA analysis (BRENDA). Using a BRENDA technique, Kakoyiannis (1984) identified up to 80 different types of *C. jejuni* and *C. coli* from 338 isolates from humans. In the same study, the authors in a PhD thesis reported that 61% of the *C. jejuni* isolates from humans had similar BRENDA patterns to isolates of *C. jejuni* from animal sources.

Karolik, Moorthy and Coloe (1995), based on an analysis of 120 isolates of *C. jejuni* and *C. coli* obtained from poultry faeces, as well as 49 isolates obtained from human stool samples, suggested that REA could be used to distinguish between these species. Kakoyiannis, Winter and Marshal (1988) first applied REA to a study of *C. coli* isolates from humans, poultry, pigs, birds and processed poultry. The authors found 18 REA profiles from 41 *C. coli* isolated from pigs. But none of these matched the 11 types from human isolates, although two human-human matches were detected. REA could be useful for distinguishing isolates of the same species, although it did not appear to separate isolates of poultry from human origin (Karolik, Moorthy and Coloe 1995).

The most benefit of this technique was that all isolates were typeable. But a major problem associated with REA is that there are too many bands to effectively resolve on normal agarose gels. Analysis by eye would be time consuming and errors may occur.

1.3.2.3 Molecular genetic techniques

1.3.2.3.1 Hybridization methods

1.3.2.3.1.1 DNA-DNA hybridisation

DNA-DNA hybridization technique was used to genetically confirm the identification of the strains of organisms. The data obtained from DNA relatedness were sufficient to identify bacteria to the species level, even in the absence of phenotypic data (Brenner *et al.*, 1982). The level of measured relatedness observed define the relationship between the test isolates and the reference isolates. Brenner *et al.* (1982) reported that a genus was defined as 40-65% relatedness between DNAs at the optimal condition, while a species has 70% or more relatedness at the optimal condition. There are several techniques for DNA-DNA hybridisation. Whatever method is used, it is absolutely critical for the success of the technique that the DNA is of high quality and purity (Brenner *et al.* 1982).

Kaneuchum and Imaizumi (1987) used DNA-DNA hybridisation analysis to demonstrate that nalidixic acid resistant, thermophilic *Campylobacter* isolated in the study was certainly *C. lari* and not an unusual *C. jejuni*. In another study, Totten *et al.* (1987) demonstrated that true hippurate hydrolysis positive *C. jejuni* isolates do exist and therefore isolates that are hippurate hydrolysis-negative should not necessarily be considered *C. coli* without other supporting tests.

1.3.2.3.1.1 Ribotyping

Ribotyping is the molecular characterization of organisms based on the recognition of restriction fragment length polymorphisms containing ribosomal RNA. The detection of rRNA DNA citrons, using a labelled probe within restriction endonuclease profiles of test DNA isolates is referred to as ribotyping (Gibson and Owen, 1998). Ribotyping requires a labeled probe that is complementary to a portion of the rRNA-DNA citron. This probe then binds to fragments of the bacterial genome containing complementary

regions and is detected by autoradiography or chemical means (Chang and Taylor, 1990).

Patton *et al.* (1991) reported that the most beneficial effect of ribotyping was to decrease the complexity of REA patterns from greater than 30 to generally less than ten (usually three to six) restriction fragments or bands produced in most experiments, thus making the interpretation of profiles simpler and complications due to plasmids are also reduced. However, Owen and Hernandez (1993) reported that ribotyping procedures were complex and time consuming to carry out.

Owen, Hernandez and Bolton (1990) determined the genomic DNA restriction endonuclease (*Hae III* and *Hind III*) total digests and 16S and 23S rRNA gene patterns for 18 isolates of *C. jejuni*. The authors suggested that *C. jejuni* isolates had both common and different bands, though this did not seem to correspond to biotypes.

In one study, ribotyping results showed that all strains of the two subspecies of *C. fetus* (subsp. *fetus*; subsp. *venerealis*) could be classified under one ribogroup implying very close relatedness (Denes *et al.*, 1997). The authors found that the *sapA* gene DNA marker, however, discriminated all the strains regardless of the subspecies when chromosomal DNA was restricted with *HindIII*, *HaeIII*, *XbaI* or *EcoRV*. These results illustrate that the *sapA* probe was potentially useful in fingerprinting *C. fetus* strains and in determining the relationships of strains for epidemiological purposes (Denes *et al.*, 1997).

1.3.2.1.2 Macro-restriction enzyme analysis

1.3.2.1.2.1 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) developed by Schwartz and Cantor (1984) is the most discriminatory DNA-based method for characterization of *Campylobacter jejuni* and sub-typing of other bacterial strains. PFGE is a molecular typing technique, which was an adaptation of the more traditional restriction enzyme analysis (REA) and

restriction fragment length polymorphism (RFLP) methods (Yan, Chang and Taylor, 1991). PFGE - typing profiles of bacterial strains from different sources can provide information about relationship of the organisms and mode of transmission (Arbeit, 1995).

PFGE was considered a robust method for characterizing genetic differences and monitoring changes which occur within *Campylobacter* species over time (Dickins *et al.*, 2001).

The development of PFGE (Schwartz and Cantor, 1984) allows the analysis of a smaller number of large molecular-weight chromosomal DNA fragments digested by so-called "rare cutting" restriction enzymes which results in simpler banding patterns more suitable for epidemiological analysis (Yan, Chang and Taylor, 1991). The DNA fragments are separated by a special electrophoretic method, PFGE, which involved the co-ordinated application of pulsed electric fields from different positions in a specially designed electrophoretic cell, in such a way that the large DNA restriction fragments were gently oriented through the agarose gel matrix (Arbeit, 1995).

Salama *et al.* (1992) isolated *C. hyointestinalis* from five members of the same family who had previously consumed raw milk. PFGE of the genomic DNAs from the five strains of *C. hyointestinalis*, after digestion with restriction endonuclease *SalI*, revealed that three strains had identical genome patterns and therefore appeared to be related, whereas the other two had completely different genome patterns and appeared to be unrelated. The study demonstrated the usefulness of pulsed-field gel electrophoresis for epidemiologic studies of this unusual campylobacter outbreak.

PFGE could also be used to detect genetic changes within bacterial strains including *Campylobacter*. Dickins *et al.* (2001) used PFGE to characterize 72 strains of *C. jejuni* isolated from retail poultry over a one-year period. Using the enzyme *SmaI*, PFGE detected genetic changes in bacterial strains after multiple serial passages in culture. Similarly, research by Odumeru (2001) found that *Campylobacter* isolates originating from the same abattoir on the same day demonstrated identical typing profiles by using

PFGE, suggesting that *Campylobacter* strains could be widely distributed within the abattoir environments.

Steinbrueckner, Ruberg and Kist (2001) investigated the rate of human intestinal infections with more than a single *Campylobacter* strain and the genetic variabilities of *Campylobacter* strains throughout an infection period by means of PFGE and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR). For 48 and 49 of 50 patients, all isolates from one sample showed identical patterns by PFGE and ERIC-PCR, respectively. Throughout an infection episode in 47 of 52 patients, the PFGE patterns of the isolates remained stable, while in one patient two different species were observed and in four patients different patterns were observed. The authors concluded that human infection with more than one *Campylobacter* strain is rare and should not significantly impair epidemiologic analyses.

Salama *et al.* (1992) found that well-separated fragments of bacterial chromosomal DNA ranged in size from 18 to 450 kilobases (Kb) and that the total genome size as determined from the sum of the *SalI* restriction fragments was approximately 1.5 megabases (mb). Such DNA fragments could be separated into a suitable number of sub-units by using PFGE with ethidium bromide and observing under UV light (Arbeit, 1995). Smith and Cantor (1987) reported the same size separation of DNAs by using PFGE ranging from 10 Kb to more than 1.5 Mb. The main disadvantage of PFGE is the lengthy preparation process for the DNA samples and its dependence on specialized and expensive electrophoretic equipment.

1.3.2.3.3 PCR

Polymerase chain reaction (PCR) devised by Mullis (1990) has become a major breakthrough in the field of molecular biotechnology. The essential feature of PCR is the ability to replicate a particular DNA sequence rapidly and exponentially. It is an enzymatic DNA technique, which allows multiple rounds of amplification. The procedure includes heating the newly synthesized DNA to separate the strands and cooling to allow the primers to anneal to their complementary sequence (Fig 1.1). PCR

requires two primer molecules to get the copying process started. The primers are short chains of the four different chemical components that make up any strand of genetic material. DNA itself is a chain of nucleotides. Under most conditions, DNA is double-stranded, consisting of two such nucleotide chains that wind around each other known as the double helix. Primers are single-stranded. They consist of a string of nucleotides in a specific order that will, under the right conditions, bind to a specific complementary sequence of nucleotides in another piece of single-stranded RNA or DNA and *vice versa*.

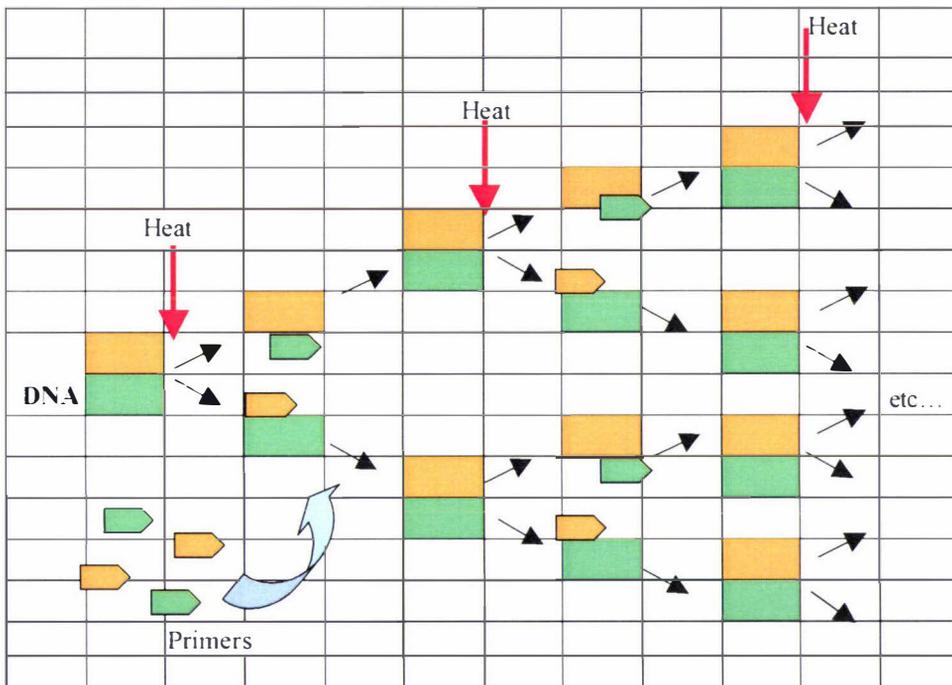


Figure 1.1. Illustration of the processes of polymerase chain reaction (PCR)

PCR is a very sensitive technique for the detection of organisms in samples from different sources and allows for the physical separation of any particular sequence of DNA, which is virtually without limit. Wegmuller, Luthy and Candrian (1993) identified *Campylobacter jejuni* and *C. coli* in milk samples and dairy products with an application of PCR, where no *Campylobacter* spp was isolated by conventional culture methods. In contrast, Giesendorf *et al.* (1992) found similar isolation rates using the PCR technique and conventional culture method. Although species-specific PCR

systems have been proposed, the main application of PCR was in the detection of pathogenicity genes (Grimont, 1999).

Giesendorf *et al.* (1992) developed a specific PCR and probe hybridisation assay based on the 16S rRNA gene sequence for the rapid detection and identification of *Campylobacter* spp in chicken products. Although, this method, which involved a combination of a short enrichment culture followed by the PCR assay could not improve the isolation of *Campylobacter* as compared with the conventional method, it did reduce isolation time to 48 hours as compared with 96 hours for culturing and biochemical identification.

The conventional phenotypic method is most commonly used technique for species and subspecies identification of *Campylobacter*. Hum *et al.* (1997) evaluated the PCR technique for identification and differentiation of *C. fetus* subspecies and compared this with the conventional phenotypic methods. Results of the PCR method suggested that *C. fetus* was often misidentified by the routine diagnostic procedures. The authors reported that the agreement between strain identification initially suggested by traditional phenotypic methods and the PCR assay was found to be 80.8%. The PCR technique proved to be a reliable method for the species and subspecies identification of *C. fetus*.

Campylobacter jejuni-specific PCR appears to be a unique molecular development based on the isolation of species-specific DNA (William, Ian and Lynn, 1997). An arbitrarily primed PCR incorporating 10-mer primers was used to generate fingerprints of *C. jejuni* M129 genomic DNA. A 486-bp fingerprint product hybridised specifically to *C. jejuni* DNA sequence and primers corresponding to three overlapping regions of the DNA probe were synthesized. In sensitivity studies using a crude M129 lysate as the template, the *C. jejuni*-specific PCR amplified the 265 bp products in a lysate with as few as 100 bacteria (William, Ian and Lynn, 1997).

Similarly Winters, O'Leary and Slavik, (1997) developed a more rapid nested PCR assay that specifically detects *C. jejuni* directly in chicken washes. This nested primer PCR method detected *C. jejuni* in approximately 80% samples of chicken washes and isolates were also confirmed by standard microbiological techniques. The nested set of primers produced a single band at 122 bp when *C. jejuni* was used as a template DNA. A single band on a 4% NuSieve agarose gel at 122 bp was apparent with *C. jejuni* cells at a sensitivity of 10^2 cfu ml⁻¹.

PCR methods have a potential to be very fast and sensitive. The methods could be designed to group-reactive or quite specific depending on what is required in a particular investigation and on which primers are chosen. PCR can also detect non-cultivable organisms and DNA of dead organisms.

1.3 Epidemiology of *Campylobacter*

Most species of *Campylobacter* cause infection of the gastrointestinal tract in human. They are the most commonly reported causes of bacterial diarrhoea in people worldwide and are widely distributed in various domestic and wild animal species. In developing countries, *Campylobacter* infections are associated with illness more often in children than adults (Anon_d, 2000). The primary source of infection is believed to be infected poultry and other animals. Most raw poultry meat has been found to be contaminated with *Campylobacter* (Evans and Sayers, 2000; Frost, 2001). Animals such as poultry, cattle, pigs, and dogs carry the bacteria in their intestines. Unpasteurized milk could also be a source of infection (Djuretic, Wall and Nichols, 1997). Person-to-person spread could also play a role in the spread of *Campylobacter* (Steinhauserova, Fojtikova, and Klimes 2000). Symptoms range from none to illness including diarrhoea (which is often bloody), nausea and vomiting, abdominal cramps, and fever. The illness usually lasts two to five days and rarely over ten days. Upon recovery, the bacterium continues to be shed in the stool for a few days to two months, if not treated with antibiotics. Species of *Campylobacter* have been isolated from cattle, deer, dogs, cats, flies, humans, milk, monkeys, poultry, rabbits, rodents, sheep and goats, pigs, wild birds

and other animal species (Annan-Prah *et al.*, 1988; Steele *et al.*, 1997; Allos *et al.*, 1998; Chuma, Hashimoto and Okamoto, 2000; Ono *et al.*, 2001). The epidemiology of *Campylobacter* in humans, domestic and wild animal species are described in the next section.

1.4.1 Human campylobacteriosis

The first human cases of infection due to *Campylobacter* were reported in two patients by Curtis (1913). In the year 1946, Levy reported the first association of microaerophilic vibrios with diarrhoeal disease in humans.

In developing countries, *Campylobacter* infections in children under the age of two years are especially frequent, sometimes resulting in death (Anon_d, 2000). In almost all developed countries, the incidence of human cases of *Campylobacter* infections has been steadily increasing for several years despite the employment of various control measures. The reason for this remains obscure.

The development and increasingly widespread use of selective media for isolation of *Campylobacter* from stool samples in the 1970s led to the recognition of *Campylobacter* as a cause of human gastroenteritis. By the end of the 1980s, it had been determined that *Campylobacter* spp were one of the most common causes of diarrhoea worldwide (Butzler *et al.*, 1973; Allos, 2001).

The major source of human infections is foods and environmental contamination (Coker *et al.*, 2002). Transmission of *Campylobacter jejuni* to humans may take the route of direct contact with animals or a sick person, but most frequently indirectly via contaminated food and water (Steinhauserova, Fojtikova and Klimes, 2000).

Domestic animals including pets could play a potential role as reservoirs and transmitters of campylobacters to people either directly or via food. Kramer *et al.* (2000) reported the prevalence of *Campylobacter jejuni* and *C. coli* in fresh bovine,

ovine and porcine liver, and chicken portions from retail outlets. The authors compared strain subtype distributions with those associated with cases of human campylobacteriosis occurring within the same period and study area. A total of 83% of the chicken samples, 73% of samples of lambs, 72% of pigs and 54% of beef were found contaminated with campylobacters. Among the human isolates, 89% were *C. jejuni* and 11% *C. coli*. These authors concluded that a significant proportion of the chicken and lamb isolates had identical subtypes to the human strains, which suggested their role as potential sources of infection (Kramer *et al.* 2000).

Infection can also result from drinking untreated water (Savill *et al.*, 2001) or from direct contact with infected animals such as puppies with diarrhoea (Bourke, Chan and Sherman, 1998). Wild and domestic animals shed campylobacters into lakes, rivers, streams and reservoirs. In Britain, defective water storage tanks were responsible for a campylobacteriosis outbreak affecting up to 250 people (Anon, 2002). It is therefore recommended that all water for human consumption must be properly treated.

Unpasteurised contaminated milk is another source of campylobacteriosis (Steele *et al.*, 1997). Hudson *et al.* (1991) reported that in some parts of the UK, people have become infected as a result of drinking pasteurised milk contaminated by magpies or jackdaws pecking through the foil tops of exposed milk bottles. Breast-feeding has been reported to play a protective role in *C. jejuni*-induced diarrhoea. It decreases the number of cases and the duration of diarrhoea (Ruiz-Palacios *et al.*, 1990). In Algeria, exclusively breast-fed infants had fewer symptomatic *Campylobacter* infections than infants who were both breast-fed and bottle-fed (Megraud *et al.*, 1990).

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 adults (12%) and children (45%) were affected. *Campylobacter jejuni* was isolated from the stools of 13 children and one asymptomatic adult. Positive persons excreted the organism for two weeks.

The infective dose of *C. jejuni* in milk to produce human campylobacteriosis is believed to be low. Robinson (1981) was able to produce classical signs of infections by ingesting 500 organisms of *C. jejuni* in 180 ml of pre-pasteurized milk. The apparent high degree of virulence of *C. jejuni*, as indicated by its apparently low infective dose, could also be valid for *C. coli* (Robinson, 1981).

In addition to food and water, other sources of *Campylobacter* are contaminated children prior to toilet training, especially in childcare centers (Goossens *et al.*, 1995). Epidemiological evidence indicates that the handling and consumption of poorly cooked chicken meat, drinking untreated water and contact with animals could be major risk factors to human (Skirrow and Blaser, 1992; Ikram *et al.*, 1994).

Campylobacter jejuni and *C. coli* cause approximately 1,375,000 –1,750,000 cases of foodborne illness and up to 500 deaths annually in the USA (Allos *et al.*, 1998). These figures are undoubtedly an underestimate because most cases of campylobacteriosis are relatively mild and are not recorded in a clinic or hospital. A trend of increase in the incidence of campylobacteriosis has been evident since the mid - 1990s. Of the total number of campylobacteriosis cases notified in 2001 in Norway, 50% of cases were acquired within the country and 50% were acquired abroad (Anon_f, 2002). In Australia, there were 13,282 and 12,643 cases of campylobacteriosis notified in 1998 and 1999 respectively (Roche *et al.*, 2001). The authors reported that the highest notification rates were found in South Australia with 161.1 per 100,000 populations. In 1998 the number of reported cases of campylobacteriosis in the UK exceeded 58,000 (Anon_g, 2002).

1.4.2 Cattle

Cattle are known to harbour campylobacters in the gastrointestinal tract (Nielsen, Engberg and Madsen, 1997). Although campylobacters do not cause substantial harm to the cattle, but loss of production, productivity or transmission to humans by direct and indirect contact has been recorded (Caldow and Taylor (1997). Whereas research reports suggest that the gastrointestinal tract of cattle is mostly contaminated with *C. jejuni* and

The prevalence of *Campylobacter* infection in clinically healthy breeding bulls was found to be 31% and in cows with reproductive problems to be 19% (Sayed *et al.*, 2001). The study suggests that clinically healthy breeding bulls could be asymptomatic carriers of *C. fetus* subsp. *veneralis*. In the same study it was reported that *Campylobacter fetus* subsp. *veneralis* was the predominant species of *Campylobacter* in these animals comprising, 80% bulls and 60% Friesian cows.

Campylobacter jejuni

In the UK, Waterman, Park and Bramley, (1984) isolated *C. jejuni* from faeces of 13% of healthy cows during the summer when they were on pasture and 51% during winter when the animals were housed. In Japan, Ono *et al.* (2001) isolated *Campylobacter jejuni* from caecal contents of 47 of 107 cattle. These cattle belonged to 20 farms in five regions. On the basis of the PCR-based randomly amplified polymorphic DNA method, isolates could be divided into 27 types. In another study, Ono, Masaki and Tokumaru (1995) examined 176 samples of caecal contents from slaughtered cattle and found 31% of the samples positive for campylobacters.

Diarrhoea may be a prominent clinical sign of campylobacteriosis in cattle but animals can also be asymptomatic intestinal carriers of *Campylobacter*. Cetin, Aytug and Kennerman (2000) isolated *Campylobacter* sp. from 21 of 100 diarrhoeic and 8 of 100 healthy calves. Of the total isolates, 79% were *C. jejuni* and 21% were *C. coli*. The authors found that the prevalence of *Campylobacter* sp. differed significantly between diarrhoeic and healthy calves.

Many studies have been carried out to understand *Campylobacter* infections in cattle. In Japan, Giacoboni *et al.* (1993) isolated *Campylobacter* in faecal samples of 97% of calves and 46% of adult cattle. *Campylobacter jejuni*, *C. hyointestinalis* and *C. fetus* were isolated from 62%, 26% and 26% respectively. However, these three species of *Campylobacter* were detected at a much lower rate ranges from 12% to 15% in adult cattle.

Nielsen, Engberg and Madsen (1997) reported that in a nationwide survey in Denmark, the prevalence of thermophilic campylobacters isolated from cattle sampled at the slaughterhouse was 47%. *Campylobacter jejuni* accounted for 83-91% of *Campylobacter* spp. in broilers and cattle; whereas 95% of the isolates from pigs were *C. coli*. In human patients with *Campylobacter* enteritis, 94% of the isolates were *C. jejuni* and 6% were *C. coli*. Heat-stable serotyping was also performed to investigate the serotype distribution in human patients, broilers, and cattle. Among human isolates, serotype O: 1,44, O: 2 and the O: 4-complex accounted for 62% of the *C. jejuni* isolates. The human serotypes were found common in samples from broilers and cattle. In pigs, *C. coli* O: 30 and O: 46 were the most common. The serotype distribution of human clinical isolates showed large overlap with those in cattle and chickens, and the authors suggested that they could be a major source of human campylobacteriosis

In New Zealand, dairy cows appear to be an asymptomatic reservoir of *C. jejuni*. Fakir (1986) found that the rate of carriage of *Campylobacter* spp by cows at Massey University No. 1 dairy farm was 24% in summer, 31% in autumn and 12% in winter. More recent research at Massey University, No.4 Dairy farm found a prevalence of *Campylobacter* spp. of 52% in dry cows, 15% in cows after calving, 81% in heifers, 37% in yearlings and 22% in calves (Wu, 2001). In another study, Ahmed (1999) isolated *Campylobacter* from 54% of dairy cattle on Massey University dairy farms and from 56% of environmental samples collected at beef and sheep abattoirs. All isolations were from apparently healthy cows.

1.4.3 Milk

The public health problems associated with the consumption of raw milk have been documented (Djuretic, Wall and Nichols, 1997). Pathogenic microorganisms could gain access to milk either from faecal contamination, particularly around the teats or by direct excretion from the udder into the milk (Little and Louvois, 1999). Houseflies could easily transmit campylobacter from one source to other host such as animal-to-

animal, animal to human or from environment to human and animals, particularly the teats of the bovine udder (Rosef and Kapperud, 1983)

Although *C. jejuni* cannot multiply in milk under either laboratory or natural conditions, Blaser *et al.* (1980) reported that milk could be a suitable biological media for the survival of *Campylobacters* and demonstrated that strains of *C. jejuni* could survive in milk for up to three weeks at 4⁰C, but for no longer than three days at 25⁰C. In another study, Tresierra-Ayala *et al.* (1999) found that all strains of *C. coli* died between 21 and 30 hours of storage in milk at 4⁰C under microaerophilic conditions. Pasteurization is designed to destroy or inactivate campylobacters in raw milk (Christopher, Smith and Vanderzant, 1982; Waterman and Park, 1982; Wright, 1983, Steele *et al.*, 1997)

Orr *et al.* (1995) isolated *C. jejuni* from unpasteurized pre-retail milk from a herd of Friesian-Holstein and Jersey cows. They examined milk by using pyrolysis mass spectrometry, Penner and Lior serotyping, biotyping, phagotyping and restriction fragment length polymorphism. The results provided evidence for direct milk excretion of *C. jejuni* by one asymptomatic cow and identified this cow as the source of milk contamination causing local cases of human enteritis. Zoghi *et al.* (1992) isolated *C. jejuni* from 0.5% of 1666 milk samples and Duzgun, Inal and Turk (2000) isolated *C. jejuni* in 1.7% of samples from tank collected milk from 2842 cows on 125 dairy farms around Izmir, Manisa and Aydin in Turkey. Research by Langoni *et al.* (1997) and El-Prince, Hussein and El-Said (1998) found 1.6% and 8% of milk samples contaminated with campylobacters respectively.

Milk is a perishable product that can be easily contaminated with *Campylobacter* spp. House flies are known to feed at the teat orifice of cows and often visit other teats of the same or other cows within a limited period of time (Rosef and Kapperud, 1983). In Hungary, Kalman *et al.* (2000) reported that campylobacters were isolated from 34 of 600 people suffering from diarrhoea following consumption of unpasteurised milk. Among them, 30 people were infected with *C. jejuni* and four with *C. coli*.

In New Zealand, only one of 111 samples of milk were found positive for *Campylobacter* (Hudson *et al.*, 1999), while research by Langoni *et al.* (1997) in Portugal and El-Prince, Hussein and El-Said (1998) in Egypt found 1.6% and 8% of milk samples respectively contained *Campylobacter* spp. In contrast, Adesiyun *et al.* (1996) in Trinidad, West Indies, Gulosano *et al.* (1993) in Catania, Italy and Decastelli, Fontana and Galipo (1991) in Novara, Italy were unable to isolate *Campylobacter* spp. from milk. Brieseman (1984) and Stone (1987) were also unable to isolate campylobacters from raw whole milk samples.

1.4.4 Water

In New Zealand, Savill *et al.* (2001) isolated *Campylobacter jejuni* from the samples of shallow ground water (75%), river water (60%), drinking water (37.5%) and roof water (29.2%). From these reservoirs *Campylobacter* are believed to spread in nature and to other hosts such as humans. These results suggest that water could act as a significant reservoir for human campylobacteriosis.

1.4.5 Deer

There are relatively few reports of isolation of campylobacters from deer populations. *Campylobacter hyointestinalis* subsp *hyointestinalis* is the species of *Campylobacter* most commonly isolated from the faecal samples of reindeer. In one study, Hanninen *et al.* (2002) isolated *Campylobacter hyointestinalis* subsp *hyointestinalis* from 6% (399) of faecal samples from reindeer in 16 herds in northern Finland. Hill, Thomas and Mackenzie, (1987) reported that *Campylobacter hyointestinalis* was found in the content of the ileum, caecum, colon and mesenteric lymph nodes from adult Moluccan rusa deer suffering from enteritis but the authors did not specify the number of isolates.

In contrast, Weber and Weidt (1986), Cerri *et al.* (1989), Donkersgoed *et al.* (1990), and Kanai *et al.* (1997) were unable to isolate campylobacters from fallow deer, mule deer and roe deer.

1.4.6 Sheep and Goats

Ovine campylobacteriosis causes severe economic losses due to abortions, stillbirths, and the birth of weak or unhealthy lambs. *Campylobacter jejuni* and *Campylobacter fetus* are the causative agents of the disease. The infection is highly contagious and may cause up to 70% of ewes to abort when the organisms are newly introduced into the flocks (Raji *et al.*, 2000). Many researchers have investigated the association of thermophilic campylobacters with colonization in the intestinal tract of sheep (Green, Wyatt and Morgan, 1992; Stanley *et al.*, 1998).

Stanley and Jones. (1998) reported that almost all sheep carry substantial populations of campylobacters in their intestines. In a study in the UK, Green, Wyatt and Morgan, (1992) reported that an outbreak of diarrhoea affected 70% of lambs in a housed flock of 375 early lambing crossbred ewes. The authors didn't mention whether the ewes were with their lambs. The affected lambs were five to seven weeks of age and *Campylobacter coli* was the only significant microorganism isolated from intestinal contents.

The Prevalence of *Campylobacter* in sheep could vary from one flock to another. Ahmed (1999) found that 44% of faecal samples from slaughter sheep were positive for *Campylobacter* spp, while research in the UK (Stanley *et al.*, 1998) showed carriage rates of 92% from the small intestine of lambs at slaughter with 88% of the isolates being *C. jejuni*. Carriage rates of *Campylobacter* in sheep from 0 – 94% have been published (Green, Wyatt and Morgan, 1990; Adesiyun *et al.*, 1992). Raji *et al.* (2000) reported isolation rates of campylobacters were 7% from sample of intestinal contents, 4% from gall bladders and 3% from vaginal swab samples. The authors could not isolate campylobacters from foetal samples.

As sheep are believed to shed high numbers of campylobacters in their faeces, these organisms could easily spread throughout the sheep population grazing contaminated pasture. About 30 – 40% of grazing sheep were found to be shedding campylobacters at pasture, which is about one third to half the intestinal carriage rate (92%) at slaughter (Jones, Howard and Wallace, 1999). However, it was reported that at pasture, the number of faecal samples positive for *Campylobacter* was much lower than the intestinal carriage rate (Stanley and Jones, 1998).

On bacteriological investigation, Abrahams *et al.* (1990) isolated *Campylobacter jejuni* from 33% apparently healthy domestic goats. Since sheep and goats commonly carry *Campylobacter* spp in the gastrointestinal tract and their carcasses regularly become contaminated at slaughter when gut contents are spilled during the process of evisceration (Abrahams *et al.*, 1990; Stanley and Jones, 1998).

1.4.7 Pigs

Pigs are known to commonly carry campylobacters in their intestinal tract and usually excrete high numbers of this microorganism in faeces. Weijtens *et al.* (1993) found that more than 85% of the sampled pigs were carrying campylobacter in their intestinal contents at all stages of fattening. But numbers of *Campylobacter* in the faeces tended to decrease as the pigs got older. There was no difference in the frequency and level of infection and with campylobacter between pigs on different farms. The feeding system (wet feed versus dry pellets) had no influence on the prevalence of campylobacter. Restriction fragment length polymorphism typing showed a high diversity of campylobacter strains at each sampling on the farm. The authors concluded that the piglets were already infected at a young age on the breeding farm

Ono, Masaki and Tokumaru (1995) examined 103 samples of caecal contents from slaughter pigs and found that 93% of the samples were positive for *Campylobacter* spp. Weijtens *et al.* (1999) studied *Campylobacter* excretion pattern of eight fattening pigs

and detected the microorganism in at least four of the six samples collected per fattening pig. Individual porkers could harbour up to eight types during the fattening period. The three types most frequently isolated from the fattening pigs were also present in the sows. All *Campylobacter* isolates were *C. coli*.

Caraivan, Potecea and Draghici (1992) isolated *C. coli* and *C. hyointestinalis* from the samples of a haemorrhagic colon and faeces of young pigs. These young pigs were from two pig farms on which many young pigs had watery diarrhoea. The authors found that the *C. hyointestinalis* strains isolated were sensitive to erythromycin, chloramphenicol, colistin sulphate and furazolidone. In another study, Aquino *et al.* (2002) isolated *Campylobacter coli* from a pig population by using a multiplex PCR-based assay. This method was used for the simultaneous identification of *C. jejuni* and *C. coli* from children with diarrhoeal disease and from domestic animals including sheep, dogs and cats in Brazil. Results showed that of the 89 *Campylobacter* isolates 41(54%) were *C. jejuni* and 35 (46%) were *C. coli*. All nine isolates from pigs were *C. coli*.

In a nationwide survey in Denmark, Nielsen, Engberg and Madsen (1997) isolated thermophilic campylobacters from 46% of pigs sampled at the slaughterhouse. A total of 95% of the isolates were *C. coli*.

1.4.8 Poultry

Campylobacter are normal components of the intestinal flora of poultry and other birds with up to 100% of chickens, turkeys, waterfowl and other wild birds found to be asymptomatic carriers of the organism (Blaser and Reller, 1981). From these reservoirs *Campylobacter* are believed to spread in nature and to other hosts including humans. Some case-control studies have reported that up to 70% of sporadic cases of human campylobacteriosis are associated with eating chicken (Anon, 2000) In Surrey, UK 80% of broiler flocks are found infected with *Campylobacter* by 42 days of age (Gibbens *et al.*, 2001). Approximately 80% of raw chickens sold in the UK (Corry and Atabay, 2001) and up to 88% of broiler chicken carcasses in the USA (Anon, 2000) are

contaminated with thermophilic campylobacters, which can be found on carcasses at levels as high as several thousands per square cm of skin. Some researchers have found that in England and Wales, most birds presented for slaughter are infected with *Campylobacter jejuni* (Evans and Sayers, 2000; Frost, 2001). There is strong circumstantial evidence that human campylobacteriosis is attributed mostly by chicken meat produced in an unhygienic manner or by cross-contamination in the home kitchen.

Live animals and the environment serve as sources of pathogenic microorganisms, which contaminate carcasses during slaughtering process and meat products during processing, storage and handling. Bacterial counts on carcasses usually increase during slaughter and the following processing steps (Stern *et al.*, 1995).

Most investigations have shown that *C. jejuni* is the most common species of thermophilic *Campylobacter* isolated from poultry (Doyle 1990; Altekruze *et al.*, 1994; Bang *et al.*, 2001; Nadeau, Messier and Qessy, 2002). A recent study in the USA identified *Campylobacter* spp. in 63% of more than 1000 chickens obtained in grocery stores (Anon, 2000). Other common species of *Campylobacter* isolated from poultry are *C. coli* and *C. lari*.

Poultry, particularly broiler chickens, may carry large numbers of these bacteria without showing any signs of illness and they constitute a major source of human infection in food (Humphrey, 1989). Poultry meat appears to be the most important source of human infection by *Campylobacter*. Prevalence of such infections can be reduced by incorporation of formic or propionic acids, or a mixture of the two ingredients into poultry feed, and improving cleaning and disinfection of broiler houses. Also chlorination of their drinking water would be of benefit (Annan-Prah and Janc, 1988; Humphrey, 1989).

1.4.9 Dogs and Cats

Many infectious diseases in humans can be acquired through contact with pets including dogs and cats. *Campylobacter upsaliensis* is the most commonly isolated species of *Campylobacter* in dogs and cats. There are considerable variations in the reported rates of isolation of thermophilic campylobacter from these animals. The thermotolerant *Campylobacter upsaliensis* was first isolated in 1983 from faecal samples of healthy and diarrhoeic dogs in Sweden (Sandstedt, Ursing and Walder, 1983) and in 1989, *C. upsaliensis* was also isolated from cats (Fox *et al.*, 1989).

In Germany, the prevalence of *Campylobacter upsaliensis* ranged from 28% in young dogs (<1 year) to 55% in adult dogs (Moser *et al.*, 2001). In the same study, it was reported that 20% of cats harboured *C. upsaliensis* and 22% of cats carried *C. helveticus*. The authors demonstrated genomic heterogeneity among *C. upsaliensis* strains by establishing genomic diversity of the isolates assessed by macro-restriction analysis with endonuclease *Sma* I and *Xho* I, using PFGE and PCR. In France, Pellerin *et al.* (1984) isolated *C. jejuni* from the faeces of 2% of pet dogs free from diarrhoea and from 1% of cats. One of the dogs but none of the cat yielded *C. coli*.

Household pets such as dogs and cats could be potential sources of *Campylobacter* infections in humans. Particularly, children who come in close contact with such pets are at risk, since campylobacteriosis may result from a very low infective dose. (Goossens *et al.*, 1990; Bourke, Chan and Sherman, 1998).

Dogs, particularly young ones, also commonly carry *C. jejuni* and *C. coli*, but high prevalence is probably limited to strays and kennelled animals (Baker, Barton and Lanser, 1999). Prevalence of campylobacters in cats is probably lower, but the pattern is similar. In Britain, about 5% of human cases of *Campylobacter* enteritis is associated with dogs and cats, mostly from the dogs (Skirrow, 1981). The author suggested that the risk of acquiring infection from normal animals is low, and can be almost eliminated by simple hygienic measures.

Bruce and Ferguson (1980) isolated *Campylobacter jejuni/coli* from 45% of apparently healthy cats, 49% of stray dogs, 39% of clinically healthy puppies and 38% of diarrhoeic puppies. The relatively high rate of isolation of *Campylobacter* was possibly due to these animals scavenging of contaminated food from a poultry processing plant. In the same study, the authors also reported the isolation of some indistinguishable strains of campylobacters from a seven-week-old puppy with diarrhoea and a child with acute enteritis in the same household.

McOrist and Browning (1982) isolated *C. jejuni* from the faeces of 16% of 62 dogs and 12% of 56 cats with diarrhoea, and 9% of 74 healthy dogs and 3% of 61 healthy cats. The same authors found that the antibiotic sensitivity pattern of strains isolated from cats and dogs was similar to that reported for human isolates.

In a study of the incidence of *Campylobacter upsaliensis* among the 225 dogs and cats suffering from acute or chronic diarrhoea, Steinhauserova, Fojtikova and Klimes, (2000) isolated *Campylobacter* spp from 23% of dogs and cats. *Campylobacter upsaliensis* comprised 7% of all strains isolated. In the same study, 15% of rectal swabs taken from a control group of 126 dogs and cats without diarrhoea yielded *Campylobacter* spp. Among these, 12% were confirmed as *C. jejuni/ C. coli* and 3% as *C. upsaliensis*. All the *C. upsaliensis* strains were isolated from dogs.

Although *C. upsaliensis* is frequently associated from dogs and cats, it is uncommon in humans (Sandsedt, Ursing and Walder, 1983). In one study, of 262 dogs and 46 cats of different ages originating from two geographically distinct regions in Germany, Mosser *et al.* (2001) isolated 109 canine *Campylobacter* isolates, of which were 80% *C. upsaliensis*. The prevalence of *C. upsaliensis* in all dogs included in the investigation varied from 28% in juvenile to 55% in adult dogs. Of the 46 cats, 48% were *Campylobacter* positive, and 41% were *C. upsaliensis*.

1.4.9 Rabbits

There are few reports of *C. jejuni* isolation from rabbits. In bacteriological examination of faecal samples of 212 rabbits, Weber, Lembke and Schafer, (1982) found 0.5% rabbits positive for *C. jejuni*. While Fakir (1986) isolated *Campylobacter jejuni* from 1% of rabbits.

Reynaud, Dromigny and Courdavault, (1993) isolated *Campylobacter*-like organisms from the caecal contents of seven rabbits but were unable to isolate these organisms from ileal contents of the same rabbits. In the same study the authors observed that isolated organisms required at least an eight-day incubation period in microaerophilic conditions at 37 °C. The strains were oxidase and catalase positive and grew at 45 °C, but not at 25 °C. The authors also conducted other biochemical test but biochemical characteristics did not permit the bacteria to be assigned to any known species.

1.4.10 Monkeys

The prevalence of enteric pathogens in monkeys was studied in Peru by Tresierra-Ayala *et al.* (1997) who found *Campylobacter* spp in 32% of rectal swab samples. All monkeys of eight different species were apparently healthy and kept as pet animals. The biovars comprised two isolates of *C. coli* biovar I, eight isolates of *C. coli* biovar II, four isolates of *C. jejuni jejuni* biovar I, and one isolate of *C. jejuni jejuni* biovar II. The authors concluded that monkeys might be an important reservoir of *Campylobacter* infection for human. In contrast, Damme and Lauwers (1983) were unable to isolate *C. jejuni* from monkeys in Zaire.

Aquino *et al.* (2002) isolated *C. coli* from 27% of rhesus monkeys and found 57% of the isolates resistant to sulfonamide, 25% to norfloxacin, 18% to erythromycin, ciprofloxacin and ampicillin, and 14% to tetracycline. All isolates were susceptible to gentamicin, chloramphenicol and cefotaxime.

Bronsdon and Schoenknecht (1988) isolated *Helicobacter pylori* from the gastric mucosa of 6 of 24 pigtailed macaques examined by gastric biopsy and culture. All isolates were morphologically and biochemically similar to the human type strain NCTC 11638, differing only in colony diameter, pigmentation, and rate of growth. The authors confirmed the identity of the isolates by whole-genomic DNA-DNA hybridization with the type strain. Colonization of the monkey stomachs was associated with hypochlorhydria and histopathology resembling type B gastritis, which can lead to peptic ulcers in humans. Infected animals showed no clinical signs of colonization, although endoscopies detected inflammation, erythema, and the presence of friable tissue in some animals.

1.4.12 Wild birds

Thermophilic campylobacters are widespread in the environment. Although intestinal carriage of campylobacters is ubiquitous in domestic and wild animals including wild birds and poultry, contamination of the environment with the bacteria in faeces is intermittent and varies seasonally, depending on factors such as stress and changes in diet (Jones, 2001). Thermophilic campylobacters are frequently isolated from a wide variety of domestic and wild birds which when infected usually excrete large numbers of organisms in their faeces.

Cabrita *et al.* (1992) isolated *Campylobacter jejuni* and *C. coli* from faecal samples of 45% sparrows. Chuma, Hashimoto and Okamoto, (2000) isolated campylobacters from sparrow faeces by the multiplex PCR method, which is able to detect type strains of *Campylobacter jejuni*, *C. coli*, and *C. lari*. Antimicrobial sensitivities of the isolated strains were determined in order to examine the role of sparrows in contamination of broilers with *C. jejuni*. The authors reported that three strains of quinolone-resistant *C. jejuni* in sparrows, must have originated from farm animals, like chickens, pigs or cattle that are frequently treated with quinolones or have these antibiotics incorporated in their feed.

Fallacara *et al.* (2001) surveyed free-living waterfowl residing in metropolitan parks in central Ohio, USA for the faecal shedding and antimicrobial susceptibility patterns of *Campylobacter jejuni*. A prevalence of 50% *C. jejuni* was observed for all waterfowl species. Antimicrobial susceptibility testing by the disk diffusion method revealed multi-drug resistance for penicillin G, lincomycin, vancomycin, erythromycin and bacitracin.

Stanley and Jones (1998) reported that on examination of a total of 2157 strains of *C. jejuni* found high rates of metronidazole resistant strains of *Campylobacter jejuni* from starlings (82%) and gulls (100%). Also strains from domestic animals were metronidazole resistant (chickens 90%, Turkeys 92%, dairy cows 19%, sheep 9% and lambs 5%).

Sommariva *et al.* (2000) isolated *Campylobacter* spp. from six faecal specimens of mallard ducks in Northern Italy. Specimens were collected during the hunting season. The authors suggested that the hunters should prevent their dogs from eating viscera of game birds in order to avoid infection. In another study, *Campylobacter thermophilus* was found in cloacal and skin swabs of 18% of city pigeon and 9% of meat pigeons (Passamonti *et al.* (2000).

Craven *et al.* (2000) isolated *C. jejuni* from wild birds with carrier rates ranging from 4% to 50%. Most of the samples collected consisted of wild bird droppings found on or near different domestic chicken housing during different seasons of the year. Isolation of the bacterial enteropathogens in wild birds near the broiler houses suggests that wild birds that gain entry to poultry houses have the potential to transmit bacterial pathogens to the birds.

1.4.13 Rodents

Available data indicate that rodents particularly mice and rats could be potential sources of *Campylobacter* transmission into pig and broiler houses (Annan-Prah *et al.*, 1988). In

Northeast Portugal, Cabrita *et al.* (1992) investigated the prevalence of *C. jejuni* and *C. coli* in black rats and found that 57% harboured campylobacters. Antimicrobial susceptibility testing showed that 5.5% of the strains were resistant to ampicillin, 5% to tetracycline, 13% to erythromycin and 23% to streptomycin.

In Japan, Kato *et al.* (1999) isolated four strains of *Campylobacter* from 5% of rats collected at a fish market and three of these strains were identified as *C. jejuni* and one *C. coli*. In the same study the authors were unable to isolate campylobacters from the 545 rats caught in restaurants. In another study, Sakai *et al.* (1989) measured antibodies to *Campylobacter jejuni* in rats using the complement fixation test. 15.8% of 342 wild rats were found positive for antibodies against *Campylobacter jejuni*.

Le-Moine, Vannier and Jestin, (1987) isolated *Campylobacter jejuni* from 16 rats (40%) and from four batches of mice in 15 pig breeding and fattening units. In contrast, Weber, Lembke and Schafer, (1982) could not isolate *C. jejuni* in faecal samples from 139 rats and 558 mice from a laboratory animal unit. The authors concluded that *C. jejuni* infections are rarely found in laboratory rats and mice.

Rubasamen (1986) isolated *C. jejuni* from 39% of apparently healthy mice using Butzler selective media (BU). In Finland, bacteriological examination revealed 34% of wild birds positive for *Campylobacter* (Pitkala *et al.*, 1992).

In New Zealand, Fakir (1986) isolated thermophilic *Campylobacter* from 22% of rats, 8% of guinea pigs, 52% of cats and 1% of rabbits, and *Campylobacter*-like organisms from 10% of mice. This study was conducted in the small animal production unit at Massey University where the animals were kept as laboratory animals. The presence of *C. jejuni* in free-living rodents has apparently not been studied in New Zealand.

1.4.14 Flies

Adult houseflies can contaminate almost anything that people use including water and food and are considered to be an important agent in the dissemination of several infectious diseases.

Few studies have been carried out to isolate *C. jejuni* from flies, either experimentally or under natural conditions (Shane, Montrose and Harrington, 1984; Rosef and Kapperud, 1983). Wright (1983) isolated *C. jejuni* from 3% of adult flies collected from three places in South-Eastern England and suggest that the potential hazard to health from the transmission of campylobacters from animals to human food is small.

Adult houseflies have been shown to be important agents in the transmission of numerous infectious agents (West, 1951).

Shane, Montrose and Harrington (1984) reported that from the sample of 32 houseflies exposed to a liquid suspension of *C. jejuni*, microorganisms were recovered from feet and ventral surface of the body of 20% of the flies, and from 70% of the viscera). Rosef and Kapperud (1983) isolated *C. jejuni* from 50.7% of flies from chicken farms and 43.2% from a pig farm. No studies of flies as carriers of campylobacters appear to have been done in New Zealand.

1.5 Aims and Objectives

In New Zealand, campylobacters cause the largest number of reported cases of food-borne illness. The number of reported cases of human campylobacteriosis has increased 40 times in the last 20 years from 270 cases in 1980 and 10054 in 2001. Campylobacteriosis is now the most common notifiable disease in New Zealand and the incidence of the highest recorded in the developed world. It is likely that farm animals as asymptomatic carriers play an important role in the epidemiology of human campylobacteriosis. An earlier study at Massey University No. 4 dairy unit found that more than 50% of dairy cows were harbouring *Campylobacter* spp, but the presence of *Campylobacter* in free-living animals (such as birds, rodents and flies) has apparently not been studied in New Zealand.

The overall objective of this study is to determine the potential role of free-living animals (sparrows, rodents and flies) as reservoirs of *Campylobacter* spp. on a dairy farm.

This study aims to:

1. determine the prevalence of *Campylobacter* spp in dairy cows.
2. determine the prevalence of *Campylobacter* spp in farm sparrows, rodents and flies.
3. determine the degree of similarity between campylobacters isolated from sparrows, rodents, flies and cattle on a dairy farm.
4. use pulsed-field gel electrophoresis in order to evaluate the hypothesis that sparrows, rodent and flies are potential reservoirs of *Campylobacter* spp on a dairy farm
5. determine the prevalence of *Campylobacter* spp. in urban sparrows and compare genetic diversity with farm sparrow isolates.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Project sites

2.1.1 No.4 Dairy Farm, Massey University

The study was carried out at No.4 Dairy Unit at Massey University (Fig. 2.1) comprising three former privately owned properties, developed by the University into one large seasonal supply dairy farm. The farm is located about 2 km west of The Massey University main complex and about 7 km south of “The Square”, in central Palmerston North.

The farm is a commercial scale (about 500 cows on 172 ha) research dairy unit using normal industry farm management practices. The farm produces 150,000 kg milk solids from about 500 Friesian cows annually. Key objectives of the farm are to study the problems inherent to large-scale dairying and operate as a profitable seasonal supply dairy farm. The farm is used to explore the opportunities and problems related to increased technical and economic efficiency, and provides a teaching resource for undergraduate and postgraduate programmes offered by the University.

Pasture production, cow condition scores, live weight and milk production are regularly monitored and data is integrated into feed budgets to assist management and provide information for teaching and extension. A large scale grazing trial is currently underway comparing late control of spring pasture with the more conventional early control system practiced by most New Zealand dairy farmers. Other recent research trials include studies of selenium and magnesium concentrations in pasture and its relationship to cow health. The property has also been used to investigate the treatment and disposal of dairy shed effluent including to what degree effluent treatment ponds reduce the nutrient loading and microbial pathogen concentration of dairy shed waste.

The present study extends previous one study on the epidemiological aspects of *Campylobacter jejuni* on this farm.

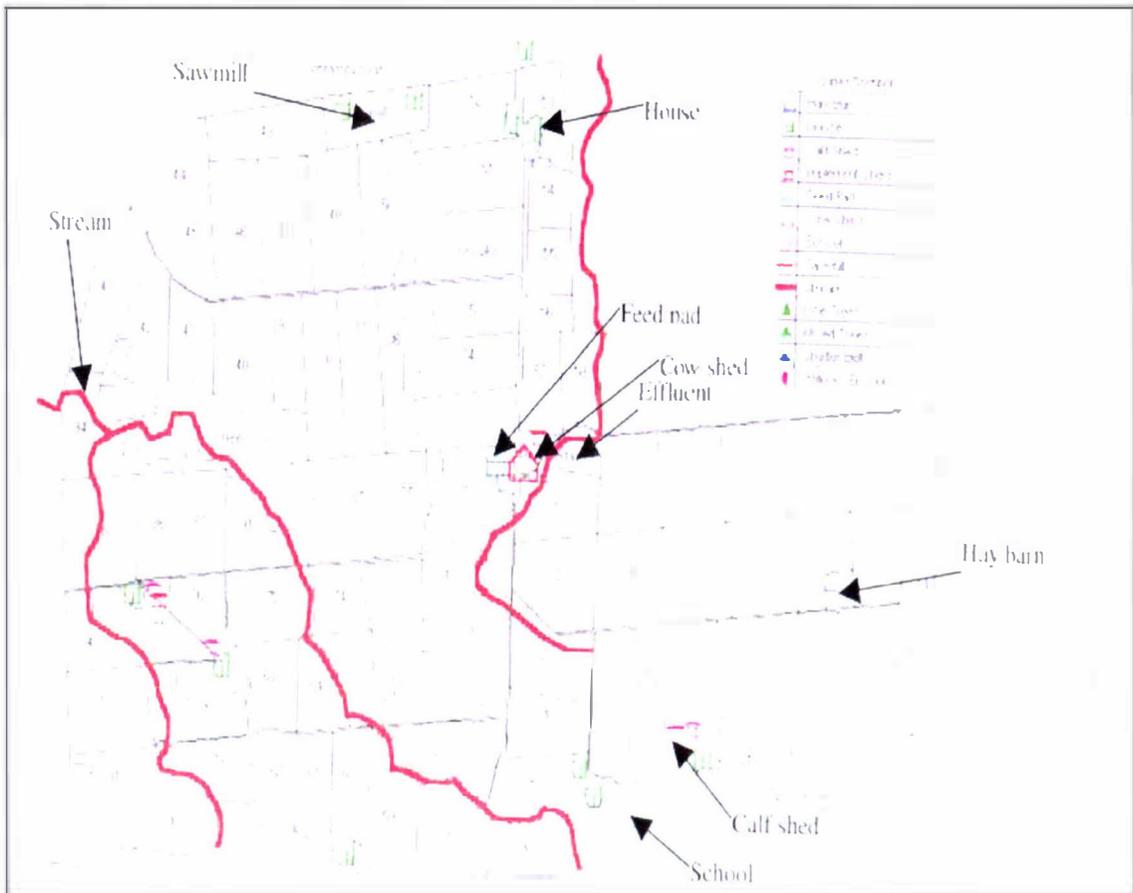


Figure 2.1. Site location of each section at No. 4 dairy farm, Massey University. Numbers denotes paddocks and red lines denotes small streams over the farm.

2.1.2 The Square, Palmerston North

Faecal samples were collected from urban sparrows roosting in trees on “The Square” in Palmerston North’s central business district (Fig. 2.2). The Square is a well-managed garden like area located in the heart of Palmerston North city about 7 km from Massey No. 4 dairy farm. In addition to sparrows the locality is also frequented by other species of birds including ducks.



Figure 2.2. The Square study site, Palmerston North city. The large trees in the background behind the pond (arrows) were used in the study to collect sparrow's droppings.

2.2 Specimen collection

All samples were collected in between 5 April 2002 to 25 May 2002

2.2.1 Dairy cows:

Rectal faeces were sampled from 52 randomly selected Friesian cows during milking time in the 36 bale rotary milking shed (Fig 2.3). Sterile disposable gloves and specimen collection containers were used for the collection of individual samples. Approximately 1 g of faecal material from each sample of rectal contents collected was inoculated into Bolton's broth (Appendix 1) on the farm and brought to the laboratory for further processing within 2 hours of collection.



Figure 2.4 A. Sample collection technique used for sparrows in the Massey No. 4 dairy farm



Figure 2.4 B. Sample collection technique used for sparrows in the city

2.2.3 Rodents:

Rectal samples from 65 rodents trapped on the feed storage premises using standard spring loaded, baited traps (Fig. 2.5) on the No. 4 dairy farm (approximately 15m from the milking shed) were collected and examined for thermophilic *Campylobacter* spp. The animals were dissected in the laboratory and rectal contents collected. Samples were placed in Bolton's broth and incubated for enrichment of *Campylobacter*. The word rodent is used here to describe mice and rats, although most of the animals trapped were mice.

2.2.4 Flies:

Fifty-six flies were captured on the dairy farm using flytraps. Collected flies were brought to the laboratory and dissected vertically and horizontally into four pieces and then inoculated into individual Bolton's broth.

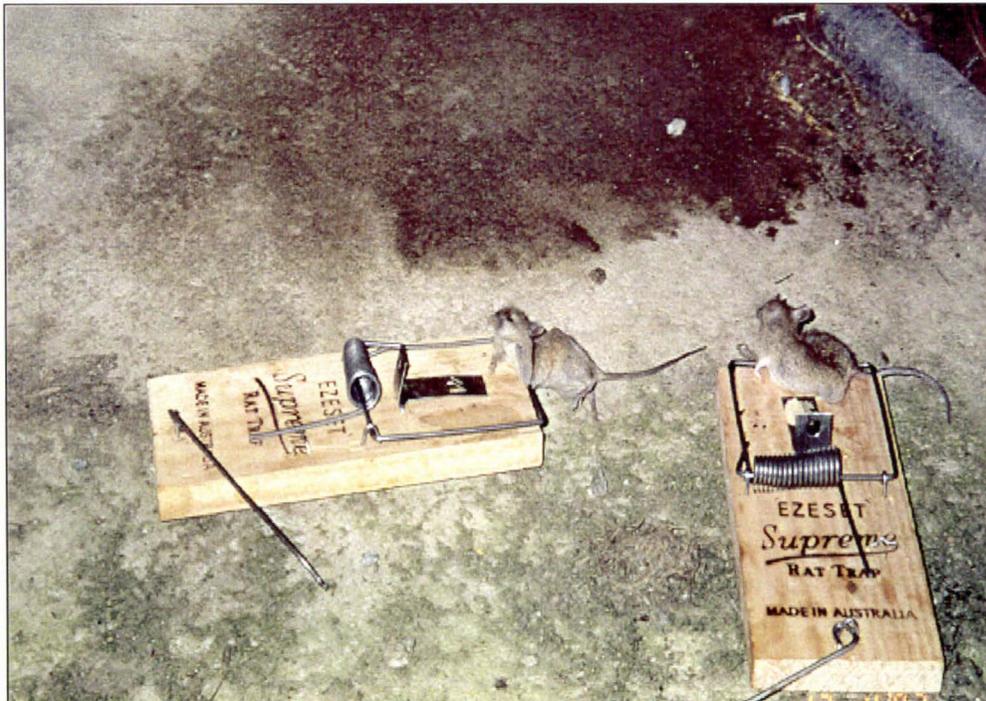


Figure 2.5. Rodents trapped in standard spring-loaded rat trap

2.2.5 Other animals:

No other animals such as farm dogs and feral animals were available for sampling during the study period on the dairy farm.

2.2.6 Other samples:

Other samples collected from around the dairy farm included five of silage (Fig. 2.6), two from aprons, two worker boots and two water samples from water troughs (Fig. 2.7) in the paddock (Fig. 2.8). The source of treated drinking water in the cattle grazing area is provided by the Massey University water supply system. The 100 ml water samples were collected in sterile containers and brought to the laboratory for further processing within two hours of collection. Sterile cotton swabs used to sample an area of about 5 cm² of workers aprons and boots were immediately placed into Bolton's broth. Five grass silage samples of approximately 1 g each were collected from the top outer layer area of silage trench using sterile forceps and immediately placed into the Bolton's broth.



Figure 2.6. Silage used as supplementary feed on No. 4 dairy farm during the time of grass scarcity



Figure 2.7. Drinking water trough in a paddock in No. 4 dairy farm

2.3 Culture and Identification of *Campylobacter* spp

2.3.1 Culture of campylobacters:

A flow diagram of culture and identification of *Campylobacter* spp is shown in figure 2.8. Approximately 1 gram of faeces was inoculated into 10 ml of Bolton's broth for enrichment of thermophilic *Campylobacter* spp. and incubated for 48 hours at 42 °C in a microaerophilic atmosphere produced by one sachet of CampyGen^R in an anaerobic campy jar. Following enrichment, the broth was streaked with a wire loop on to mCCDA (Fig. 2.9A, Appendix II) and incubated for 48 to 72 hours. The plates were examined for typical greyish, moist, spreading and mucoid colonies. Colonies with *Campylobacter*-like morphology were examined by Gram staining (Appendix III) and by motility. In the hanging drop method, a loopful of culture is emulsified on a glass slide in a drop of saline and observed under high power (100 x 10) under a microscope.

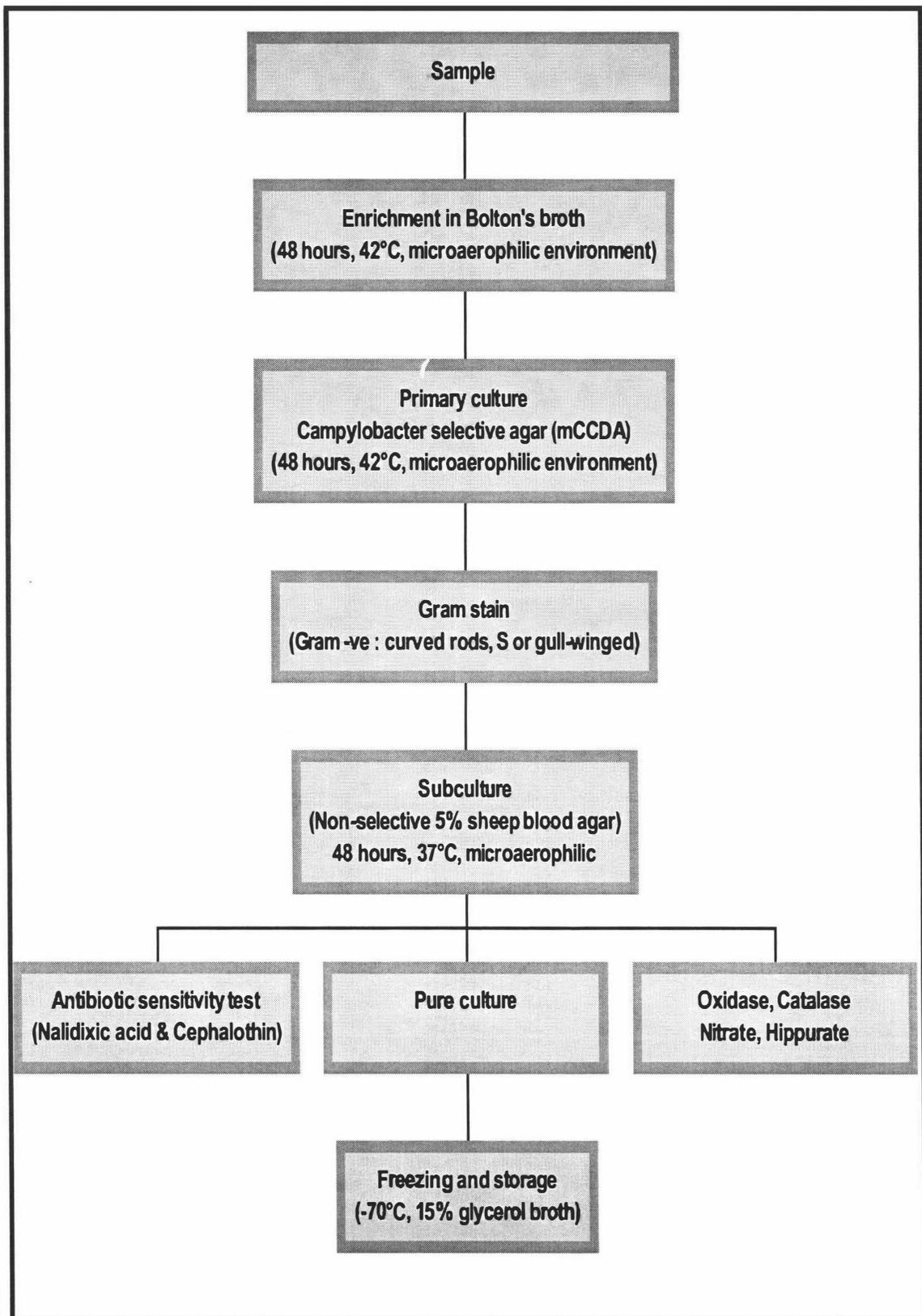


Figure 2.8. Flow diagram of procedures for *Campylobacter* spp isolation, identification and storage

Morphologically typical colonies, if found to be Gram negative curved rods, were plated on non-selective sheep blood agar (Fig. 2.9B, Appendix IV) and incubated microaerophilically at 37 °C for 48 hours as described. The *Campylobacter* spp were identified by oxidase, catalase, nitrate, hippurate test and sensitivity testing with cephalothin and nalidixic acid.

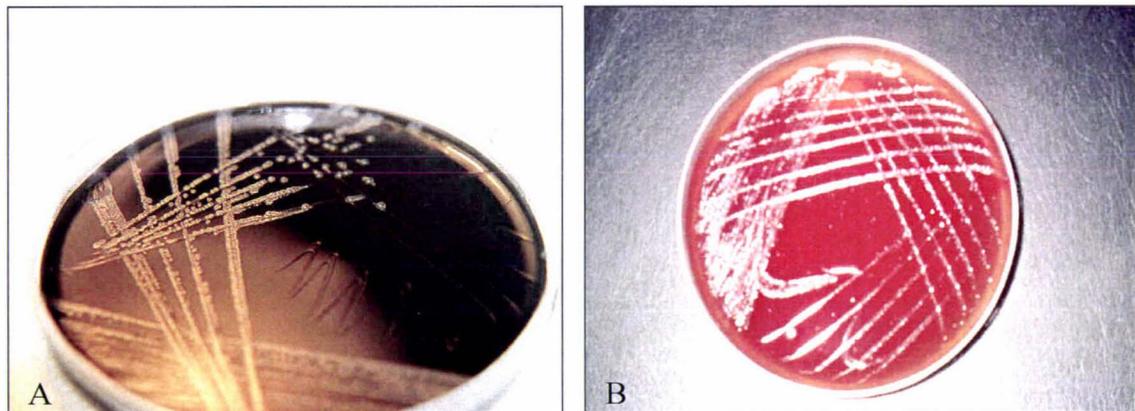


Figure 2.9. A- *C. jejuni* colonial morphology on selective mCCDA; B - *C. jejuni* colonial morphology on non-selective blood agar

2.3.2 Identification of campylobacters

2.3.2.1 Presumptive identification of campylobacters:

2.3.2.1.1 Gram stain

Gram staining was performed as described in Appendix III. Development of pink colour was interpreted as Gram-negative and, development of a purple colour was interpreted as Gram-positive.

2.3.2.1.1 Oxidase test

A drop of oxidase reagent (BBL Oxidase) was placed on the colonies to be tested directly on the plate. After 10 seconds the reaction was read. Development of a purple colour was interpreted as positive reaction. Negative reactions remained colourless or turned light pink or light purple after 30 seconds. *Campylobacter* spp are all oxidase positive.

2.3.2.1.2 Catalase activity

The catalase test was performed by placing one drop of 3.5% solution of hydrogen peroxide on a microscope slide and adding a small amount of growth with a loop and emulsified. Catalase converts hydrogen peroxide into oxygen and water providing effervescence and bubbles within a few seconds in positive reactions. Catalase positive species of the campylobacters are *C. jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. lari* and *C. sputorum*.

2.3.2.2 Confirmative identification of campylobacters

2.3.2.2.1 Nitrate reduction test

A swab was moistened in nitrate broth and used to pick up all the colonies from pure culture on a blood agar plate and emulsified these into the nitrate broth (Appendix V). The broth was incubated in microaerophilic atmosphere for 48 hours at 37 °C. About 0.2 ml of the nitrate solution “A” plus 0.2 ml of the nitrate solution “B” was added to the cell suspension in the nitrate broth and watched for an immediate reaction. A change in colour from yellow to pink/red indicated that the organisms reduce nitrate to nitrite (Fig. 2.10). Except *Campylobacter fennelliae* and *C. jejuni* subsp *doylei*, other species of *Campylobacter* reduce nitrate to nitrite.

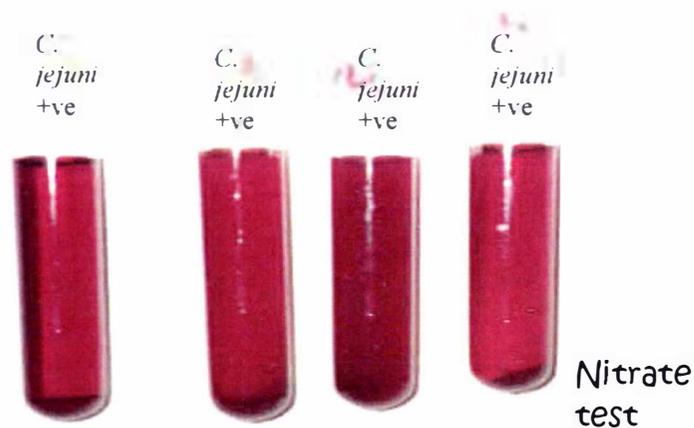


Figure 2.10. A colour change from yellow to pink/red indicates the organism reduces nitrates to nitrites.

2.3.2.2.1 Sensitivity to antibiotics

Disc diffusion tests were performed to evaluate antibiotic sensitivity using 30-microgram nalidixic acid and cephalothin discs. The sheep blood agar plate was inoculated with the nitrate broth pure culture cell suspension using a spread technique. A nalidixic acid (NA30) and a cephalothin (C30) antibiotic disc (Fig. 2.11) were placed on the inoculated blood agar plate and incubated for 48 hours in a microaerophilic environment. The absence of a clear zone of inhibition around the disc was indicative of resistance. Cephalothins resistant campylobacters are *C. jejuni*, *C. cinaedi*, *C. concisus* and *C. lari*. Nalidixic acid sensitive campylobacters are *C. jejuni*, *C. coli*, *C. cinaedi*, *C. fennelliae*, *C. jejuni* subsp *doylei*, *C. nitrofigillis* and *C. upsaliensis*.



Figure 2.11. Antibiotic sensitivity test using nalidixic acid disc (NA30) and cephalothin disc (C30) in blood agar. A zone of inhibition of growth of *C. jejuni* around the nalidixic acid disc (NA) indicating sensitivity of the isolate to nalidixic acid, whereas *C. jejuni* growth at C disc is indicative of resistance against Cephalothin.

2.3.2.2.2 Hippurate hydrolysis test

A swab was moistened in distilled water and used to pick up half of the plate of pure *Campylobacter* culture growth and was emulsified into a cloudy smooth suspension in a tube. A hippurate disc was added to the tube and incubated in a water bath at 37⁰C for 2

hours. Then 0.2 ml of ninhydrin reagent (Appendix VI) was added to the suspension before re-incubation for 10 minutes. A positive hippurate reaction was indicated by the formation of a deep purple colour (Fig. 2.12). Except *C. jejuni*, all *Campylobacter* spp. provides a negative hippurate reaction. But the records of hippurate reactions for the *C. cinaedi*, *C. fennelliae*, *C. crryaerophila*, *C. jejuni* subsp *doylei*, *C. nitrofigillis* and *C. upsaliensis* were not available.

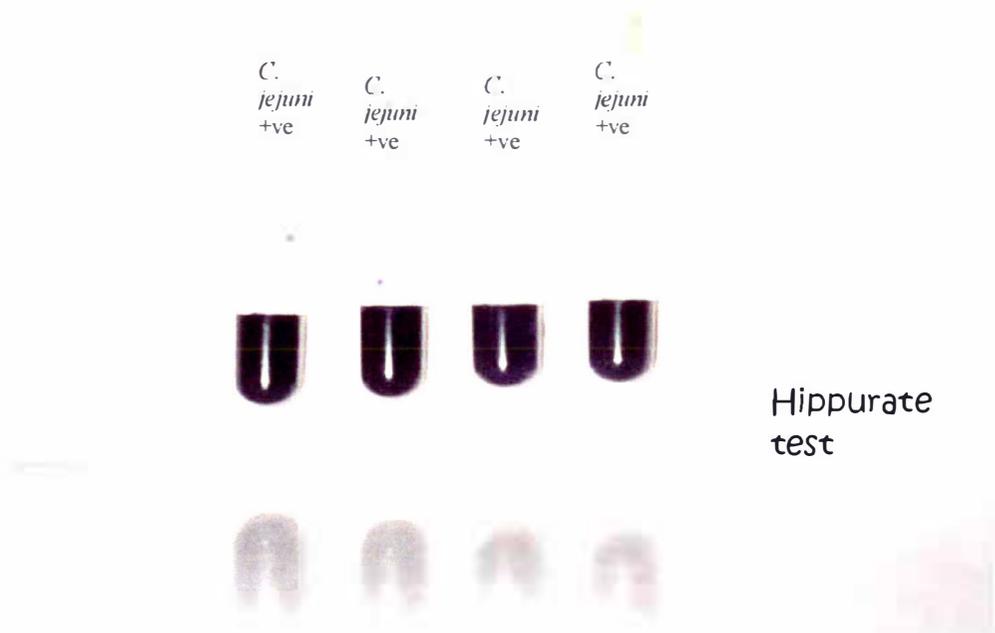


Figure 2.12. A deep purple, crystal violet-like colour indicates the presence of glycine from the hydrolysis of hippurate by *C. jejuni*.

2.4 Storage:

Stocks of *Campylobacter jejuni* isolated from subsequent pure culture in sheep blood agar were preserved in 15% glycerol broth and stored at -70°C for later molecular characterization.

2.5 Pulsed-field gel electrophoresis of *Campylobacter*

All 88 isolates of *C. jejuni* obtained during the study were subjected to pulsed-field gel electrophoresis after digestion with the restriction enzyme *Sma* I. Of the 88, only 61 isolates showed visually countable restriction band patterns. Unclear band patterns were discarded in the analysis of PFGE profiles. Of the 61 clear band patterns, 14 were obtained from dairy cows, 19 from farm sparrows, 10 from urban sparrows, seven from rodents, five from flies, one from silage, two from water, two from boots and one from an apron. The reason for unclear band patterns even after repeated PFGE is unclear but could be associated with the quality of enzymes or other reagents applied. Details of the PFGE process for typing campylobacters are given in chapter 3 (section 3.4).

2.5.1 Preparation of Plugs – Day 1

Campylobacter jejuni colonies were incubated microaerophilically for 48 hours at 37⁰ C on 5% sheep blood agar, were swabbed gently without disturbing the agar surface using sterile cotton swabs and suspended in 2-3 ml of brain heart infusion (BHI) broth. The optical density (OD) of each of the bacterial suspensions in BHI broth was measured using a Helios Alpha ® (Unicam, Cambridge, UK) spectrophotometer at 610 nm and adjusted to an OD of 1.4 (200µl of cells x 1.4 /OD reading). A 200µl aliquot of each bacterial suspension was placed into an Eppendorf tube and centrifuged at 8,000 revolutions per minute (rpm) for five minutes. The supernatant was removed and the cells resuspended in 150 µl of cold PETH IV buffer (1M NaCl, 10 mM Tris-HCl (BDH Laboratory Supplies, Poole, England) [pH 8.0] and 10 mM ethylenediamine tetra-acetic acid (EDTA) (BDH laboratory supplies, Poole, England) [pH 8.0], Appendix VII A, VII I and VII J) and centrifuged for five minutes at 8,000 rpm. The supernatant was pipetted out and the bacterial pellets resuspended in 50 µl of PETH IV buffer.

Low-melt agarose (1%) was prepared (Appendix VII B). A 100µl aliquot of molten agarose was added to the cell suspension and gently pipetted up and down 5-6 times to mix. The molten agarose was held at 55 °C for a few minutes before mixing it with the cell suspension. Following mixing, 100 µl of cells-agarose suspension was immediately

dispensed into the wells of reusable plug molds by 200µl pippette. One plug was made from each sample. Plugs were allowed to cool and solidify on ice at 4 °C for an hour. Lysis buffer was prepared (Appendix VII C). Plugs were placed into 1 ml of lysis buffer in Eppendorf tubes and incubated overnight at 56 °C in a water bath.

2.5.2 Plug washing – Day 2

After incubation overnight at 56 °C in a water bath, the buffer was removed and the plugs were transferred into 50 ml universal plastic tubes containing 10 ml of 10:1 TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], Appendix VII D, VII I and VII J). The plugs were incubated in ice for one hour on a rocking machine. The TE buffer was replaced with 10 ml of fresh TE buffer and incubated in ice for a further one hour on a rocking machine. The wash process was repeated another three times. Plugs were stored in 2 ml TE buffer at 4 °C until ready for restriction digestion. Plugs remain useable for several months if stored in plug wash TE buffer at 4 °C.

2.5.3 Restriction endonuclease digestion with *Sma* I – Day 3

Each plug was removed from the storage tube and placed on a clean sterile glass slide. With the help of a flamed scalpel, one third of the plug was sliced off and placed into 100 µl of restriction buffer (Appendix VII E) and equilibrated on ice for 45 minutes. The remaining two-thirds of the plug was placed into 1 ml of TE buffer in a microcentrifuge tube and stored at 4 °C until required. Ethanol was used to sterilize the glass slide and scalpel after each specimen.

Cutting buffer was prepared as shown in appendix VII F. Restriction buffer was removed and replaced with 100 µl of cutting buffer. Plugs were equilibrated on ice for 45 minutes before incubating overnight at 25 °C in a water bath.

2.5.4 Gel running for PFGE –Day 4

A wide and long 1% pulsed-field certified agarose (PFC) was prepared as shown in appendix VII G. The PFC agarose (1%) was poured into the gel-casting tray, and was allowed to solidify for one hour before careful removal. The gel tank was filled with 2.3L of 0.5 × TBE buffer (Appendix VII H), and circulated for about one hour at 14 °C. The gel was pre-electrophoresed at 6V/cm, 5-5 sec., for 1-1.5 hours using CHEF MAPPER[®] apparatus (Bio-Rad laboratory, CA, USA). The gel was removed from the tank and the remaining buffer removed from the wells. The plugs were inserted into wells and pushed down to the bottom of the well using a ‘hockey stick’ (a small glass rod with one end bent in to a curve to prevent damage to the plugs when pushing plugs down into the wells). A low-molecular-weight marker (New England Biolabs, MA, USA) and a Lambda –marker (New England Biolabs, MA, USA) were also loaded into the wells. The gel was placed in the electrophoresis tank and run using the following parameters (Table 2.1).

Table 2.1. Gel running parameters

Parameters	Conditions
Initial Switch Time	0.5 seconds
Final Switch Time	40 seconds
Run Time	22 hours
Angle	120 ⁰
Gradient	6V/cm
Temperature	14 ⁰ C
Ramping factor	Linear

2.5.5 Staining and photographing the gel – Day 5

The gel was removed from the tank and stained in ethidium bromide solution (800 ml of distilled water with 80 ml of 10 mg/ml of ethidium bromide) for 10 minutes. The gel was then rinsed briefly with sterile Milli – Q (MQ) water and photographed under UV light using the Gel Doc 2000 (Bio-Rad). A dendrogram was then generated using a diversity database to show the level of similarity between PFGE patterns of *Campylobacter* from various sources.

CHAPTER THREE: RESULTS

A total of 290 samples were collected during this study from dairy farm and urban sources. *Campylobacter* spp were isolated from 88 (30%) samples. All the *Campylobacter* isolates were *Campylobacter jejuni*.

3.1 Isolation of *campylobacter* spp. from cattle, sparrows, rodents and flies

Campylobacter jejuni was isolated from 28 of 52 (54%) apparently healthy cows sampled from No. 4 dairy farm, Massey University. No other species of *Campylobacter* was isolated in this study. *Campylobacter jejuni* was isolated from 20 (38%) of the 53 faecal samples from farm sparrows. A total of 56 flies were trapped around the milking shed and five (6%) of the samples were found to be positive for *Campylobacter jejuni*. Faecal samples from 65 rodents (mice and rats) trapped in the farm feed storage area were collected and examined for thermophilic *Campylobacter* spp. *Campylobacter jejuni* were isolated from 7 (11%) of these samples.

Results show that two of five silage, two of two worker's boots, one of two aprons and two of two water samples from farm troughs were found positive for *C. jejuni*. The results of isolation are summarised and presented in Table 3.1 and Table 3.2.

Table 3.1. Summary of *Campylobacter* spp. isolation from farm and urban sources (5 April 2002 to 25 May 2002)

	Dairy cows (n=52)	Urban Sparrows (n=53)	Farm Sparrows (n=53)	Rodents (n=65)	Flies (n=56)	Other samples (farm)			
						Silage (n=5)	Boots (n=2)	Aprons (n=2)	Water (n=2)
<i>C. jejuni</i> positive samples	28	21	20	7	5	2	2	1	2
<i>C. jejuni</i> negative samples	24	32	33	58	51	3	0	1	0
Prevalence	54%	40%	38%	11%	9%	Positive	Positive	Positive	Positive

Of the 52 cows sampled, eight were common in the present study and the study by Wu (2001). Table 3.2 shows the status of *Campylobacter jejuni* in common cows. Results of the present study show that there is a continuous presence of *Campylobacter jejuni* in milking cows at Massey No. 4 dairy farm. Cows 101, 125, 198 and 356 were still positive for *C. jejuni* at least 24 month after the study by Wu (2001) of the same group of cows (Table 3.2). These cows could have been carrying *Campylobacter* in the intestines continuously or acquired infection intermittently or been intermittently excreting the organisms through contamination of the farm environment. Cows 44 and 145 were found negative for *C. jejuni* in both studies. However, cow 90 was carrying *C. jejuni* in the study by Wu (2001) and found negative in the present study. Also Cow 239 was negative for the organisms in 2001 study but found positive in the present study.

Table 3.2. Comparison of *Campylobacter* carriage by cows samples in the present study and in the study by Wu (2002)

Cow ID	2001 study	2003 study
101	+	+
125	+	+
198	+	+
356	+	+
44	-	-
145	-	-
90	+	-
239	-	+

3.2 Isolation of *campylobacter* spp. from urban sparrows

A total of 53 faecal samples were collected from sparrows at The Square, Palmerston North city, of which 21 (40%) were found positive for *C. jejuni*. It can be seen from the results presented in Table 3.1 that there is not much difference between the rates of isolation of *C. jejuni* in urban and farm sparrows.

3.3 Descriptive statistics study

3.3.1 Prevalence

3.3.1.1 Prevalence of *C. jejuni* in cows, sparrows, rodents and flies on the farm

The distribution of prevalence of *C. jejuni* in different animals in dairy farm is shown in Figure 3.1. The dairy cows showed highest prevalence (54%) followed by sparrow (38%), rodents (11%) and flies (9%). The prevalence of *C. jejuni* in urban sparrows was 40%, almost similar results that carried by farm sparrows (Fig. 3.2).

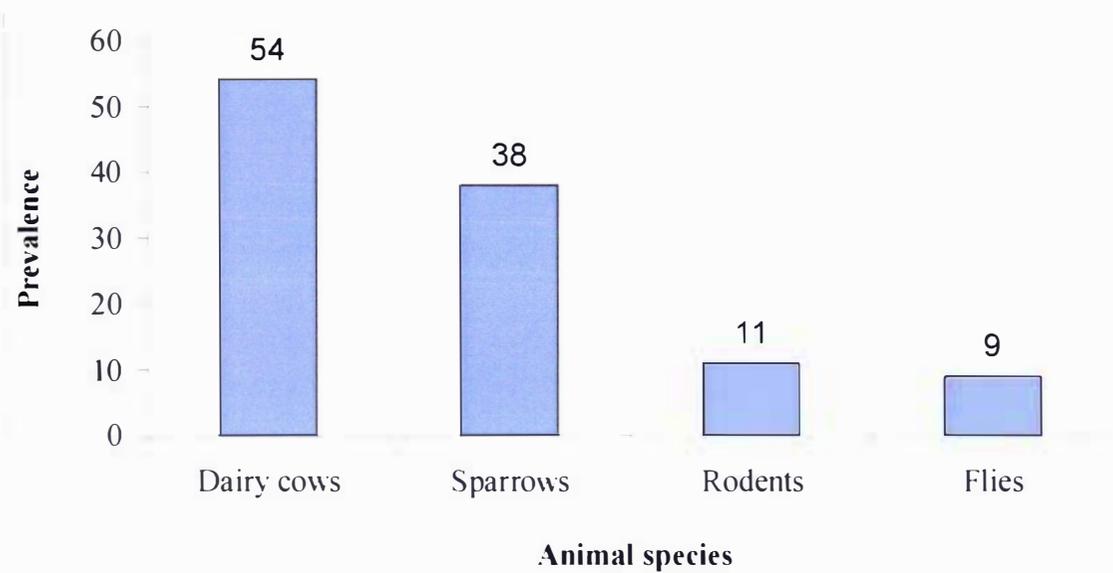


Figure 3.1. Prevalence of *C. jejuni* in farm sources

3.3.1.2 Prevalence of *C. jejuni* in farm and urban sparrows.

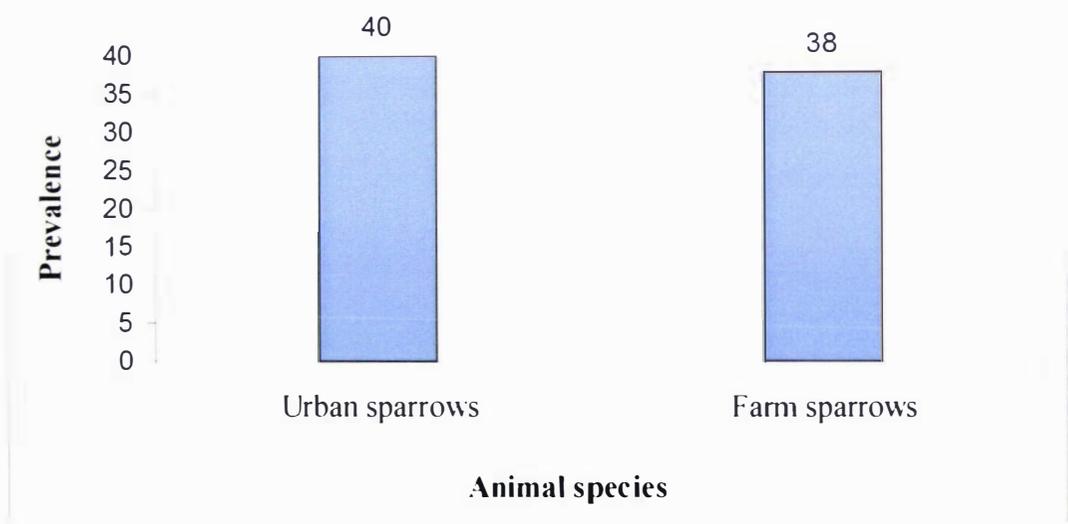


Figure 3.2. Prevalence of *C. jejuni* in urban and farm sparrows

3.3.2 Confidence intervals

From sampling events and sample size, a 95% confidence interval for prevalences in different populations was calculated. Binomial distribution can be made from sampling events. If repeated samples of the same number of individual n were selected, the calculated rate p would vary from sample to sample (Martin, Meer and Willeberg, 1987) and this inconsistency is described by the standard error (SE) of the mean, which is estimated from the sample.

$$SE(p) = [p(1-p)/n]^{1/2}$$

[Where p denotes for proportion of test positive samples (e.g. measured prevalence $p = X/n$, X denotes binomial count)]

$$\text{Variance} = p(1-p)/n$$

Therefore, confidence interval for a proportion will be $p \pm z [p(1-p)/n]^{1/2}$

[Where z value will be 1.65 for 90%, 1.96 for 95% and 2.567 for 99%]

In this way the 95% confidence interval could be formed to allow for a 2.5% chance that the population proportion is lower than the lower confidence limit and a 2.5% chance that the population proportion is higher than the upper confidence limit (Pfeiffer, 1999). Table 3.3 was obtained by calculating total measured prevalences for each sample, variance, standard error and, finally upper and lower confidence limit. One of the examples is given below for dairy cows.

Sample size = 52, *C. jejuni* positive animals = 28, Total measured prevalence for *C. jejuni* (p) = $28/52 = 53.8\% = 0.538$

Variance = $[0.538(1-0.538)/52 = 0.004779$, $SEp = (4.779 \times 10^{-3})^{1/2} = 0.06913$

95% confidence interval (upper and lower) = $0.538 \pm 1.96 \times 0.06913 = 0.538 \pm 0.135$

Consequently, at the 95% confidence interval, the prevalence of *Campylobacter jejuni* carriage by the population of dairy cows ranges between 40.3% and 67.3%.

Table 3.3. Calculation of 95% confidence intervals for the prevalence of *C. jejuni* in different populations.

Source type	Milking cows	Farm sparrows	Urban sparrows	Rodents	Flies
Sample size	52	53	53	65	56
Total number of samples positive	28	21	20	7	5
Total measured prevalence for <i>C. jejuni</i>	53.8%	39.6%	37.7%	10.8%	8.9%
Variance	0.004779	0.004512	0.004431	0.001482	0.001447
SEp	0.06913	0.06717	0.06656	0.03849	0.03804
95% Confidence intervals for prevalence ($p \pm 1.96 \text{ SEp}$)	0.538 ± 0.135	0.396 ± 0.132	0.377 ± 0.130	0.108 ± 0.075	0.089 ± 0.075
Upper confidence limit	67.3%	52.8%	50.7%	18.3%	16.4%
Lower confidence limit	40.3%	26.4%	24.7%	3.3%	1.4%

Finally, it can be concluded that at the 95% confidence interval, the prevalence of *Campylobacter* carriage in the population of milking cows ranges between 40.3% and 67.3%. The prevalence of *Campylobacter* carriage in the population of farm sparrows ranges between 26.4% and 52.8%. The prevalence of *Campylobacter* carriage in the population of urban sparrows ranges between 24.7% and 50.7%. The prevalence of *Campylobacter* carriage in the population of rodents ranges between 3.3% and 18.3%. The prevalence of *Campylobacter* carriage in the population of flies ranges between 1.4% and 16.4%.

3.4 Pulsed-field gel electrophoresis

3.4.1 Pulsed-field gel electrophoresis of *C. jejuni* isolates

Genomic DNA from 61 *C. jejuni* isolates were characterised by PFGE digestion with restriction enzyme *Sma* I during the study (Figs. 3.3, 3.4 and 3.5). The gels were analysed visually for band pattern differences. Bands were identified numerically from the highest molecular weight downwards. Bands were also described by their sizes in kilobases relative to one of the molecular weight markers. *Campylobacter jejuni* isolates were then classified into A to V patterns on the basis of three band differences.

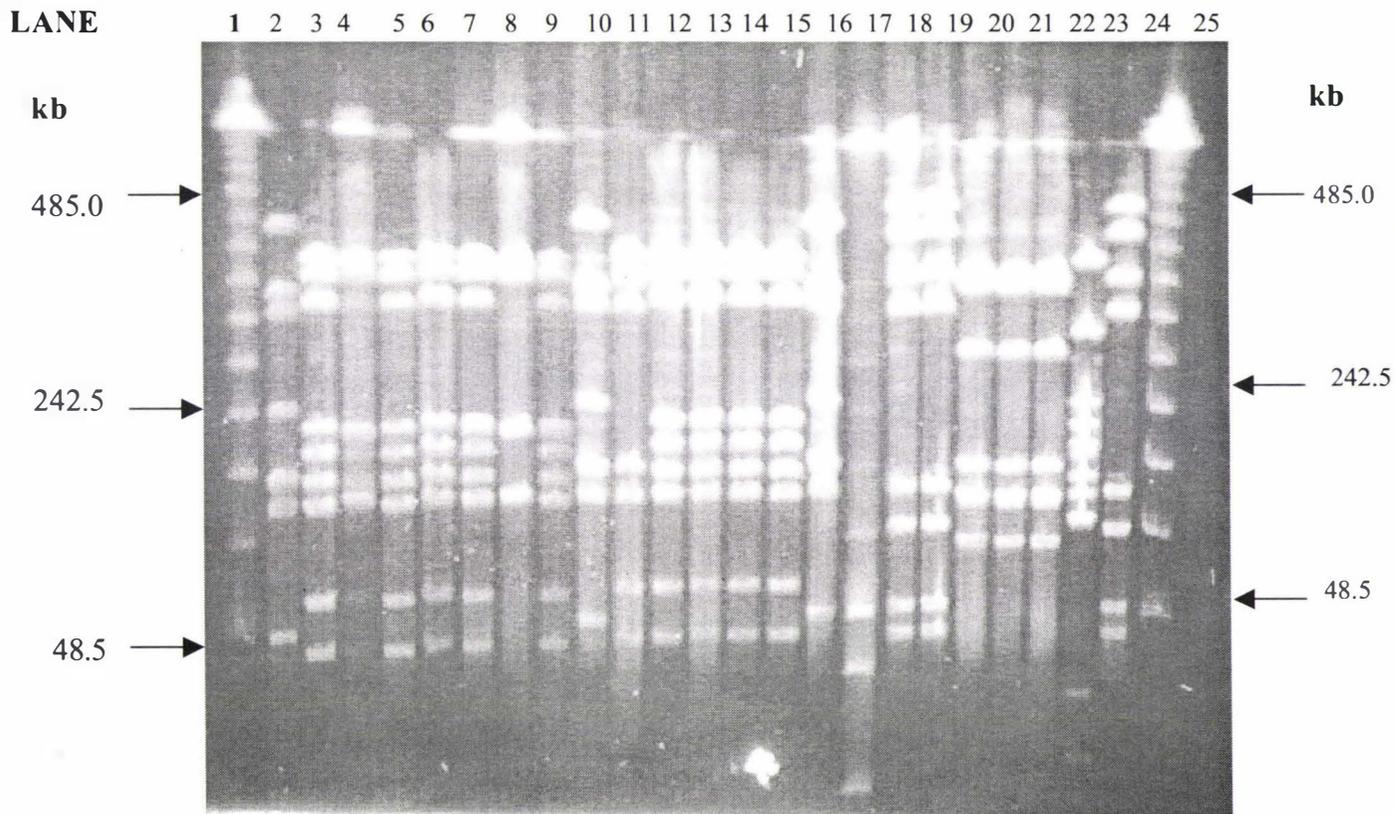


Figure 3.3. *Sma* I pulsed-field gel electrophoresis (PFGE) restriction patterns of 22 isolates of *C. jejuni* genomic DNA.

Lane 1 and 25: Lambda ladder marker, Lanes 2 – 16: farm sparrows, Lane 17: low molecular weight marker, Lane 18 and 19: Water trough, Lane 20 and 22: Boots, Lane 21: apron, Lane 23: fly, Lane 24: Rodent

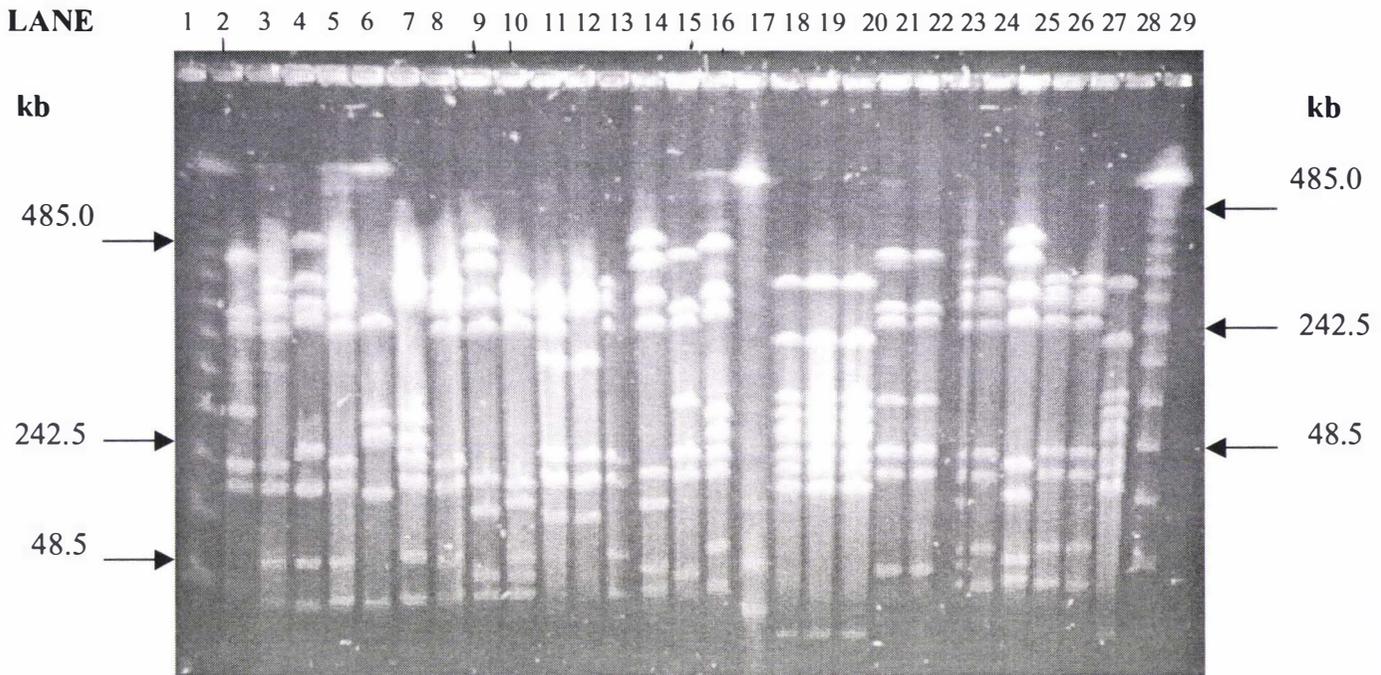


Figure 3.4. *Sma* I PFGE restriction patterns of 25 isolates of *C. jejuni* genomic DNA.

Lane 1 and 29: Lambda ladder marker, Lanes 2 – 9: Dairy cows, Lane 10 - 15: Farm rodents, Lane 16 – Silage, Lane 17 - Low molecular weight marker, Lane 18 – 24: Dairy cows, Lane 25 – 28: Farm flies, Lane 23: unclear

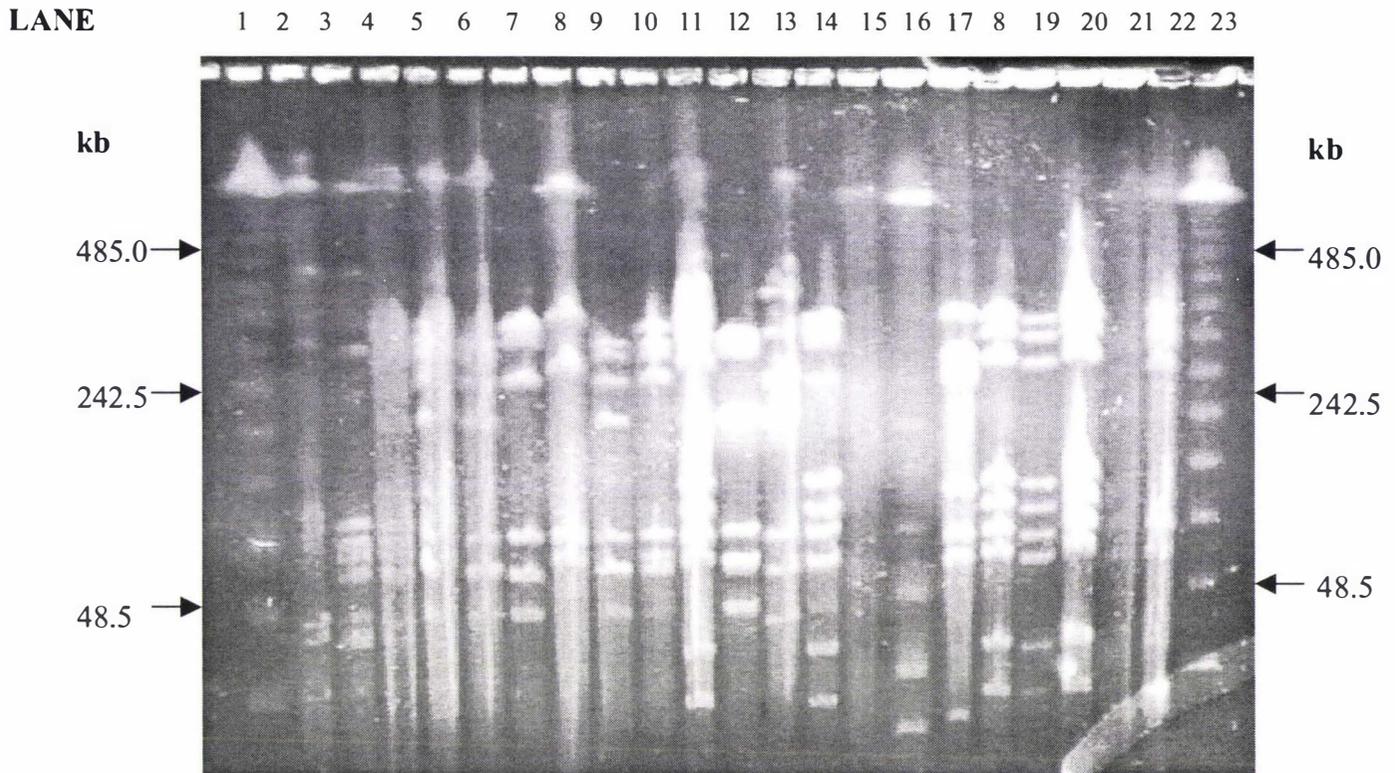


Figure 3.5. *Sma* I PFGE restriction patterns of 20 isolates of *C. jejuni* genomic DNA. (see section 2.5 for detail methodology)

Lane 1 and 23: Lambda ladder marker, Lanes 2 – 15: urban sparrows isolates, Lane 16: low molecular weight marker, Lane 17 – 20: farm sparrow isolates, Lane 2,4,8, 15, 21: unclear, Lane 22: fly isolates

Twenty-two patterns were obtained, of which eight patterns were common to more than one source. Table 3.4 shows that the highest subtype diversity index was found in urban sparrows (0.89), followed by rodents (0.83), flies (0.5), dairy cows (0.46) and farm sparrows (0.28). The subtypes of *C. jejuni* obtained during this study were compared to those analysed by PFGE in the Wu (2001) study. It is interesting that the subtype diversity index for dairy cows in the present study and in the earlier study by Wu (2001) is the same (0.46) in both studies, which may suggest that the subtype diversity of *C. jejuni* in dairy cows at No. 4 dairy farm could be similar or just coincidence.

Table 3.4. PFGE restriction patterns and the subtype diversity index of *C. jejuni* from different sources.

Patterns	Number of <i>Campylobacter jejuni</i> isolates								
	Cattle (n=14)	Farm sparrow (n=19)	Urban sparrow (n=10)	Rodent (n=7)	Flies (n=5)	Silage (n=1)	Water (n=2)	Boot (n=2)	Apron (n=1)
A	1								
B	3				2				
C		1							
D	1			2	1		2		
E			1						
F	3	3		1					
G			1						
H			1						
I			1						
J			1						
K			1						
L			2	1					
M				1				2	1
N		1							
O			1						
P	1								
Q				1					
R	4	1		1	2				
S						1			
T	1		1						
U		1							
V		12							
No. of subtypes	7	6	9	6	3	1	1	1	1
Diversity index*	0.46	0.28	0.89	0.83	0.5	Undefined	0	0	Undefined

* $\frac{\text{No of subtypes}-1}{\text{No. of isolates}-1}$

PFGE of *C. jejuni* isolated from dairy cows

The phenotypically identical *C. jejuni* isolates from dairy cattle were characterised into seven different types, which reflects the high degree of discrimination obtained by PFGE and the diversity of the organism within a limited geographical area. The resolution of *Sma* I digested DNA yielded from five to eight fragments based on a difference of one or more bands with sizes ranging from 48.5kb to 485 kb. Of the seven cattle restriction patterns, five patterns (B, D, F, R and T) were shared by more than one source. These were farm sparrows, rodents, flies and water (Table 3.4). Restriction patterns A and P were only associated with cattle.

PFGE of *C. jejuni* isolated from farm sparrows

Genomic DNA of 19 isolates of *C. jejuni* obtained from farm sparrows were characterised by PFGE. Digestion with restriction enzyme *Sma* I yielded four to nine bands on PFGE ranging in size from 48.5 – 485 kbp. On the basis of genomic DNA analysis, 15 isolates of *C. jejuni* could be classified into six different restriction patterns (Table 3.4). Of the six, two restriction patterns (R and F) were indistinguishable from patterns of isolates from urban sparrows, rodents and cattle (Table 3.4). Restriction patterns C, F, U and V were only seen in farm sparrows.

PFGE of *C. jejuni* isolated from urban sparrows

Genomic chromosomal DNA obtained from 10 isolates of *C. jejuni* from urban sparrows was digested with restriction enzyme *Sma* I. PFGE analysis of digested DNA provided five to eleven fragments ranging size from 48.5 – 485 kbp. The isolates could be grouped into nine different restriction patterns from the dairy farm (Table 3.4) based on PFGE band differences. Of the nine, two restriction patterns (L and T) were indistinguishable from cattle and rodent patterns (Table 3.4). Restriction patterns E, G, H, I, J, K and O were observed in urban sparrows but not any of the farm sources.

PFGE of *C. jejuni* isolated from rodents

Genomic DNA from seven *C. jejuni* isolates from rodents trapped on the farm feed storage premises was digested with the restriction endonuclease enzyme *Sma* I and analysed by PFGE. This provided five to nine distinct bands ranging in size from 48.5 – 485 kbp. The isolates could be grouped into six different restriction patterns (Table 3.4) based on their PFGE profiles. Five patterns (patterns D, F, L, M and R) were indistinguishable from farm cattle, sparrows, flies, boot and apron patterns and urban sparrow patterns (Table 3.4). Restriction pattern Q was only observed in the farm rodents.

PFGE of *C. jejuni* isolated from flies

Genomic chromosomal DNA from five *C. jejuni* isolates obtained from flies was digested with the restriction enzyme *Sma* I. PFGE of digested DNA yielded eight bands for each isolates ranging size from 48.5 – 485 kbp. These isolates could be grouped into three different restriction patterns (Table 3.4) based on band position and size. All three patterns (patterns B, D and R) were common to farm cattle, sparrows, flies, rodent and water isolates (Table 3.4).

PFGE of *C. jejuni* isolated from other samples

Genomic chromosomal DNA from five *Campylobacter jejuni* isolates obtained from farm silage, water, worker's boots and apron sample was digested with the restriction enzyme *Sma* I. The analysis of digested DNA provided five to nine bands ranging in size from 48.5 – 485 kbp. These isolates could be classified into three different restriction patterns (patterns D from water, S from silage and pattern M common to boot and apron). Restriction pattern S was only observed in farm silage and from no other source.

3.4.2 Analysis of common restriction patterns of *C. jejuni* isolates from different source

The common PFGE pattern of *C. jejuni* isolates from cattle, farm sparrows, urban sparrows, rodents, flies and water are shown in Table 3.5. Cattle restriction pattern B is shared by 40% fly isolates of *C. jejuni* making it a common pattern. Cattle restriction pattern D was also seen in 29% rodent isolates, 20% fly isolates and one water isolates making it a common restriction pattern. Restriction pattern F includes farm sparrows and rodent isolates, which can be consider a common restriction pattern with respect to cattle isolates. Restriction pattern R was observed in *C. jejuni* isolates from 28% cattle, 5% farm sparrows, and 14% rodents. Also 10% urban sparrow isolates of *C. jejuni* belonged to pattern T, indicating a common source of origin with 7% cattle isolates. Sparrows are non-migratory, generally sedentary birds. Non-breeding sparrows in America had a range of 3.2 km compared to nesting sparrows that seldom travelled more than 30 m from the nest (Will, 1973). A few range sparrow movements (65 – 300 km) have been reported in New Zealand (Heather and Robertson, 1997). Table 3.4 shows the number of *C. jejuni* isolates in each PFGE restriction profiles.

Table 3.5. Percentage *C. jejuni* from different sources having PFGE patterns indistinguishable from cattle

Indistinguishable PFGE patterns	Percent of <i>C. jejuni</i> isolates from different sources					
	Cattle (n=14)	Farm sparrow (n=19)	Urban sparrow (n=10)	Rodents (n=7)	Fly (n=5)	Water (n=2)
B	21	–	–	–	40	–
D	7	–	–	29	20	100
F	21	16	–	14	–	–
R	29	5	14	–	–	–
T	7	–	10	–	–	–

A dendrogram (Fig. 3.6) showing the similarity among the observed PFGE patterns by the unweighted pair group method using arithmetic averages (UPGMA) analysis using diversity database (Bio-Rad) software. Alphabets A to V on the right side of Fig. 3.5 indicates PFGE restriction patterns from different sources.

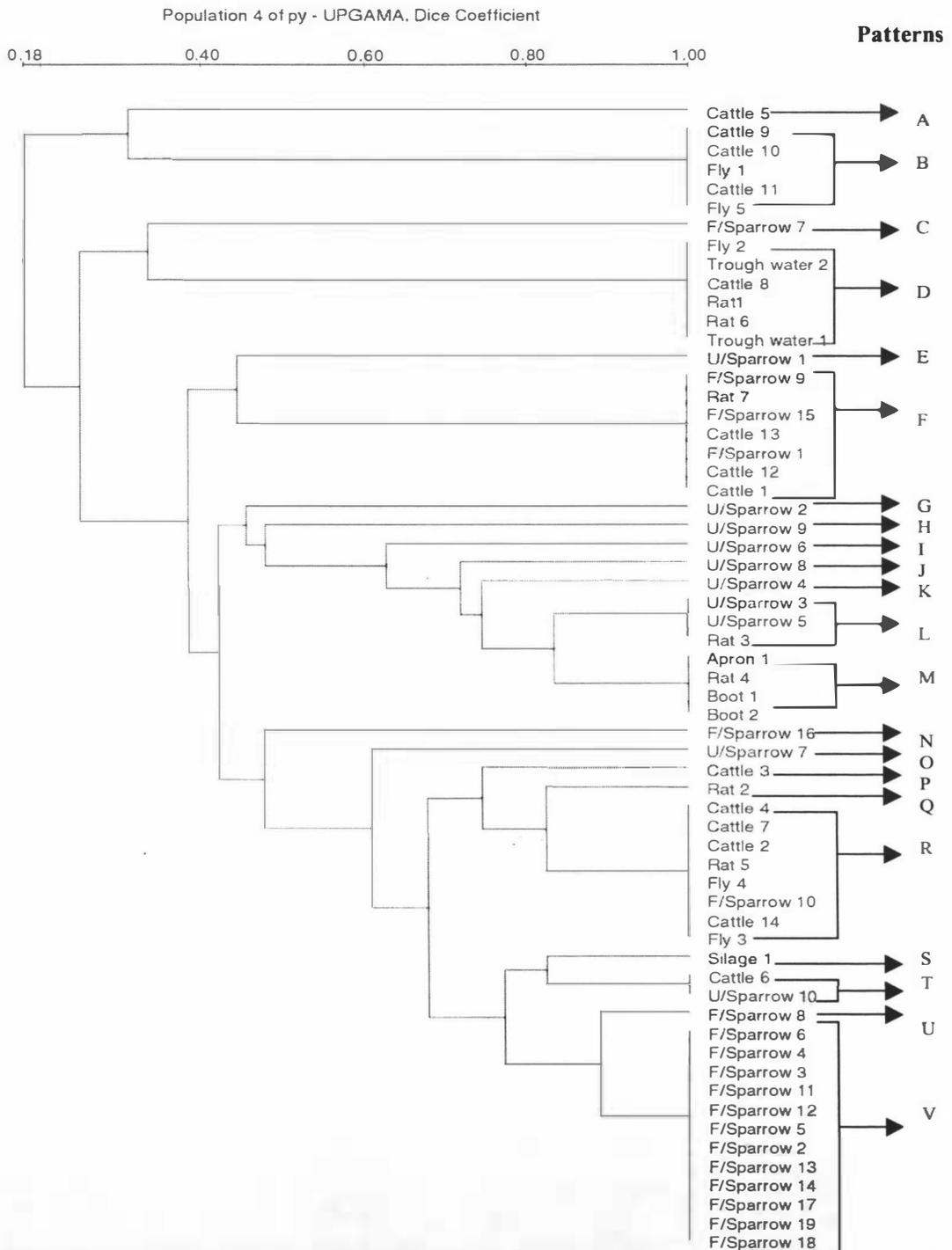


Figure 3.6. Dendrogram of similarity between 61 *C. jejuni* PFGE patterns. A percentage scale of similarity is indicated at the top. U/sparrow- Urban sparrow, F/sparrow- Farm sparrow

Restriction patterns produced by three pulsed-field gels with the genomic DNA of 61 *C. jejuni* isolates from different sources (e.g. dairy cows, farm sparrows, urban sparrows, rodents, flies and other farm environment samples) were analysed visually for the assessment of band differences.

A total of 22 different restriction patterns reflect the high degree of diversity among the organisms within a limited geographical area. However, the majority of the isolates from cattle could be grouped into six predominant patterns (patterns B, D, F, M, R and V). The other 16 types were found to be represented by only one or two isolates each. The predominant pattern in farm sparrows (pattern V) was not detected in cattle at all.

In this study, the PFGE typing of *C. jejuni* isolates provided a total of seven (32%) restriction patterns common to more than one source (patterns B, D, F, L, M, R and T) and 15 distinguishable patterns (A, C, E, G, H, I, J, K, N, O, P, Q, S, U and V) from one source only. Restriction pattern B observed in 21% cattle isolates and 40% of fly isolates shows the widespread presence of *C. jejuni* of the same clonal type in this farm environment. Restriction pattern D was found in six *C. jejuni* isolates from four different sources including 7% cattle isolates, 29% rodent isolates, 20% fly isolates and two water isolates. Restriction pattern F represented by 21% cattle, 16% farm sparrows and 14% rodent isolates provides evidence of identical clones infecting cattle, sparrows and rodents.

The L pattern in PFGE profile is common to 20% (2) of the urban sparrows and 14% (1) of the farm rodent isolates of *C. jejuni*. The other eight isolates of *C. jejuni* from the urban sparrows showed different restriction patterns except for pattern T, which is also seen in 7% of cattle isolates.

3.4.3 On-farm comparisons of PFGE profiles of *C. jejuni* over time

A dendrogram of similarity between 47 phenotypically identical *C. jejuni* isolates from dairy cows (14), farm sparrows (19), rodents (7), flies (5) and other farm environment samples (6) revealed 14 different PFGE restriction patterns including five indistinguishable patterns (Fig. 3.7). The level of similarity of *C. jejuni* isolates from the present study and that by Wu (2001) 24 months earlier on the same farm is shown in Figure 3.8. Some of the *C. jejuni* isolates between these two studies show indistinguishable band patterns (Table 3.7).

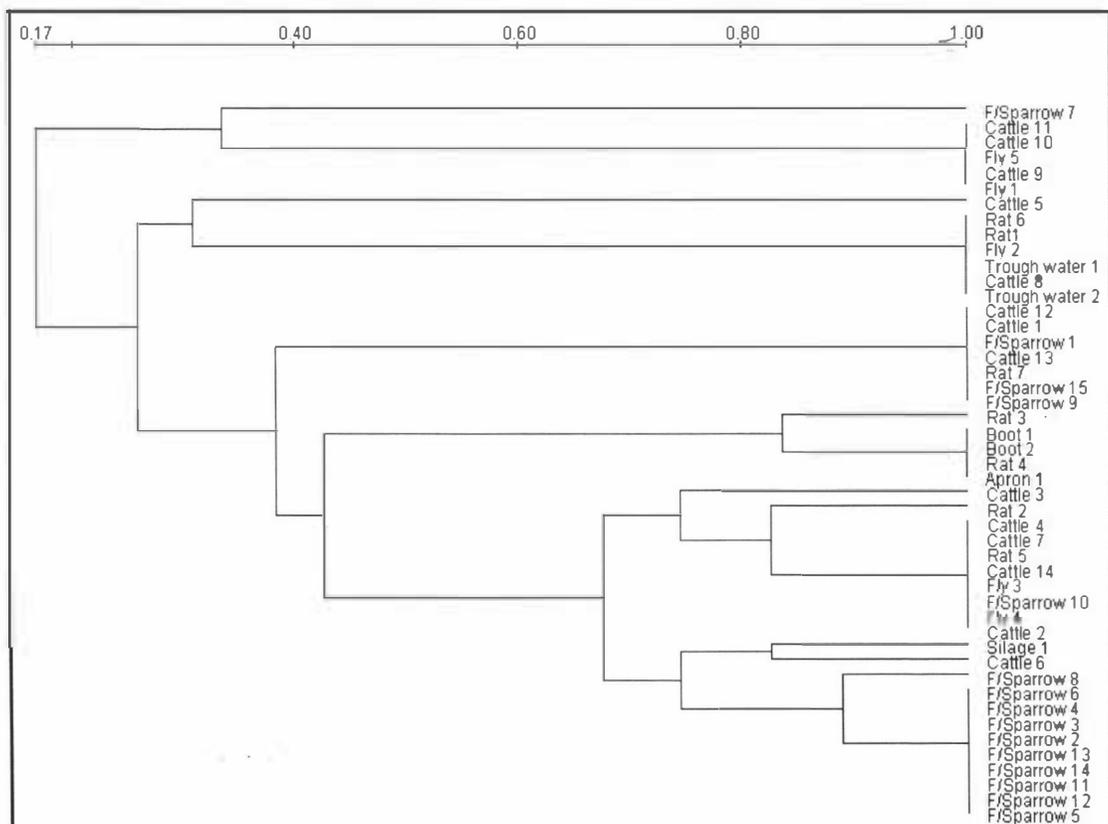


Figure 3.7. Dendrogram of similarity between PFGE patterns of 47 *C. jejuni* genomic DNA determined by the UPGMA cluster analysis diversity data base (Bio-Rad). A percentage scale of similarity is indicated at the top. F/sparrow- farm sparrow.

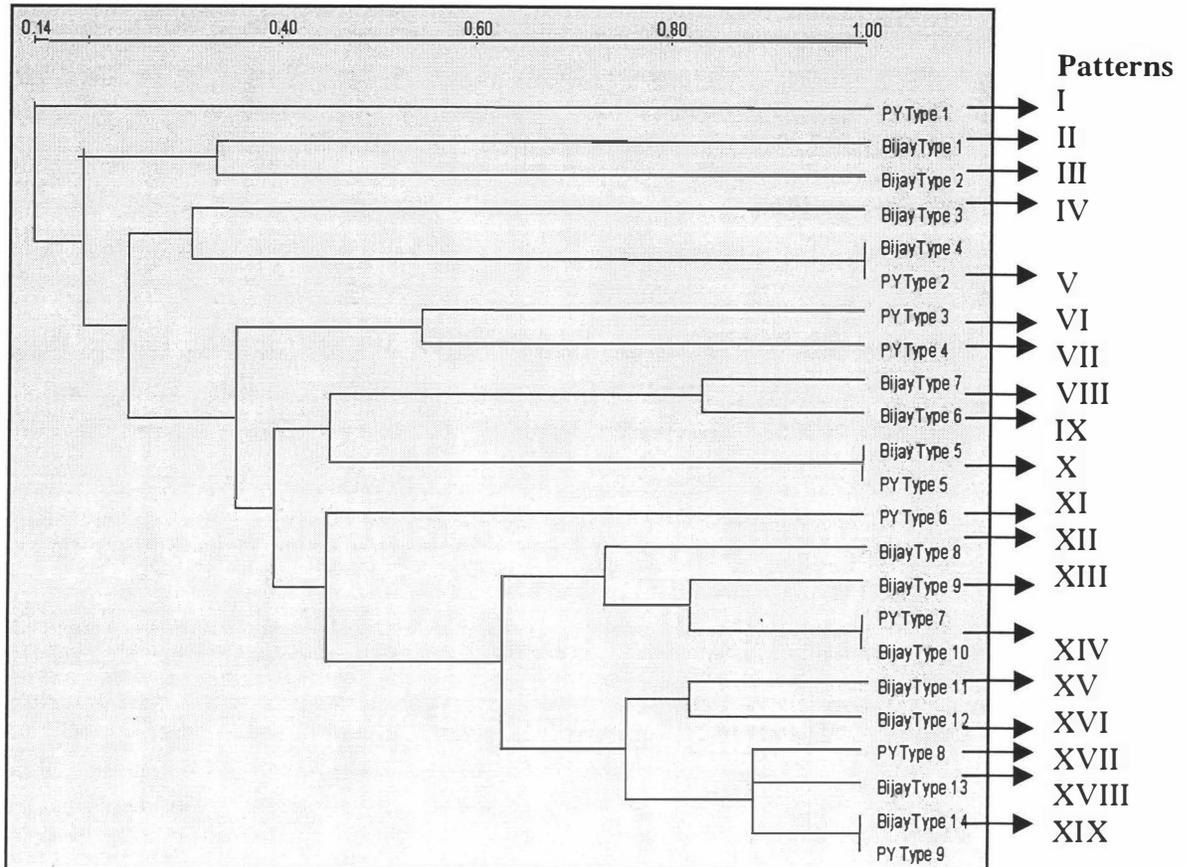


Figure 3.8 Dendrogram comparing PFGE restriction patterns of *C. jejuni* genomic DNA in the present study and an earlier study on the same farm (Wu, 2001). Present study indicated by Bijay type and study by Wu (2001) indicated by PY Type.

Table 3.6. Indistinguishable PFGE patterns of *C. jejuni* isolates in the present study and study by Wu (2001) on the same farm and their sources.

Type	Sources	Type	Sources
Bijay Type 4	Cattle, rodent, fly, Trough water	PY Type 2	Cattle
Bijay Type 5	Cattle, farm sparrow, rodent	PY Type 5	Pond water
Bijay Type 10	Cattle, farm sparrow, rodent, fly	PY Type 7	Cattle
Bijay Type 14	Farm sparrow	PY Type 9	Cattle

CHAPTER FOUR: DISCUSSION / CONCLUSION

DISCUSSION

Campylobacter jejuni is recognised as one of the most common causes of enteritis and acute diarrhoea in humans worldwide. Despite its importance as a human pathogen, relatively little is understood of the epidemiology of campylobacteriosis and the mechanisms of *C. jejuni*-associated disease in people and animals. The disease is usually seen with a mild attack of diarrhoea lasting 24 hours and in case of severe illness may last five to seven days before recovery. A chronic complication of the disease includes the Guillain-Barré syndrome (Ang *et al.*, 2001), the Miller-Fisher syndrome and Reiter's syndrome (Rees *et al.*, 1995).

The aim of the present study was to investigate the potential role of free-living animals such as sparrows, rodents and flies as reservoirs of *Campylobacter* spp. on a dairy farm. The study also examined the genetic diversity of campylobacters isolated from various sources on the farm. Other aims include a comparison of the prevalence and genetic diversity of *Campylobacter* isolated from sparrow populations on the farm, and from an urban environment, using of PFGE typing

In the present study only *Campylobacter jejuni* was isolated. The results found the prevalence of 54% of *Campylobacter jejuni* in dairy cows at No. 4 dairy farm. This is higher than the 12% reported by Wu (2001) in milking cows at the same time of the year on the same farm but comparable with the 52% total prevalence of campylobacters reported by her in subsequent sampling. She isolated a wider range of species of *Campylobacter* (*C. jejuni*, *C. coli*, *C. hyointestinalis* and *C. upsaliensis*) and reported a 26% and a 7% prevalence of *C. jejuni* in dry cows and milking cows in the subsequent sampling during winter and spring season respectively. Overall the results show that the dairy cows at No.4 dairy unit are commonly asymptomatic carriers of *Campylobacter jejuni* for at least 24 months (the period between the two studies). Cows may carry *Campylobacter* in intestine continuously or acquired infection intermittently from contamination of the farm environment.

Results of this study show that there was a continuous presence of *Campylobacter jejuni* in some milking cows at Massey No. 4 dairy farm. Cows 101, 125, 198 and 356 were still positive for *C. jejuni* in the 2002, after also being positive in 2000 study.

The study also determined the prevalence of *C. jejuni* in the farm sparrows (38%) and urban sparrows (40%). A similar observation was made in a Portuguese study, which found a prevalence of 45% *Campylobacter* in sparrows (Rodrigues, Sousa and Penha-Goncalves, 1998), which is slightly higher than the 38% found in the present study. Craven *et al.* (2000) isolated *C. jejuni* from wild birds with carrier rates ranging from 4% to 50%. Most of the samples collected consisted of wild bird droppings found on or near different domestic chicken housing during different seasons of the year. In our study, the 38% prevalence of *Campylobacter* in farm sparrows may not be a surprising result since the dairy cows, calves and heifers had been reported to be asymptomatic carriers of campylobacters (Wu, 2001). The reason for the similar prevalence of *Campylobacter jejuni* in urban and farm sparrows is unclear, but could be related to contacts with other wild birds and duck populations inhabiting nearby ponds or via other environmental contamination.

Little is known about the role of sparrows in the transmission of *C. jejuni* to livestock in New Zealand but overseas studies have suggested that wild birds may be an important source of *C. jejuni* (Cabrita *et al.*, 1992). Since sparrows commonly roost and feed around cowsheds and nearby pasture, chances of environmental contamination by faecal droppings must be high. Contamination of silage and stored concentrate and minerals fed to livestock are also potential links in the transmission of *Campylobacter* from wild animal reservoirs to livestock.

The knowledge of the epidemiology of *Campylobacter* on farms is likely to be complex and transmission routes are unclear. Figure 4.1 shows some possible routes of *Campylobacter* transmission.

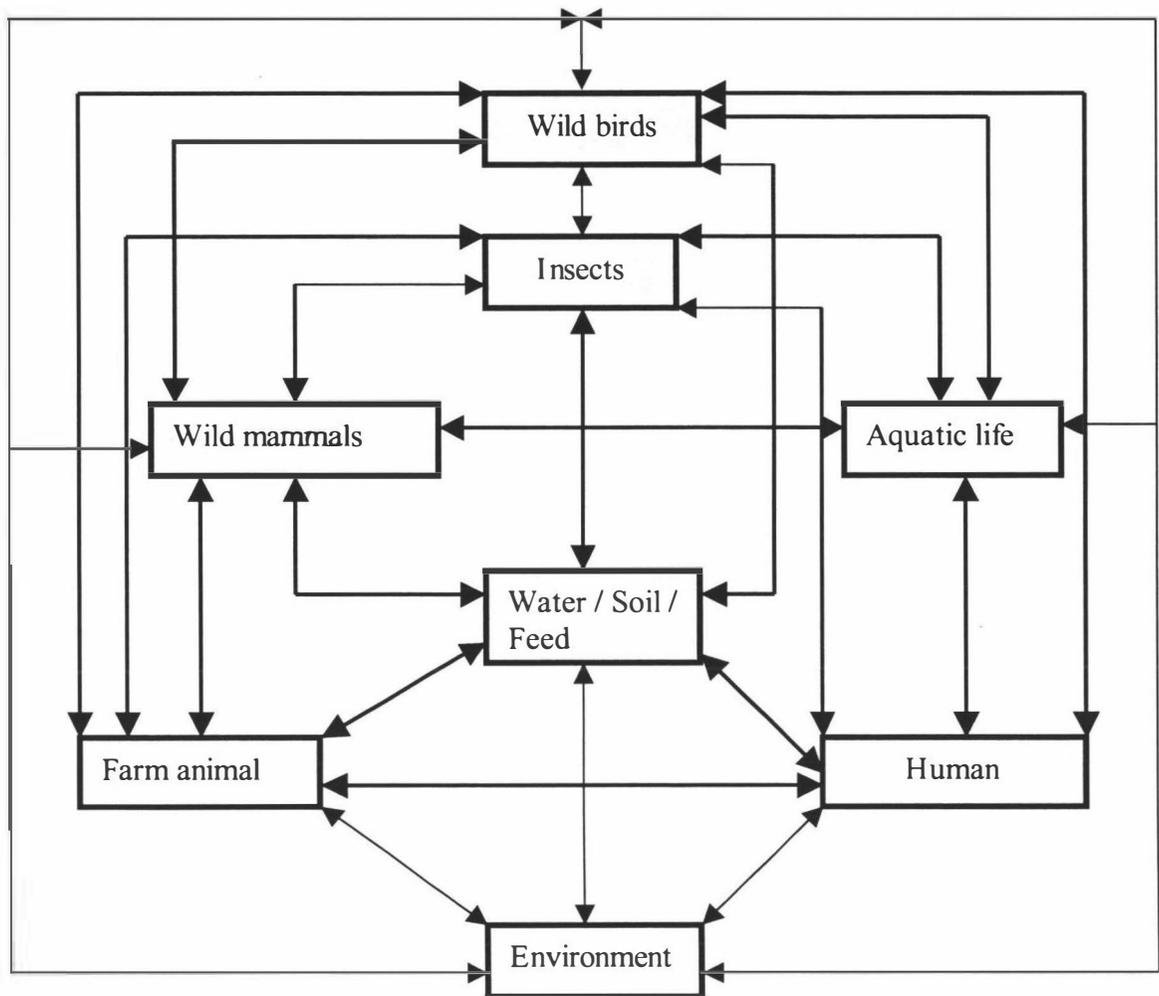


Figure 4.1. Potential transmission routes of *Campylobacter*

The prevalence of *C. jejuni* in rodents and flies tested in this study was found to be 11% and 9% respectively. Apparently, this is the first reported of isolation of *C. jejuni* from these animals on a dairy farm New Zealand. Studies in Portugal and Norway found the prevalence of *Campylobacter* in rats to be 57% (Cabrita *et al.*, 1992) and in flies upto 51% (Rosef and Kapperud, 1983) respectively. The difference in prevalence between countries could be due to variations in geography, ecology, season, age, crowding of animals, culture conditions and media use (Stern, 1981).

Environmental samples from the dairy farm, including grass silage, worker's boots and aprons, and water from drinking troughs in paddocks were also positive for *C. jejuni*.

Although the water supplied to the troughs in the paddocks is from the Massey University chlorinated, reticulated supply system, wild birds have access to the troughs and could easily contaminate the water. Chlorine breaks down when exposed to UV radiation in sunlight and chlorine gas readily leaves the water, reducing the bactericidal effect (Anon_j, 2002).

At the time of silage feeding and when the silage cover is removed, a considerable number of wild birds, particularly sparrows were seen roosting on the top layer of the silage. Silage samples taken from the outer layer were found to be positive for *C. jejuni*, most likely contamination by sparrows or other wild birds.

Effluent management is an important environmental issue for the dairy industry. In the context of successful application of deferred effluent irrigation on Massey No. 4 dairy unit, an interim report stated that no *Campylobacter* were detected in samples taken from the anaerobic pond. Eventually *Campylobacter* was detected in drainage water after deferred effluent irrigation. This warrants further detailed investigation of *Campylobacter* in the farm ecology including soil and pasture (Hedley and Horne, 2001).

The management of animal manure has become a problem for agriculture in New Zealand. With the increase in organic production of fruit and vegetables there is a potential for more manure application onto these crops, and therefore increased risk for the transfer of pathogens to humans. A study in the USA reported *Campylobacter* in fresh fruits and vegetables, which were believed to have originated from animal manures (Anon_k, 1996).

In this study, the only *Campylobacter* spp isolated from dairy cows, sparrows, rodents and flies was *C. jejuni*. This may be a surprising result because the other species of *Campylobacter* (*C. lari*, *C. hyointestinalis* and *C. upsaliensis*) have been previously isolated on this farm (Wu, 2001). The reason for this remains obscure, but could be associated with seasonal variance, microbial competition, strain dominance in wildlife

reservoirs or perhaps variations in the microbiological methods applied. In the study by Wu (2001), the methods of isolation of *Campylobacter* included the use of Preston broth but in the present study Bolton's broth was used. Minor differences in isolation technique would appear highly unlikely to account for differences in the species of *Campylobacter* isolated in the two studies.

PFGE analysis of isolates of *C. jejuni* from the farm environment yielded a total of 14 restriction patterns, of which five were common to more than one source, whereas a study by Wu (2001) reported nine restriction patterns, of which three were common to more than one sources. These 14 restriction patterns in the present study were obtained from dairy cows, sparrows, rodents, flies and other environmental samples (silage, water worker's apron and boots) whereas in the former study the nine restriction patterns were obtained from cattle and pond water samples. The PFGE profiles of isolates from two dairy cows in restriction pattern IV and X (Table 3.7 and Fig. 3.8) in the present study were indistinguishable from the PFGE profiles of two dairy cows reported in the previous study on the same farm. This suggests that some of the strains of *C. jejuni* are persisting in the farm environment for at least of two years. The subtype diversity index of *C. jejuni* for dairy cows in this study and earlier study (Wu, 2001) was 0.46. Similar subtype diversity index in both studies would suggest that similar clonal diversity of *C. jejuni* exist in the farm environment over time.

Most of the isolates (63%) from farm sparrows shared in one restriction pattern (pattern V), which was at least 60% similarity with *C. jejuni* isolates from urban sparrow 7 and 70% similarity with urban sparrow 10. Otherwise the urban isolates of *C. jejuni* showed different PFGE subtypes, which indicate clonal diversity within a limited geographical area.

In general, the observations in this study found considerable variability in the prevalence and PFGE restriction patterns of *Campylobacter jejuni* on the dairy farm. The occurrence of *C. jejuni* in the faeces of milking cows, rodents, flies and sparrows

from both urban and farm sources recognises the likely importance of these species in the epidemiology of campylobacteriosis in New Zealand.

Conclusions:

Campylobacter jejuni is recognized as one of the most common causes of foodborne enteritis in humans worldwide and is a major public health and economic burden. Despite its importance as a human pathogen, relatively little is understood of the mechanisms of *C. jejuni*-associated disease in animals and people.

This study suggests that dairy cows, rodents, sparrows and flies could be potential reservoirs of *Campylobacter* on the dairy farm. The high prevalence of *C. jejuni* found in cows would be sufficient to maintain infections within the dairy farm ecology via environmental contamination. The high level of asymptomatic carriage of *C. jejuni* by dairy cows is a potential source of contamination of the human food chain. Some of the dairy cows sampled were carrying *C. jejuni* in both studies suggest further longitudinal study on the farm to understand the presence of *Campylobacter* in this animal over time.

PFGE analysis of *C. jejuni* shows a high degree of diversity of the organisms within a limited geographical area. Common restriction patterns have provided evidence of some identical clones infecting cattle, sparrows, flies and rodents probably originating from a common source of infection. However, the number of campylobacters shed by a cattle defaecating approximately 25 kg of fresh faeces per day (Matsuzaki, 1975) should greatly exceed the environmental bacteria compared to faecal shed of bacteria by sparrows or rodents. This could be due to the concentration of organisms and volume of faecal matter could affect its significance as a source of environmental contamination.

The incidence of *Campylobacter* as a leading cause of enteritis in people worldwide has increased significantly over the past 20 years. The current trend in this country could result in *Campylobacter jejuni* posing a potential threat to the New Zealand's meat and

milk industry, because of its asymptomatic presence in food animals. Although this is a global problem, pasteurisation and proper cooking readily kill the organisms.

Campylobacter jejuni has been isolated from poultry with high prevalence rate worldwide. However, poultry are probably not the only important source of human campylobacteriosis as it is well documented that carriers of zoonotic campylobacters are common among many other animal species, e.g. sheep, pigs, cattle and free-living birds and mammals (Stanley *et al.*, 1998). Consumption of poorly cooked meat, consumption of contaminated water, overseas travel and animal contact with domestic animals and pets are known risk factors (Anon_h, 2002).

Campylobacters could enter the human food chain as a result of certain agricultural practices and products, and at slaughter and processing of animals. Raw milk and poorly treated water supplies are also important sources of *Campylobacter* infections.

Finally, it is suggested that the high level of asymptomatic carriage of *Campylobacter* by dairy cows is a potential source of contamination of the human food chain. Dairy cows, sparrows, rodents and flies can be considered potential reservoirs of *Campylobacter* on the dairy farm. To ascertain the most likely routes of transmission, further studies on the epidemiology of *Campylobacter* in the farm ecology are needed. Also to achieve the significance of the various pathways for *Campylobacter* transmission needs further investigations. In future studies, the use of multiple *Campylobacter* culture media including different pre-enrichment broths would provide better understanding.

APPENDIX I

Bolton Broth (Code: CM0983)

Preparation of Bolton selective enrichment broth

Ingredients:	gm/litre
Meat pepton	10.0
Lactalbumin hydrolysate	5.0
Yeast extract	5.0
Sodium choride	5.0
Alpha – ketoglutaric acid	1.0
Sodium pyruvate	0.5
Sodium metabisulphite	0.5
Sodium carbonate	0.6
Haemin	0.01
PH 7.4 ± 0.2	

Bolton broth selective suppliment

Vial contents (Each vial is sufficient to suppliment 500ml of medium)

Cefoperazone	10.0mg
Vancomycin	10.0mg
Trimethoprim	10.0mg
Natamycin	12.5mg

Procedures:

Dissolve 13.8g of Bolton broth (CM938) in 500ml of distilled water. Sterilise by autoclaving at 121⁰C for 15 minutes. Cool to 50⁰C. Aseptically add 25 ml laked Horse Blood and one vial of Bolton selective suppliment, reconstituted as directed below. Mix well and distribute into sterile screw top containers. To reconstitute Bolton broth selective suppliment, add aseptically 5ml 50:50 ethanol/water to one vial of suppliment. Mix gently to dissolve.

APPENDIX II

Preparation of *Campylobacter* blood-free selective agar (mCCDA)

Ingredients:	g/litre
Pepton blend	25
Bacteriological chrcoal	4
Sodium chloride	3
Sodium desoxycholate	1
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar No. 2	12

Procedure:

Weigh 45.5 grams of powder, disperse in 1 litre of deionised water and allow soaking for 10 minutes. Swirl to mix, then add 2 vials of K112 supplement, mix well and pour into Petri dishes. Continuously mix whilst pouring to prevent the charcoal setting.

APPENDIX III

Gram stain

Procedure:

Mark an area on the underside of the slide with a black wax pencil. Within the marked area, place a colony culture directly onto the slide. Allow the film to dry in the air, then fix the film by passing through a burner flame three to four times. Cover the slide with 0.5% crystal or methyl violet. Leave for 30 seconds. Wash with tap water and drain off excess water. Cover with Gram's iodine. Leave for 30 seconds. Using a pair of forceps hold the slide at a steep slope, wash off the iodine under a running tap. Decolorized by pouring acetone alcohol over the slide from the upper end and allow to run down over the slide. Immediately wash under the running tap. Decolorisation is very rapid and should take no longer than 2-3 seconds. Counterstain with 0.5% Safranin for 1 minute. Wash off the stain and blot dry gently.

APPENDIX IV

Preparation of Blood Agar

Ingredients	g/L
Pancreatic digest of casein (Oxoid)	14.0 g
Agar (Gibco BRL)	12.5 g
NaCl	5.0 g
Bacto-peptone (Gibco BRL)	4.5 g
Yeast extract (Gibco BRL)	4.5 g

Procedure:

1 litre of distilled/ deionised water was added to the above ingredients, mixed thoroughly and pH adjusted to 7.3 ± 0.2 at 25°C before autoclaving. Basal media was cooled to $45\text{-}50^{\circ}\text{C}$ before the addition of 70 ml sterile, defibrinated sheep's blood and media poured into Petri dishes.

APPENDIX V

Preparation of Nitrate Broth (Reagent)

Ingredients	Composition
Solution A:	
Glacial acetic acid	30 ml
Distilled water	120 ml
Sulfanilic acid	0.5 gm
Solution B:	
Glacial acetic acid	30 ml
Distilled water	120 ml
1,6-Cleve's acid	0.5 gm

Procedure:

Add the water to the Cleve's acid and warm with frequent shaking until most of the compound is dissolved. Almost all the compound will dissolve in reasonably warm water. Do not boil. Till the solution cool, then add the acetic acid. Store reagents A, and B in refrigerator.

APPENDIX VI

Ninhydrin reagent

Ingredients	Composition
N-Ninhydrin	3.5 g
Acetone	50 ml
Butanol	50 ml

Procedure:

Mix up 50 ml acetone and 50 ml butanol. Add 3.5 g N-Ninhydrin powder and dissolve to this solution to become 3.5% solution of Ninhydrin.

APPENDIX VII

Preparation of agarose and buffers

A) PETT IV

For 100ml mix the following and autoclave

1 M NaCl	20ml of 5M NaCl
10mM Tris-HCl pH8.0	1ml of 1M Tris-HCl
10mM EDTA pH 8.0	2ml of 0.5M EDTA
	Make up to 100ml with MQ water

B) Preparation of molten agarose

Add 4ml of PETT IV buffer to 40mg of Low Melt agarose (Bio-Rad Laboratories), California, USA)

Heated in a boiling water bath until the agarose has dissolved. Allow agarose to cool down.

C) Preparation of Lysis buffer

0.5M EDTA	20ml of 0.5M per 20ml buffer
Sodium lauroyl sarcosine 1%	200mg per 20ml buffer
Proteinase K 0.1%	20mg per 20ml buffer

Buffer can be stored at -20°C until use

D) 10:1 TE Buffer

For 100ml mix the following and autoclave

10mM Tris-HCl pH 8.0

1 ml of 1 M Tris-HCl

1mM EDTA pH 8.0

200µl of 0.5M EDTA

Make up to 100ml with MQ water

E) Preparation of restriction buffer (*Sma*I NEB)

For each plug add the following and mix

12µl of restriction buffer (10X NE Buffer 4)

88µl sterile MQ water

F) Preparation of cutting buffer:

For each plug add the following and mix

10µl restriction buffer (10X NE Buffer 4)

30 Units *Sma*I (1.5µl of 20U/µl)

Sterile MQ water to 100µl

G) Preparation of 1% PFC Agarose :

Prepare 80ml agarose for standard mould and 140ml for wide/long mould.

e.g. for 80ml mould

0.8g agarose and 80ml 0.5X TBE buffer

Heat for around 3 minutes in microwave until agarose is dissolved. Place agarose at ~ 50°C to cool for ~ 10 minutes.

H) 0.5X TBE Buffer

For 2L mix the following

Add 200ml of 5X TBE to 1800ml of MQ water and mix

I) 1M Tris-HCl pH 8.0

Dissolve 12.1g of Tris base in 80ml MQ water, adjust the pH to 8.0 with **concentrated** HCl. Let the buffer equilibrate for at least 5 minutes and then re-check the pH. Make volume up to 100ml and autoclave.

J) 0.5M EDTA pH 8.0

Dissolve 18.6g of disodium EDTA in 70ml MQ water, adjust the pH to 8.0 with NaOH pellets. Let the buffer equilibrate for at least 5 minutes and re-check the pH. Make the volume up to 100ml and autoclave.

K) 5X TBE Buffer

For 2L mix the following and autoclave

0.45M Tris Base	108g of Tris
0.45M Boric acid	55g of Boric acid
10mM EDTA	40ml of 0.5M EDTA
	Make up to 2L with MQ water

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