A Longitudinal Study of *Campylobacter* Spp. on a New Zealand Dairy Farm

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ABSTRACT

Although *Campylobacter* is a common cause of gastroenteritis in humans in New Zealand, the source of infection usually remains unknown. However, the high frequency of human infection may be due to the relatively low infectious dose. *Campylobacter jejuni* and some other *Campylobacter* species are commonly found as commensals in livestock including cattle which may be reservoirs for a number of *Campylobacter* species.

The objective of this study was to estimate the prevalence of *Campylobacter* carriage in healthy dairy cows at the study farm. The combined epidemiological and microbiological investigation was useful in conducting a longitudinal study of Massey University No. 4 Dairy Farm in this project. The project surveyed cows of different ages in the herd at different times over the study period. In order to determine whether strains of *C. jejuni* isolated from the cows were identical, Pulse-Field Gel Electrophoresis was applied to examine the similarities among *C. jejuni* isolates.

Based on the results of an initial pilot study, selecting a suitable sample size of dairy cows for planned sampling events saved time and cost in estimating the *Campylobacter* prevalence. In this study, on a basis of the results of pilot study, a sample size of about 60 animals was selected in order to estimate 90 % confidence level within 10% accuracy. Finally, the results of prevalences of *Campylobacter* at different samplings were applied to calculate 95% confidence intervals for prevalences in different populations.

The survey of different age groups of the same herd at different times within the period 8/6/00 to 5/10/00, found significant differences in isolation rates. For example, the prevalences of both *C. jejuni* and other *Campylobacter* species during dry off period were higher than before calving and after calving. The prevalence of *Campylobacter* carriage by heifers had the highest ranges between 72.1% and 91.0% compared with other populations. Yearling group had relatively prevalence of *C. jejuni* infection but the

prevalence of other *Campyloabcter* species was 35%, but the reason is unclear. Moreover, *C. jejuni* strains 74 and 75 was isolated from the pond of this study farm display distinct restriction patterns and are different from the 15 strains from cattle. Although some identical strains occurred across the 1st and 2nd samplings, variation within and between sampling events was evident. In addition, wild birds may be important reservoirs of *C. jejuni* infection of cows. Thus, in this study *Campylobacter jejuni* has a very complex ecologic cycle involving water and animals. Another significant explanation is that some animals may be recovering or recovered carriers or *Campylobacter* may be present in only localized areas or shed intermittently in faeces. Table 10 shows the changes in *Campylobacter* status within different stages of sampling. However, from the present study, there is insufficient evidence to indicate which is a major contributory factor in the causation of infection with *Campylobacter*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

1.1 INTRODUCTION

Campylobacter spp. have probably caused human illness for centuries, but were not recognized as human pathogens until the 1970s (Owen and Gibson, 1994; Ketley, 1995; Ketley, 1997; Altekruse *et al.*, 1998). In 1886, Theodor Escherich made drawings depicting of spirally curved rods (Altekruse *et al.*, 1998). In 1913, *Campylobacter* were first isolated and were thought to be associated with sheep abortions (Lior, 1994). Since 1977 *Campylobacter* have been recognized as a leading cause of diarrhoea and an important zoonosis (Lior, 1994). *Campylobacter* infections were first made notifable in New Zealand in 1980 (Faoagali, 1984). In the United Kingdom and other developed countries, *Campylobacter* is the predominant cause of diarrhoea. *Campylobacters* also have been related to milkborne and waterborne outbreaks in several countries (Melby *et al.*, 1991; Lior, 1994).

Cattle, sheep, horses, goats, rodents and monkeys as well as domestic cats and dogs have shown to be colonised with *Campylobacter* (Skirrow, 1990; Moreno *et al.*, 1993; Hald and Madsen, 1997). *Campylobacter* has shown to be a cause of diarrhoea in calves, lambs, monkeys, dogs and cats (Prescott and Bruin-Mosch, 1981). Birds are reservoirs of *Campylobacter* species that cause human diarrhoea. Up to 100% of chickens, turkeys and wild birds have been known to harbour this organism (Blaser and Reller, 1981).

In most cases carriers do not exhibit symptoms but may have acquired immunity through an earlier *Campylobacter* infection (Park *et al.*, 1991). These organisms may survive in the environment for several weeks. Water can harbor *Campylobacter* and act as a source for reinfection (Hanninen *et al.*, 1998; Waage *et al.*, 1999). Eleven of 57 reported outbreaks of *Campylobacter* infection in the United States between 1978 and 1986 were waterborne (Tauxe, 1992). Raw milk was the first documented source of human

campylobacteriosis. Contamination with faeces is a major cause of milk contamination, but *C. jejuni* mastitis is also reported to be a source of contamination (Humphrey and Beckett, 1987; Altekruse *et al.*, 1994). Contact with infected pets can be a potential source of human infection (Saeed *et al.*, 1993). Thus, human infection may occur following consumption of contaminated, untreated surface water, unpasteurized milk and incompletely cooked meats or handling meat or companion animals (Altekruse *et al.*, 1994).

Campylobacter species are causative agents of human enterocolitis (Ketley, 1997). *C. jejuni* infection is responsible for a major public health and economic burden (Tauxe, 1992). It is estimated that over 2.5 million *Campylobacter* cases occur each year in the United States (Tauxe, 1992). Campylobacteriosis is also the most frequently reported gastrointestinal disease in New Zealand. The current incidence of 285.2 cases per 100,000 per annum is around four times that of giardiasis and 7.5 times that of salmonellosis. However, because *Campylobacter* spp. are ubiquitous in the environment, cases are sporadic for the most part, and outbreaks are rare, tracing of sources has proved to be difficult (Hudson *et al.*, 1999; Wassenaar *et al.*, 2000).

Agricultural research has been developed to a high degree in New Zealand. Grassland management has enabled support of high stock numbers. Rainfall and plentiful sunlight enable pasture grasses to grow well, and in the mild climate livestock are able to live outside throughout the year and feed almost entirely on grass. Rotational grazing is often managed in New Zealand (Anon. 1995a). However, these systems probably provide favorable conditions for *Campylobacter* transmission within farms.

1.2 MICROBIOLOGY

1.2.1 *CAMPYLOBACTER* MORPHOLOGY AND TAXONOMY

1.2.1.1 MORPHOLOGY

The term *Campylobacter* is derived from the Greek words for curved rod and was given to this microorganism because of its spiral or S-shaped cell morphology (Penner, 1988). *Campylobacter* are Gram-negative and in size between 0.5-8μm long and 0.2-0.5μm wide. The cells usually possess a polar flagellum at one or both ends (Penner, 1988; Bolton *et al.*, 1992). One species, *C. pylori*, has one to six sheathed flagella located at one end of the cell (Penner, 1988). It may be presumed that the flagellum and spiral cell morphology impart a high degree of motility to the cell (Ketley, 1995; Ketley, 1997). Some species can be differentiated by the characteristic wavelength of helical cells (Griffiths and Park, 1990). Round or coccoid forms occur in old cultures, in adverse environments, in some species, or are induced by certain inhibitory agents (Griffiths and Park, 1990; Bolton *et al.*, 1992; Ketley, 1995). Round forms may occur in long-phase cultures (Walker *et al.*, 1986).

Campylobacter spp. have a small genome of 1.6-1.7Mbp of AT-rich DNA; the GC content is approximately 30%, although the *C. upsaliensis* genome has a size of 2 Mbp. Extrachromosomal elements in the form of both conjugative plasmids and bacteriophages have been reported in *Campylobacter* spp. (Ketley, 1997).

1.2.1.2 TAXONOMY

In 1963, the term *Campylobacter* was introduced by Sebald and Veron to describe spiral or S-shaped members of the genus *Vibrio* consisting predominantly of "comma" shaped bacteria e.g. *Vibrio cholerae* (Lior, 1994) although *Vibrio* genus is separated now. Taxonomy is a dynamic science. The taxonomy of the genus *Campylobacter* has been revised extensively, particular over the last ten years. *Campylobacter* spp. represent a taxonomically heterogeneous group. This original genus contained just two species,

Campylobacter fetus and *C. bubulus* (Penner, 1988); at present, 15 *Campylobacter* species and six subspecies are recognized (Bolton *et al.*, 1992; On, 1996). Reevaluation of their phylogenetic relationships has resulted in the current classification scheme of *Campylobacter*, *Helicobacter*, and *Arcobacter* species (Al Rashid *et al.*, 2000). Current nucleotide-based classifications place the genus *Campylobacter* in rRNA superfamily VI. (Altekruse *et al.*, 1998). Most species seem pathogenic and associated with diseases in humans and animals (Bolton *et al.*, 1992). *Campylobacter jejuni* and related species have been known to be causative agents of human enterocolitis (Ketley, 1995). *C. fetus* was probably first recognized and isolated (Walker *et al.*, 1986; Griffiths and Park, 1990; Ketley, 1995).

1.2.1.3 ISOLATION AND IDENTIFICATION OF *CAMPYLOBACTER*

Isolation techniques involve pre-enrichment and enrichment steps. Pre-enrichment involves incubation of samples in a non-selective medium at 37-42 °C for 2 hours, to allow recovery of injured cells. This procedure can improve isolation rates of *Campylobacter* from contaminated river water and milk (Griffiths and Park, 1990). Many enrichment systems have been proposed but the most widely used medium is Preston broth consisting of Nutrition broth No.2 (OXOID) supplemented with ferrous sulfate-sodium metabisulfite-sodium pyruvate [F.B.P.] (OXOID), Preston selective supplement (OXOID), and 5% defibrinated horse blood (Goossens and Butzler 1992). *Campylobacter* are generally isolated from faecal samples, which are collected into sterile plastic containers and then into enrichment broth, incubated at 37-42 °C in a microaerobic atmosphere for 24-48 hours. Then swabs are inoculated onto *Campylobacter* selective medium incubated at 37-42 °C in a microaerobic atmosphere for 24-48 hours (Garcia *et al.*, 1985).

Most *Campylobacter* species are microaerophilic (Ketley, 1995; Griffiths and Park, 1990; Bolton *et al.*, 1992). Oxygen is usually necessary for the growth of *Campylobacter* but it is toxic at concentrations in the air. Generally, *Campylobacter* require a 3-5% O_2 concentration, but some species are aerotolerant. (Ketley, 1995; Griffiths and Park, 1990) Carbon dioxide is essential for growth and the growth of some species is improved in the

presence of hydrogen. *Campylobacter jejuni* is primarily microaerophilic and grows well in an environment that contains 10% CO_2 and 5-6% O_2 (Bolton *et al.*, 1992).

Campylobacter can grow at a wide range of temperatures (Penner, 1988). *C. jejuni* and *C. coli* are thermophilic , growing best at 42°C (Ketley, 1995; Harvey and Leach, 1998). This probably reflects adaptation to the temperatures, which are found in their normal habitats, the intestines of warm-blooded animals and birds (Ketley, 1995; Ketley, 1997). *C. laridis* and *C. upsaliensis* are also thermophilic (Penner, 1988; Bolton *et al.*, 1992). *C. hyointestinalis* may also be considered thermophilic, but it grows better at 37°C than at 42°C (Penner, 1988). *C. fetus* grows at 25°C and 37°C, while *C. cinaedi* and *C. fennelliae* both have an optimum growth temperature of 37°C and do not grow at 25°C or 42°C (Penner, 1988; Griffiths and Park, 1990; Ketley, 1995). *Campylobacter* selective agar is incubated in a microaerophilic atmosphere for 2-3 days at 37-42°C (Goossens and Butzler 1992). Morphology is an important characteristic that can aid the differentiation of some species (Bolton *et al.*, 1992). Typical colonies are opaque, shiny, and tend to spread along the growth line or to expand to the whole surface of the medium (Humphrey, 1995; Zanetti *et al.*, 1996) They can be confirmed as *Campylobacter* by their morphology in Gram-stained smears (Taylor, 1983).

Campylobacter are biochemically less active than most bacteria. However, tests for catalase, oxidase, sensitivity to 30μg of nalidixic acid and cephalothin disks, hippurate hydrolysis, H_2S production, indoxyl acetate hydrolysis, nitrate reduction and urease production are carried out for the routine identification of *Campylobacter* spp. (Bolton *et al.*, 1992; Penner, 1988; Garcia *et al.*, 1985). *Campylobacter* are oxidase positive and reduce nitrates. They have a respiratory type of metabolism and use amino acids and intermediates of the tricarboxylic acid cycle. Because they are unable to ferment or oxidize carbohydrates, identification is based on only a few tests. (Penner, 1988; Griffiths and Park, 1990; Bolton *et al.*, 1992).

There are still some difficulties in identification of *Campylobacter* species (On *et al.*, 1996). For example, *C. jejuni* may be distinguished from *C. coli* by its ability to ferment

hippurate (Taylor, 1983). It seems that hippurate hydrolysis is a useful test, but it cannot be relied upon to distinguish between all *C. coli* and *C. jejuni* strains, due to the existence of hippurate-negative *C. jejuni* strains. Even more sensitive hippurate tests have been developed but hippurate-negative *C. jejuni* strains are still encountered occasionally (Griffiths and Park, 1990). At present, it is confirmed that a few strains of *C. jejuni* were indeed hippurate negative (Penner, 1988). Harvey and Greenwood, in their examination of a few of these isolates, found that four of five hippurate-negative isolates serotyped by antisera against hippurate-negative serostrains were closely related in DNA hybridization tests to *C. coli* but one isolate was clearly related to *C. jejuni* (Harvey and Greenwood, 1983). Lin *et al.* observed that *C. coli* of hippurate-negative reaction and *C. jejuni* of hippurate-positive reaction occasionally both have some weak reactions in conventional hippurate test tubes (Lin *et al.*, 1986). Thus, hippurate test was insensitive in some cases because they did not prove or disprove the existence of hippurate-negative *C. jejuni* or hippurate-positive *C. coli*. However, rapid and reliable identification of different *Campylobacter* species is not always possible by conventional procedures that are based on selective plating, biochemical identification and serotyping. The DNA-based methods should be developed and improved for the detection and identification of *Campylobacter* species in food, in clinical and environmental samples although they also have several limitations (van Doorn *et al.*, 1999).

1.2.1.4 PCR DETECTION OF *CAMPYLOBACTER*

The Polymerase Chain Reaction (PCR) is an extensively used genetic approach for detecting infectious agents and a number of PCR assays for detecting *Campylobacter* cells have been developed during the past few years. These assays have been used to detect *Campylobacter* cells in poultry, faeces, dairy products, sewage and water (Waage *et al.*, 1999).

1.2.1.5 TYPING OF *CAMPYLOBACTER JEJUNI* BY PULSED-FIELD GEL ELETROPHORESIS

Different typing systems have been widely used in the characterization of *Campylobacter jejuni* isolates from sources such as human diarrhoeal stools, animal fecal samples, food samples, and water samples (Hanninen *et al.*, 1999). Typing systems are valuable in epidemiological studies providing information on relationships between isolates, sources of the organism and modes of transmission, and the extent of disease outbreaks (Arbeit, 1995). If putative disease outbreaks are corroborated by detailed epidemiologic investigation, then molecular typing studies can effectively serve to verify that the isolates represent an outbreak due to a single strain. In this scenario, typing methods are used to confirm a strong clinical and epidemiologic hypothesis. Moreover, a number of different animals have been identified as reservoirs, so subtyping is essential to compare these isolates with human isolates to evaluate the possible role of these animals as sources of human infection. It should be possible to use typing systems to differentiate between pathogenic and nonpathogenic *Campylobacter* strains in the future (Patton *et al.*, 1992).

Typing methods include serotyping, biotyping, phagetyping and molecular typing techniques, such as Pulsed-Field Gel Electrophoresis (PFGE), random amplified polymorphic DNA, ribotyping, restriction endonuclease patterns, plasmid profile analysis and whole cell protein profile (Patton *et al.*, 1991; Lior, 1994). Several molecular typing methods have been used to support studies of the epidemiology of *Campylobacter* infections during the 1990s. Of these, PFGE pattern analysis has been shown to be a highly discriminatory method (Rivoal *et al.*, 1999; Hanninen *et al.*, 2000).

PFGE has been shown to be of value in epidemiological investigations of a range of bacterial species (Gibson *et al.*, 1994) and could be an alternative and useful method for differentiating *C. jejuni* from *C. coli* in epidemiological investigations (Lior, 1994; Owen and Gibson, 1994). *C. jejuni* genomic DNA digested with *Sma*I always possessed at least one fragment in the range of 400-500 kb. On the contrary, *Sma*I did not produce restriction fragments of > 250 kb from *C. coli* DNA. Thus these distinct patterns can be used as a criteria for identification of *C. jejuni* and *C. coli* (Yan *et al.*, 1991).

PFGE involves the digestion of chromosomal DNA into large fragments using cutting restriction endonucleases that yield simple banding patterns suitable for epidemiologic comparisons (Yan *et al.*, 1991). A restriction endonuclease cuts DNA at a specific nucleotide recognition sequence. Both the recognition sequence of the enzyme and the composition of DNA influence the number and sizes of the generated restriction fragments. Thus, an enzyme whose recognition sequence is composed of only guanine (G) and cytosine (C) will cut DNA with a low G+C content less frequently and consequently will generate fewer and larger restriction fragments than an enzyme recognizing sequences of only adenine and thymine. An enzyme that recognizes a sequence of 6 bp (a 6-bp cutter) typically has more recognition sites than an 8-bp cutter, will digest DNA more frequently, and consequently will generate more and smaller restriction fragments (Arbeit, 1995). These enzymes have an average of fewer than ten recognition sites per megabase and cut the chromosomal DNA into a few large fragments (Owen and Gibson, 1994). Such fragements can be separated by size by using constant-field agarose gel electrophoresis and pattern can be detected by staining the gel with ethidium bromide and examining it under UV light (Arbeit, 1995). PFGE allows the size separation of DNAs ranging from 10,000 bp (10kb) to more than 1.5 million bp (1.5 Mb) (Smith and Cantor, 1987). The size of the *C. jejuni* genome would also be similar to those of *Lactococcus lactis* and *Streptococcus salivarius*, which have sizes of 1.75 to 2.5 Mb as determined by PFGE. But the genome size of *C. jejuni* is only about 40% of the size of the *E. coli* chromosome (Chang and Taylor, 1990). All bacterial isolates are theoretically typeable by PFGE, and results are highly reproducible. The relative simplicity of the restriction profiles greatly facilitates the analysis and comparison of multiple isolates (Arbeit, 1995).

Smith and Cantor (1987) made the following observations relevant to PFGE:

1. All DNA molecules move with the same average velocity in the presence of an electrical field in PFGE solution.
2. Fractionation of DNA molecules occurs because molecules moving in an electrical field are sieved through an agarose matrix. Agarose is used for DNA in the size range of 500 bp to 20 kb because it has large pores.

3. A small molecule will fit through all pores and can travel through the gel in a straight line. A large molecule will be unable to enter most pores and have to travel via a circuitous and much longer route. Thus its net translation velocity through the gel will be much smaller.
4. DNA molecules above 20 kb long usually can not be separated by gel electrophoresis because they are larger than the pore size of the matrix, however, DNA molecules are not rigid. They are spherical coils and can travel through a gel matrix by deforming their shape to pass through the pores. Thus in an electrical field in a gel, large DNA molecules appear to behave like highly extended coils and adapt their size and shape to match the gel pores.
5. In PFGE, the electrical field is applied alternately in three directions. This change in field direction forces DNA molecules to orient themselves in three directions alternately. The timing of the actual field change is effectively instantaneous relative to DNA motion. The time spent in each direction is called the pulse time.
6. For different length molecules, the optimal pulse time for fractionation will be different. If the pulse time is too long, the DNA molecule will effectively reorient itself rapidly to the new field and move by ordinary electrophoresis, thus no size fractionation will occur. If the pulse time is too short, the molecule will not have time to reorient at all. In general, large molecules will be optimally fractionated at longer pulse time. At long pulse times, small molecules can completely reorient and will not be fractionated. In contrast, at short pulse times large molecules will only see the average field.
7. PFGE with short pulse times (30sec) only resolves lower molecular weight chromosomes ranging in size from 250 to 600 kb, while longer pulse times (80sec) are required to separate the larger chromosomes. Thus pulse times used with PFGE can be tuned such that maximal separation of particular sized molecules is obtained.

Pulse-Field gels are usually run at higher voltage than standard gels and can generate considerable heat. Thus they often run in special buffers (Finney, 1988).

In general, analyzing the restriction digest patterns resolved by PFGE and differentiate strains is straightforward. Some points are briefly described as below:

1. Strains with identical PFGE patterns are considered to be clonal.

2. Strains with one or two band shifts consistent with a single genetic event (such as a point mutation resulting in the loss or gain of a restriction site, an insertion, a deletion, or a chromosomal inversion) are considered to be clonally related.
3. If there are a very limited number of band shifts which are not explained by a single genetic event, the strains are considered to be very closely related but distinct.
4. Strains that differ at three or more bands are considered to represent independent strains, although strains with multiple or distinctive similarities may have a common ancestry.
5. Epidemiologically related strains are, with few exceptions, clonally related.

(Anon. 1993; Gibson *et al.*, 1995)

1.2.1.6 *CAMPYLOBACTER JEJUNI*

C. jejuni is a slender, curved Gram-negative rod, 0.2-0.5μ wide and 0.5-5μ long and with a polar flagellum (Altekruse *et al.*, 1998). Antigenically similar 41-45 kilodalton (kd) surface-exposed proteins are the major outer membrane protein in all *C. jejuni* (Cover and Blaser, 1989). The organism may be identified by its colonial morphology on solid medium after 48-hour incubation in microaerophilic conditions (5-6 % O_2 and 10 % CO_2). The optimum temperature for growth of the thermophilic *C. jejuni* is 42°C) Colonies are greyish, non-haemolytic, flat, irregular and spreading (Skirrow and Benjamin, 1980; Humphrey, 1995; Ketley, 1997).

C. jejuni is found in the intestinal mucosa and contents or in the faeces of a number of species of animals (Taylor, 1983). *C. jejuni* can cause enteritis in dogs, cats and pigs, mastitis in cattle, and hepatitis in poultry (Adesiyun *et al.*, 1992). It is also an important cause of bovine abortion but uncommonly reported in aborted fetuses of dogs, goats, mink and humans (Van Donkersgoed *et al.*, 1990; Giacoboni, G.I. *et al.*, 1993). The other species such as *C. upsaliensis*, *C. hyointestinalis* and *C. laris* also infect humans. However, recent scientific research interest has been directed mainly towards *C. jejuni*. (Ketley, 1997).

1.3 EPIDEMIOLOGY

1.3.1 RESERVOIRS AND TRANSMISSION OF *CAMPYLOBACTER*

The natural habitat of most *Campylobacter* species is the intestines of birds and other warm-blooded animals (Jones and Telford, 1991; Ketley, 1997). Wild rodents and birds are important reservoirs of infection for domestic animals (Cabrita *et al.*, 1992; Altekruse *et al.*, 1994). In the United Kingdom, one third of seagulls excrete *C. jejuni* and could contribute to the contamination of reservoirs (Jones and Telford, 1991). *C. jejuni* and other *Campylobacter* species also can cause human gastroenteritis, but each species seems to have a favoured reservoir. For example, *C. jejuni* is the most common species and is important in poultry (Altekruse *et al.*, 1998); *C. coli* infection in piglets was recognized as the main *Campylobacter* species of pigs (Adesiyun *et al.*, 1992; Altekruse *et al.*, 1998). *C. fetus subsp. fetus* causes sporadic abortion in cattle and sheep (Giacoboni *et al.*, 1993). In addition, houseflies and beetles can carry *Campylobacter* on their exoskeletons and perhaps serve as vectors (Altekruse *et al.*, 1994; Altekruse *et al.*, 1998). Puppies and kittens have occasionally been vectors in infections but there is no evidence to suggest that they are important sources (Griffiths and Park, 1990; Altekruse *et al.*, 1994). Moreno *et al.* (1993) reported that some household pets are reservoirs of *Campylobacter* and a potential source of *Campylobacter* infections in humans and identical serotypes have been isolated from humans and their pets.

Campylobacter species can be resistant to atmospheric oxygen tensions and low nutrient environments such as river water. In adverse environments, it may change to round or coccoid cell forms in order to adapt its metabolism and prolong survival. Viable and nonculturable forms occur and may be responsible for *Campylobacter* infection (Harvey and Leach, 1998; Ketley, 1997; Leach *et al.*, 1997). *Campylobacter jejuni* can survive in faeces for extended periods and surface water may be a persistent source of *C. jejuni* due to faecal run-off (Altekruse *et al.*, 1994). *Campylobacter* cells can survive at ambient

temperature (Curtis *et al.*, 1995) and remain viable at 4°C for up to 3 weeks in faeces, 4 weeks in water, and 5 weeks in urine (Wesley *et al.*, 2000).

1.3.2 *CAMPYLOBACTER* IN HUMANS

Some *Campylobacter* spp. are important pathogens for humans and animals (Van Donkersgoed *et al.*, 1990; Blaser, 1997; Giacoboni, G.I. *et al.*, 1993). As few as 500 cells have been shown to cause diseases in humans (Curtis *et al.*, 1995; Wesley *et al.*, 2000). The incubation period is 1-3 days after infection and the first clinical signs are a rise in rectal temperature to 41°C and the passage of loose faeces in which there are strings of mucus containing blood. The course of the disease from the onset of clinical signs to complete clinical recovery may be 10-20 days (Taylor, 1983). It is clear that *Campylobacter enteritis* is a major public health problem in industrialised countries (Ketley, 1995; Ketley, 1997). Warm blooded animals and birds are thought to be the most likely reservoirs (Owen and Gibson, 1994). Transmission to humans occurs by ingestion of contaminated food of animal origin, contaminated water that has not been treated, and by direct contact with infected animals, especially pets carrying the microorganism in the intestinal tract (Saeed *et al.*, 1993; Blaser, 1997). *Campylobacter jejuni*, *C. coli* and *C. lari* are recognized as causes of human diarrhoea and their presence in food implies a potential health risk. Recent reports show that the less common species of *Campylobacter*, e.g. *C. fetus subsp. fetus*, *C. hyointestinalis*, *C. sputorum* and *C. upsaliensis* are increasingly being implicated in human disease (Fennel *et al.*, 1986; Goossens *et al.*, 1990; Lindblom *et al.*, 1995). However, the most important of these currently recognized *Campylobacter* in human disease is *Campylobacter jejuni* (Blaser, 1997). *C. jejuni* is estimated to be responsible for over 95% of human *Campylobacter* infection in New Zealand (McNicholas *et al.*, 1995). Hudson *et al.* (1999) described that poultry appeared to be a major source of *C. jejuni* infections in humans and that 49.7% of the human isolates typed were indistinguishable from poultry isolates.

On a global scale *Campylobacter* is identified as one of the most common causes of bacterial gastroenteritis in humans. About 4000,000 cases of human campylobacteriosis

are reported annually in the United States. The Centers for Disease Control and Prevention (CDC) estimates that true figure is 2.5 million cases of campylobacteriosis with over 200 deaths annually in the United States (Mandal *et al.*, 1984; Altekruse *et al.*, 1998). In Canada, the number of isolates reported increased from about 2,000 in 1983 to 12,815 laboratory reports in 1992 and since 1989 surpassed *Salmonella* as the most common pathogen of diarrhoeal disease (Lior, 1994). In Australia, there are around 10,000 cases of *Campylobacter* infection each year (Stafford, *et al.*, 1996). In the United Kingdom, *Campylobacter* has become the most common single cause of gastrointestinal infections with over 50000 reported in 1997 (CDSC, 1998). The incidence of this infection in Denmark has recently shown a marked increase (Anon., 1995b). Since 1985, *Campylobacter* has been more prevalent than *Salmonella* in Scotland (Stringer, 1994). The few published reports on enteritis from countries such as Malaysia and Singapore give a low isolation rate for *Campylobacter* but the true incidence may be 5-10 times greater than the industrialized countries. The incidence of *Campylobacter* enteritis in Mexico and Thailand is 40,000 per 100,000 for children (Saleha *et al.*, 1998).

Campylobacteriosis is the most frequently notified disease in New Zealand (Brieseman, 1990). Since 1993 campylobacteriosis in this country has consistently exceeded 200 cases per 100,000 population per year. This is high by world standards and represents a major increase over the rate of 13.7/100,000 in 1981 (Withington and Chambers, 1997). Nationally the incidence rose steadily to a peak of 2921 cases in 1987. Christchurch has maintained the highest number of notifications and the highest rate within New Zealand with a peak rate 261.9 cases per 100,000 population in 1987 (Brieseman, 1990). New Zealand's recent notification rates for campylobacteriosis have been twice those of England and three times those reported Australia and Canada (Eberhart-Phillips *et al.*, 1997). In New Zealand, the number of reported cases of campylobacteriosis has increased steadily to approximately 7-8000 cases each for past 1994-1998 (Brieseman, 1998). Eberhart-Phillips *et al.* (1997) mentioned that direct costs for reported cases of campylobacteriosis in New Zealand have been estimated at NZ $ 4.2 million per year.

Withington and Chambers (1997) mentioned that *Campylobacter* enteritis is generally a mild and self-limiting illness (Faoagali, 1984; Altekruse *et al.*, 1994); serious sequelae include reactive arthritis and Guillain-Barré syndrome (Owen and Gibson, 1994; Eberhart-Phillips *et al.*, 1997; Blaser, 1997; Hudson *et al.*, 1999). Campylobacteriosis is predominantly sporadic, but outbreaks have been described (Harrington *et al.*, 1999). Most outbreaks are associated with raw milk or surface water, whereas sporadic illnesses are often associated with mishandling and consumption of undercooked foods (Altekruse *et al.*, 1998). Routes of transmission in sporadic infections are thought to be predominantly foodborne, but are rarely established, since a wide range of zoonotic and environmental risk factors have been identified (Harrington *et al.*, 1999). Thus, the need for epidemiologic tools to determine the source of the infectious agent and its route of transmission to the infected patients should be emphasized (Yan *et al.*, 1991). Transmission from animals to humans may happen by contact with immature or diarrheic pets or livestock (Altekruse *et al.*, 1998). Serotyping, biotyping, and bacterial restriction endonuclease DNA analysis (BRENDA) have identified the main sources of human infection to be associated with the consumption of contaminated food and food products, water, raw milk, or contact with faeces of animals (Van Donkersgoed *et al.*, 1990).

Overt disease is usually restricted to children, and particularly, infants and young people have the highest infection rates (Eberhart-Phillips *et al.*; 1997 Ketley, 1997). In developed countries including New Zealand, 0-4 year age group is reported with the highest rate of infection (Brieseman, 1990; Stafford *et al.*, 1996). These differences are probably due to higher rates of exposure and infection early in life, resulting in a different pattern of immunity (Ketley, 1997). In New Zealand, the peak incidences occur in spring and summer and there is a high incidence amongst males (Brieseman, 1990). In New Zealand and U.K., it has been reported that the expected incidence of infection in rural communities would be higher than in residential areas (Brieseman, 1990; Stafford *et al.*, 1996). It is not surprising that *Campylobacter* infection in humans are a problem in New Zealand as there are nearly 20 domestic or farm animals for every human being (Brieseman, 1998).

1.3.3 *CAMPYLOBACTER* IN RUMINANT ANIMALS

Campylobacter are important pathogens in ruminant animals. *C. mucosalis* and *C. faecalis* are associated with intestinal adenomatosis and enteritis in calves, respectively (Giacoboni *et al.*, 1993). *C. fetus subsp. fetus* causes sporadic abortion in cattle and sheep (Lior, 1994). *C. fetus subsp. venerialis* causes bovine genital campylobacterosis and *C. jejuni* causes infertility and abortion in cattle and sheep (Giacoboni *et al.*, 1993). In particular, *C. jejuni* is a common cause of abortion in sheep and follows ingestion of the organism by ewes (Prescott and Bruin-Mosch, 1981); it has been reported to cause stillbirth, weak lambs and neonatal deaths in sheep (Prescott and Munroe, 1982). The prevalence of *C. jejuni* and *C. coli* in dairy cattle ranges from 5 to 53%, depending on methods of isolation, age of animal, season and number of samples analyzed (Wesley *et al.*, 2000).

Most sheep carry substantial populations of *Campylobacter*, mainly *C. jejuni* in their intestines (Stanley *et al.*, 1998c). Recently, Jones *et al.* (1999) pointed out that *C. jejuni* was the main species isolated and could survive for up to 4 days in sheep faeces; that lambs became colonized by *Campylobacter* within 1-5 days of being born; that ewes were not shedding *Campylobacter* prior to lambing but started to shed after lambing; and that ewes that were shedding low numbers of *Campylobacter* before lambing, increased the numbers of bacteria being shed after lambing. *Campylobacter* are also harbored in the intestines of healthy calves and cattle (Giacoboni *et al.*, 1993). Healthy cattle may be reservoirs for a number of *Campylobacter* species, including *C.jejuni* (Wesley *et al.*, 2000). In addition, rodents and wild birds spread *Campylobacter* strains in nature. Exposure to their excrements or consumption of contaminated water is believed to constitute the main routes for infection of domestic animals (Cabrita *et al.*, 1992). *C. jejuni* can occasionally cause mastitis in ruminant animals (Taylor, 1983; Humphrey, 1995), thus occasionally cows excrete the organisms into their milk (Skirrow, 1990).
In New Zealand, *C. fetus* is an important cause of abortion in sheep and every year some farms experience major outbreaks, sometimes affecting over one third of the animals in a flock. The reason for the variation in annual incidence and for the sporadic occurrence of

major outbreaks is not clear (Collins and Ross, 1984). Once an animal is infected it can excrete *Campylobacter* for several months, seasonally, or even for its whole life (Hanninen *et al.*, 1998). The organisms can be easily isolated from gall bladders and intestinal contents of cattle and sheep (Garcia *et al.*, 1985).

1.3.3.1 CATTLE FAECES

Campylobacter spp. seem to be present everywhere and are frequently found in the faeces of healthy wildlife and domestic animals (Cabrita *et al.*, 1992). They have been readily isolated from the faeces of domestic farm animals such as beef cattle and dairy cows, sheep, pigs and poultry (Lior, 1994; Stanley *et al.*, 1998a; Stanley *et al.*, 1998b).

Campylobacter are present on most farms (Altekruse *et al.*, 1998) and harboured in the intestines of healthy calves and cattle (Giacoboni *et al.*, 1993). Particularly *C. jejuni* is a common inhabitant of the intestinal tract of cattle (Altekruse *et al.*, 1998). The growth of *C. jejuni* is restricted by its microaerophilic nature and inability to grow outside a narrow temperature range, and therefore, the intestines of host animals is a critical site of amplification in the contamination cycle of this organism (Stanley *et al.*, 1998a).

Healthy cattle may be reservoirs for a number of *Campylobacter* species, including *C. jejuni* (Wesley *et al.*, 2000). In most cases the host is a carrier that does not exhibit symptoms, but it may have acquired immunity through an earlier *Campylobacter* infection (Waage, *et al.*, 1999). Robinson (1982) concluded that faecal shedding of *Campylobacter* within dairy herds is intermittent. Young animals are more likely to be infectious than older animals (Altekruse *et al.*, 1998; Altekruse *et al.*, 1999). Taylor (1983) mentioned that calves might be infected at birth from the faeces of dams by infected diarrhoeic animals of any age, or from clinically normal recovered carrier animals. Raw milk is also a common vehicle in outbreaks of *C. jejuni* in humans and furthermore milk can be contaminated with bovine feces (Griffiths and Park, 1990; Altekruse *et al.*, 1998).

1.3.3.2 EFFLUENT FROM DAIRY FARMS AND SLAUGHTER HOUSES

The significance of *Campylobacter* colonization of cattle relates not only to the potential for contamination of milk at the farm and the carcass at slaughter, but also for environmental and water contamination during disposal of abattoir effluents and slurries to land (Stanley *et al.*, 1998a).

Cattle, sheep, and pigs commonly carry *Campylobacter* in the gastrointestinal tract and their carcasses regularly become contaminated at slaughter when gut contents are spilled during the process of evisceration (Skirrow, 1990; Stanley *et al.*, 1998b).

In the United Kingdom, there have been no reports of contamination with human effluent, but there have been incidents of *Campylobacter* infection of water through contamination with faeces from domestic or farm animals (Jones and Telford, 1991). In Finland, Hanninen *et al* (1998) observed that more cows were found to be *Campylobacter* positive in summer or in autumn after the grazing period than after the winter, when the animals were inside and their drinking water source was municipal chlorinated tap water.

1.3.3.3 WATER

Outbreaks of *Campylobacter* infection have been traced to contaminated water sources in Europe, North America, New Zealand and elsewhere (Merritt *et al.*, 1999). In Europe, waterborne outbreaks have occurred when drinking water has become polluted by human sewage (Jones and Telford, 1991). *Campylobacter* have also been isolated from surface water, rivers, lakes and seawater (Hanninen *et al.*, 1998). Water plays an important role in the epidemiology of *C. jejuni*. Surface water may be a reservoir for *Campylobacter* (Altekruse *et al.*, 1998). *Campylobacter* may enter the environment in faeces from animals, wild birds and infected humans, or from run-off from pasture after rainfalls, or from sewage plants (Waage *et al.*, 1999; Hanninen *et al.*, 1998). *Campylobacter* can survive for about 15 days in water (Jones and Telford, 1991).

Campylobacter in surface water can undergo a morphologic transition to round or coccoid forms and become viable but nonculturable. Such changes may be important in transmission cycles (Altekruse *et al.*, 1998; Altekruse *et al.*, 1999). It appears to survive for less than 72 hours in meat, gut and faeces but may survive for quite longer periods in water (Taylor, 1983). Several studies have shown that *Campylobacter* can survive in surface water and their survival in water appears to be seasonally dependent and affected by sunlight and temperature. If in autumn the water temperature decreases to less than 10°C, which may aid the survival of *Campylobacter* (Hanninen *et al.*, 1998) Thus water temperature may be an important factor in the life cycle of *Campylobacter* (Altekruse *et al.*, 1999). In addition, domestic animals, wildlife, human and pets may carry *C. jejuni*, and *C. jejui* may also contaminate untreated surface water and soil (Blaser, 1997; Harvey and Leach, 1998).

1.3.4 SEASONAL PREVALENCE OF *CAMPYLOBACTER*

The disease in humans is strikingly seasonal in temperate climates. In the United Kingdom, the number of clinical infections peaks sharply in the early summer of May and June, and with a lower peak in the autumn. In Northern Europe the peak occurs in mid-late summer, but summer peaks occur in the United States of America and some other European countries (e.g. Italy and Spain). Spring and summer peaks have been reported in New Zealand, Australia and South Africa. It has been suggested that the seasonality of human infections may reflect important differences in the ecology of the poultry and bovine reservoirs of *Campylobacter* (Skirrow, 1990; Tauxe, 1992; Stanley *et al.*, 1998a).

It is also possible that the observed seasonal trends may be affected by other factors such as increased travel during summer holidays or increased consumption of poultry during the summer months (Stafford *et al.*, 1996). There is a clear seasonal change in the incidence of *Campylobacter* infection in New Zealand. Most reported cases are sporadic and tend to occur in the spring and summer in New Zealand (Eberhart-Phillips *et al.*,

1997). The incidence falls during winter but at this time there is still a considerable infection rate of about one third of the peak incidence (Ikram, *et al.*, 1994).

In England, the prevalence of *Campylobacter* infection in dairy cattle has been reported to be high in the summer and low in the winter (Meanger and Marshall, 1989). In New Zealand, Brieseman (1990) reported that the seasonal incidence of *Campylobacter* in animals peaks in August and September with smaller peaks in February and March. In other words, there is a difference in the seasonal pattern between human and dairy cows (Brieseman, 1990; Meanger and Marshall, 1989).

Wesley *et al.* (2000) also mentioned that the incidence of *C. jejuni* in cattle may be seasonal, with peak shedding occurring in either the winter or the summer. A bimodal trend with fecal shedding occurring in spring and autumn has also been observed. Human campylobacteriosis outbreaks associated with consumption of contaminated milk or water occur in the fall and spring. This seasonal trend may reflect peaks in either fecal shedding in the bovine reservoir or exposure to a common source of contamination.

1.4 OVERVIEW OF THE DAIRY INDUSTRY IN NEW ZEALAND

1.4.1 GENERAL DESCRIPTION

Friesian cattle were first imported to New Zealand from Holland in 1884. The number of herds increased until 1930 and since then has decreased. The total number of cows increased until 1970 and has remained relatively constant afterwards. The number of cows per herd also increased rapidly until 1970, and since then has continued to increase gradually (Holmes *et al.*, 1987). Many changes have taken place on dairy farms in New Zealand over the last decades. From 1981 to 1997, the average herd size increased by 90 cows, milkfat production per cow increased by 24 kg, and there was a shift in breed from Jersey to Holstein-Friesian. Overall the stocking rate increased by 0.5 cows per hectare (Xu and Burton, 2000).

There are two dairy management systems within the industry in New Zealand: seasonal supply dairying and town supply dairying. The former is characterised by cows calving in the springtime and not producing milk in winter. The latter is characterised by cows calving in spring and in autumn, or throughout the year and the farmer supplying a specified quota of milk daily throughout the year. Most cows calve in the springtime and most milk is manufactured into dairy products. Grazed pasture is the main diet of all cows and generally stocks are not housed at any time of year (Holmes *et al.*, 1987).

The New Zealand dairy industry is relatively small when compared with some other countries and is characterised by its co-operative structure of the dairy companies. The farmers who supply milk to the companies are also shareholders of the companies. The New Zealand Dairy Board is responsible for all export marketing of dairy products and constituted as the industry's organization body, to make sure that good quality milk is available for human consumption. The Milk Board includes representatives from the producers, processors, vendors and consumers of milk, and from the government; it also

has a representative from the New Zealand Dairy Board. The 'share-milking' system is a vital component of the New Zealand dairy industry. It allows young farmers with limited amounts of capital for the purchase of land to progress within the industry. The farmer's family provides most of the labour on the farm (Holmes *et al.*, 1987).

Mechanical milking is used in New Zealand and some automated sheds can milk more than 350 cows in an hour. Milk is then cooled and pumped to vats from which road tankers collect it once or twice daily. About 330,000 tonnes of milk fat result annually, around 13% being consumed as milk or fed to stock (including beef calves and pigs). The rest is used for the dairy products, casein and caseinates of which New Zealand is the world's largest exporter. These include milk powders (skim milk, whole milk, and buttermilk powder), cream products (butter, anhydrous milk fat and ghee), and cheeses, mainly cheddar but with a growing number of French-style speciality cheeses. All these products together provide 20% of New Zealand's export income (Anon., 1995a).

Research and development produces new products, targets them to specific markets, and modifies existing products to meet similarly specialised markets. The New Zealand Dairy Research Institute is one of the world's most advanced organisations in this field, and based near Palmerston North. It is financed primarily through the Dairy Board and secondarily by the government's Foundation for Research, Science and Technology.

1.4.2 SEASONAL DAIRY MANAGEMENT IN NEW ZEALAND

1.4.2.1 MANAGEMENT OF FEEDS

Milk production on the seasonal supply dairy farms starts in spring, but planning and preparations should have begun in autumn, at the end of previous lactation.

Seasonal supply dairy herds start to calve in early spring. The herd's calving date can influence the cows' level of feeding in early lactation and the length of lactation. These factors can influence the herd's milk production. Some pasture can be saved for hay and silage during winter and stored in the paddocks for consumption in spring. Thus the herd can be removed from the main farm for a period during the winter, so as to fatten the

cows and to allow the pasture on the main farm to recover before spring. For drying-off cows, a herd's feed requirements can be reduced suddenly and significantly. The feeding of supplements such as hay or silage or crops during late lactation may be adopted in order to save pasture and to reach the required target average pasture cover in spring (Holmes *et al.*, 1987; McGrath, 1999). Supplementary feed will increase cows' body condition, increase current daily milk yields and result in pasture being spared (McGrath, 1999).

Gareth Evans (Farm Supervisor in the study farm, personal communication) pointed out that in New Zealand, the seasonal dairy management usually depends on the balance of the expenses between feed demand and feed supply. From mid October to December, when the feed surplus (feed supply is over feed demand) usually occurs, silage or hay is fed to dairy cows; from December to March or July, when the feed shortage (feed demand is over feed supply) happens, feed supplement, such as turnip crop or grass silage is fed to the animals. Thus farmers may adopt two routines to solve this problem. One is that they often will reduce the number of cows, restrict the feed intake, and send dry cows off farm in order reduce feed demand. Thus dry cows are grazed off for the winter; replaced animals or young animals are grazed off anytime in the year. However, when cows are sent off the farm to graze, some diseases such as bovine tuberculosis can be transmitted among the farms. Another approach is to increase feed supply. The common supplements used are: maize silage for winter and spring; grass silage for winter and summer; turnip for summer; hay (low energy, low quality, low protein) given to dry cows in winter; and urea (about 46% nitrogen) available all year.

1.4.2.2 WATER REQUIREMENTS

Generally lactating cows must have access to clean drinking water at all times of year. They drink very little in cool, wet weather in winter or spring, but it is preferable to provide a source of water in every paddock. If they have access to unlimited water twice daily at the milking shed, lactating cows grazing on pasture can drink until satisfied. In addition, lactating cows for which diets are being supplemented with hay or other dry feeds may need unrestricted access to water at all times. Dry cows on winter and spring

pasture do not need access to drinking water, even though they will drink if it is available. But if their diet contains more than 0.3 bale of hay per cow daily, or they are grazing on the dry summer pasture, then they will need drinking water (Holmes *et al.*, 1987).

1.4.2.3 STOCKING DENSITY

Dairy farming is mainly a North Island industry with 90% of the dairy cattle in New Zealand in the North Island. Over 14,000 dairy herds, totalling 3.5 million cattle, are farmed in New Zealand. Herds are large, averaging 140 milking cows since on the best land 3.5 cows per hectare can be carried, and mechanical milking was pioneered in New Zealand to cope with this. About sheep farming, the best-known New Zealand statistic is the ratio of sheep to people: 20 to one at the height of the season. There are about 24,000 farms in New Zealand with stock, mainly sheep, occupying over 11 million hectares that together support 60 million sheep, in flocks averaging about 1800. Depending on the quality of the land, one to 25 sheep can be run per hectare. Beef cattle numbers have been slowly increasing in recent years and there are now about 5 million beef cattle in New Zealand. Two-thirds of beef exported is high-specification manufacturing quality aimed at North American food processors. Other animals such as goats and deer have been developed as commercial animals over the past few years. Pigs are farmed for domestic consumption. (Anon., 1995a)

1.5 SUMMARY

Both domestic and wild animals are known to be asymptomatic carriers of thermophilic *Campylobacter* spp. Due to the asymptomatic carriage of *Campylobacter jejuni* by cattle, the discharge of treated or untreated dairy farm effluent into rivers and streams can pose a hazard to humans and livestock. The economic significance of infection in animals is uncertain. Its importance to veterinarians lies in the possibility that the diarrhoeic syndrome may be confused with salmonellosis or coccidiosis or contribute to other diarrhoeic syndromes in cattle of any age. *Campylobacter* is also a public health hazard in humans as its transmission can be via contaminated food or water. The rate of human campylobacteriosis is relatively high in New Zealand as compared with other countries and is a major problem in this country. However, a recent national study of sporadic cases did not find drinking water to be a major risk factor (Bohmer, 1997).

CHAPTER II

LONGITUDINAL STUDY OF *CAMPYLOBACTER* IN CATTLE

2.1 INTRODUCTION

2.1.1 PROJECT BACKGROUND

2.1.1.1 HISTORY OF THE STUDY FARM

The property of W.J. Brogden (111.3 ha) and the property of L.L. Lovelock (50.6 ha) were purchased by Massey University in April 1973 and July 1973 respectively. These two properties were combined and developed into a large seasonal supply dairy farm. The neighboring property of G.W. Perry (55.24ha) was also purchased. The current No. 4 Dairy farm is developed from them.

It is planned and managed as a profitable, large scale, commercial seasonal supply dairy farm. It also can provide a teaching resource for undergraduate and postgraduate programs and a link between the University and Agribusiness.

Its area is about 184ha effective and its altitude is 80m above sea level. The average daily maximum temperature is 18.5°C in January and the average daily minimum temperature is 7°C in July. The average annual rainfall is about 1000mm. The soil predominately consist of Tokomaru Silt Loam with poor natural drainage. The soils have moderate natural fertility and tend to dry out in summer. The farm facilities contain a 36 bail turn-style rotary cowshed and concrete feeding pad (300 cow capacity). The farm is subdivided into approximately 80 paddocks of about 2.3 hectare each, all with race access. The breeds of cattle are restricted to Friesian and Friesian crossbreeds.

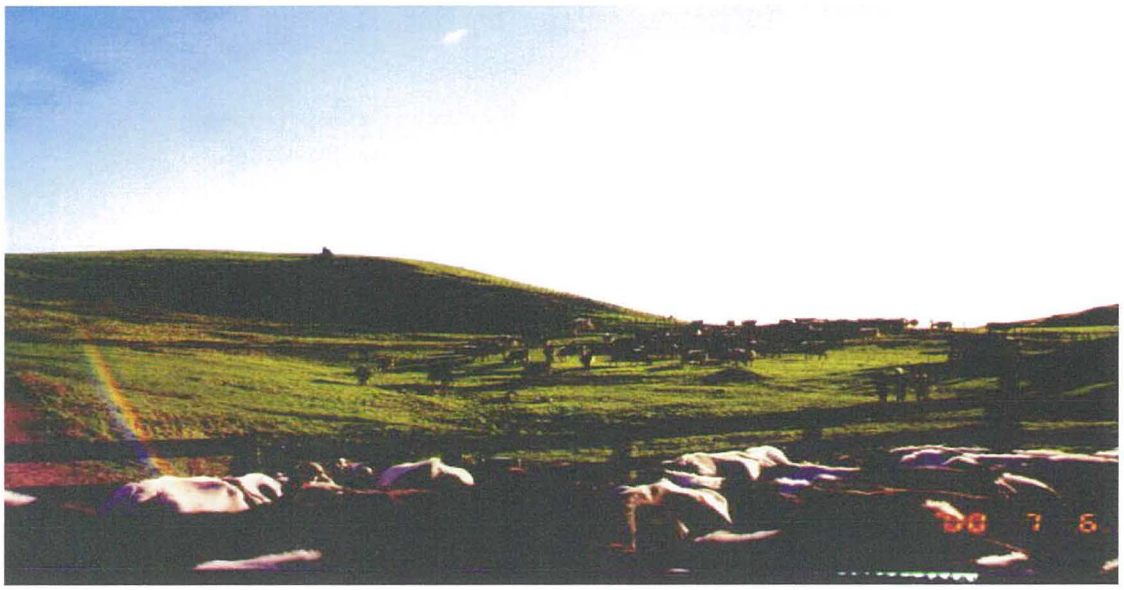

Figure 1. Massey No.4 Dairy Farm has characteristics typical of a temperate climate and pasture-grazed animal husbandry.

2.1.1.2 GENERAL MANAGEMENT

This section is based largely on information provided by David Lawton, lecturer in Animal Health information at Massey University and Gareth Evans, farm supervisor at the study farm:

Massey University Number 4 Dairy Farm is a 500-cow spring-calving seasonal supply dairy herd. Heifers and some calves are grazed off farm. Some cows are also grazed off as required during the dry period. At present it is anticipated that about 120 cows will be retained on the farm all winter. The farm has 184 hectare of pasture that is used for grazing of the milking herd and for wintering stock (Figure 1). Farm income is generated by the sale of milk, surplus calves and cull cows.

In NZ about 95% of dairy herds operate a seasonal system whereby calving is concentrated in either the spring or autumn. This production system has developed as pasture forms the main component of the lactating and dry cow diet for most herds i.e. by

2.1.1.2.1 CALVING MANAGEMENT

In the year 2000 calving season, the mean and median calving dates were August 16 and August 11 respectively. All cows were calved by October 11, 2000. Approximately 500 animals (390 cows and 120 heifers) are to calve. Before they calve, cows are managed in several mobs, some of which are grazed off farm, although all cows return during July for calving. During the calving period, individual cows are drafted from the dry mob into a "close-up" mob on the basis of udder development and due calving date. On average most cows spend between 10 and 14 days in the "close-up" mob. Mobs of cows are given a fresh break of grass (part of paddock) daily to meet their feed requirements. Typically, a portion of the daily feed requirement is provided as supplement (hay and/or silage). Daily movement of cows enables management to control the allocation of feed while providing fresh pasture and a clean calving environment.

Within 24 hours after calving, both the dam and calf are drafted from the "close-up" group. The cow is placed in a fresh cow mob for four days before she enters the milking herd. The fresh cow mob includes those cows that have calved in the last four days. During this period, the cows' milk is considered to be colostrum and is withheld from sale to the dairy factory. Milk collected over this time is saved and fed to calves. All cows are milked twice a day following calving.

2.1.1.2.2 CALF REARING

Within 24 hours of birth calves are transferred to the calf rearing facility. This is located on the farm and includes two sheds, each divided into several pens. Calves with similar birth dates are grouped together in pens of between 10 and 20 animals. Initially, calves are taught to drink from a nipple feeding system and are fed 2L of fresh colostrum twice daily. Most calves have adapted to the new feeding system within 24-48 hours. At this time, in addition to milk, calves are provided with fresh water, barley straw and a high protein and energy meal. The amount of straw and meal provided to each group of calves is increased daily to match that groups demand, so as to provide unrestricted access while maintaining freshness. The amount of milk offered per calf is increased progressively to

3L twice a day over the first week. At the same time the composition of the milk fed is progressively changed from colostrum to whole milk (untreated).

Calves that are to be reared on the farm are moved from the calf rearing shed onto pasture in groups at between two and three weeks of age. They continue to be fed milk until they are weaned at about seven weeks of age. Meal and straw continue to be offered until the calves lose interest in these. For most calves this occurs at between 9 and 12 weeks of age, and is influenced by the quantity and quality of pasture offered. Once they have been fully weaned onto pasture, calves are grazed off farm until they return at about 22 months of age.

Only those heifer calves that are likely to provide suitable replacement stock are kept on the farm. These are the heifers born from artificial mating to sires from the Livestock Improvement Corporation, Hamilton, New Zealand. All male calves and heifer calves from natural mating are usually sold within the first four days of life to be reared for beef or to be slaughtered as bobby calves. In most years about 120 female calves are reared.

2.1.1.2.3 HEALTH MANAGEMENT IN YOUNG STOCK

All calves are vaccinated against infection with *Clostridia* and *Leptospira* with 7 in 1 UF (CSL (NZ) Ltd) before they leave the farm. Further booster vaccinations are given four weeks later, at about 12 and 20 months of age.

Anthelmintics are used to control internal nematode parasites, most notably *Ostertagia ostertagi*, *Cooperia oncophora* and *Dictyocaulus vivipara*. Drenching frequency depends on the season, although this is typically monthly from 4-10 months of age. Products used to treat calves born in 1999 included oxfenbendazole (Oxfen C, Ancare NZ Ltd) and moxidectin (Cydectin, Fort Dodge Animal Health). All rising two-year olds are treated as considered necessary. Those that entered the milking herd in the year 2000 were treated in December 1999 and in June, 2000 with abamectin (Genesis Pour On, Ancare NZ Ltd).

To protect stock against sporidesmin toxic damage following ingestion of spores of the fungus *Pithomyces chartarum*, young stock are treated with zinc oxide. Usually this involves administration of a capsule of zinc oxide in autumn (Time Capsule, Agri-feeds Ltd).

2.1.1.2.4 GRAZING MANAGEMENT OF MILKING HERD

Four days after calving, cows enter the milking herd and are milked twice daily in a 36 bale rotary cowshed. Cows are milked twice daily at approximately 6 a.m. and 4 p.m. Between milkings, cows graze a portion of the farm. The area of the farm offered to the herd between each milking varies on a daily basis throughout the season, and is dependent on several factors e.g. number of cows in the herd, stage of lactation, amount of additional supplement fed, pasture mass and quality, pasture growth rate. Management aims to maximise the amount and quality of pasture eaten while optimising pasture growth. To achieve this, the amount and nature of supplement fed daily, the area of the farm offered to the herd and the time between subsequent grazings of the same paddock are regularly adjusted.

Pasture growth rate is the major factor influencing the interval between consecutive grazings of the same paddock. This interval is known as the rotation length. Typically it is longest during the winter (e.g. about 80 days) and shortest during the spring (e.g. about 20 days) when growth rates are at their highest.

When grazing by the herd does not remove an adequate amount of pasture from the farm, a portion of the farm is taken out of the rotation and the pasture grown on that area either ensiled or made into hay. When grass growth fails to meet the demands of the cows the rotation is slowed (less area per cow per day is offered) and the feed deficit met through the use of supplements. The amount of supplement fed varies between 0 and 8 kg per cow per day. The main supplement used on the farm in early lactation is maize silage. In summer, a 50:50 mixture of maize and grass silage is commonly used. Turnips are often grown as an additional form of supplement over the summer.

The farm has a concrete feed pad with feeding bins. For periods throughout the year, particularly when the paddocks are too wet to get the silage trailer over them, the supplement offered is fed out on the feed pad.

2.1.1.2.5 MILKING HERD MANAGEMENT

When the herd size exceeds about 350 cows, the herd is split into two smaller herds, with up to 250 cows in each. Each herd is grazed and milked in succession but independently. During the milking season, the milk produced by each cow and its composition is determined using the Livestock Improvement Corporation's herd testing scheme on four occasions. The information that is derived from these tests is used to assist with management decisions.

The availability of feed is an important determinant of lactation length. Poorer producing cows or those that produce milk of a poor quality (high somatic cell count) are selectively dried off to reduce the demand for feed. This enables high producing cows to be preferentially fed. Furthermore, cows that have been dried-off can be grazed off farm, further reducing feed demand. The herd is thus progressively dried off between February and June, resulting in considerable variation in lactation length. The mean (±SD) lactation length for the 1999-2000 milking season was 271±63 (±SD) days. The number of cows that are retained on farm over winter is determined by the availability of feed.

Twenty-three percent (110-115) of the adult-cow herd is replaced by pregnant rising 2-year olds each year. Most of these animals (45-50%) replace mixed aged cows that have failed to conceive during the mating period (October 19 to December 31). Other important reasons for removal include low production, mastitis, lameness, milk quality and illness or death. These animals are replaced by the remaining heifers.

2.1.1.2.6 HEALTH MANAGEMENT IN MILKING HERD

1. Minerals

To minimise the incidence of milk fever and grass staggers, the herd is supplemented with magnesium from three weeks prior to calving to three months after calving. This is offered as either magnesium oxide that is dusted onto pasture immediately before grazing or magnesium sulphate that is dispensed into the water supply or top dressed onto supplement (e.g. silage). The form that is used at any particular time will depend on the use of supplements, weather and other factors.

In order to compensate for the low calcium content of maize silage, when this supplement is fed to lactating cows, lime flour is added to the supplement at the rate of 40g per cow per day.

The herd's copper and selenium status is monitored and additional copper or selenium given by injection as required. The herd was treated with selenium (Deposel, Novartis NZ Ltd) during the 1999-2000 season.

Zinc sulphate is included in the water supply throughout the autumn to provide some protection against facial eczema.

2. Intramammary antibiotic treatments

At the time that cows are dried off, individual animals are selected for dry cow therapy using a number of criteria, the most important of which include their mastitis history and somatic cell counts throughout the lactation. At the end of the 1999-2000 milking season 248 cows were selected for treatment with Dryclox DC (500mg cloxacillin and 250mg ampicillin, Bomac laboratories Ltd). During that lactation, 138 cows with clinical signs consistent with mastitis were treated with one of several lactating-cow intramammary products.

3. Parenteral antibiotic treatments and vaccinations

During the year from July 1999 to July 2000, 192 antibiotic treatments were given for a variety of reasons. In many instances treatment followed examination of the animal by a veterinarian. All cows were vaccinated against leptospirosis in June (Leptosheild vaccine, CSL (NZ) Ltd).

2.1.1.2.7 WATER SUPPLY AND EFFLUENT DISPOSAL

The water that used on the study farm is obtained from the Palmerston North Municipal Water Distribution Centre. This water has been treated so as to be fit for use by the urban population. Using an on farm pumping system, water is distributed to drinking troughs in each paddock. Each paddock has two water troughs. There are also a number of small streams or creeks on the farm (see Figure 2) that flow through several of the paddocks. These provide an alternative source of water when cows are in those paddocks, as does surface water after heavy rain. At pre-calving, water sources are predominantly untreated. Calves have unlimited access to water from Massey University water source.

Effluent from the dairy shed is washed away after each milking to a series of settling and oxidation ponds. Water flowing out of the last pond enters the stream that flows close to it (see Figure 2). This effluent disposal system has been inspected and given approval by the local authorities.

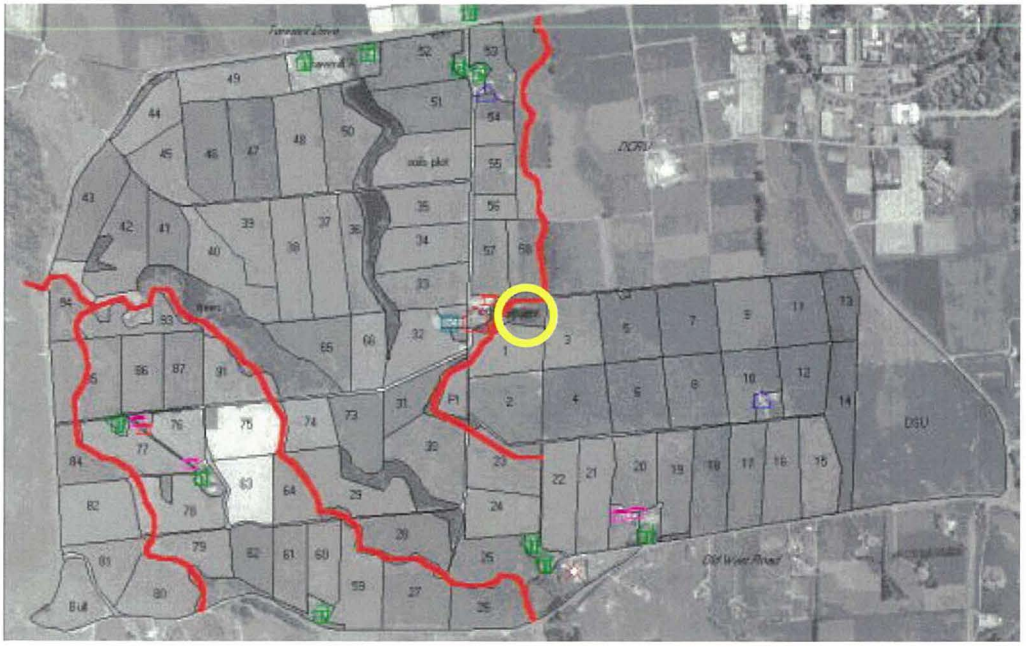

Figure 2. Aerial photograph of Massey University No. 4 Dairy Farm. Numbered sections correspond to individual paddocks. The red lines indicate the small streams that flow over the farm. The yellow circle indicates the location of the effluent ponds.

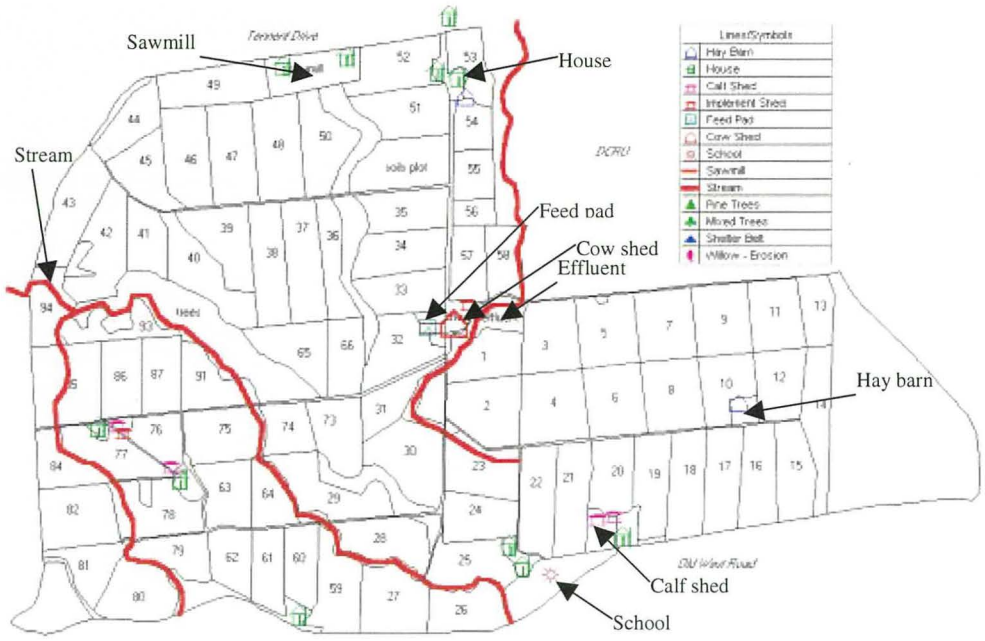

Figure3: Outline of the Massey No.4 Dairy Farm. Showing the location of feed pad, cow (milking) shed and other features mentioned in the text.

2.1.2 OBJECTIVES OF THIS STUDY

Human campylobacteriosis is an important problem in New Zealand, and multiple sources of human infection have been identified. Healthy cattle may be reservoirs for a number of *Campylobacter* species, including *C. jejuni*, but there is little informaion available about the epidemiology of *Campylobacter* infection in cattle in New Zealand. In particular there is a lack of information about the dynamics of *Camplyobacter* infection in dairy cattle populations with respect to overall prevalence of faecal shedding of the organisms, and the predominant species and sub-types present. When designing studies to address these questions across an industry, it is usually necessary to make some assumptions about the epidemiology within herds (e.g. expected prevalence). The objective of this study is to provide preliminary data about the prevalence and distribution of faecal shedding of *Campylobacter,* and particularly *C. jejuni*, in different age-groups of cattle and in adult milking cows over a period of time involving major management and phyiological events (late lactation, through drying off and subsequent calving and onset of lactation). The main purpose is to document the variabity in prevalence, and species and sub-types isolated, in order to provide a framework for effective sampling in future epidemiological and ecological studies.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Between May and October 2000, faecal specimens were collected from milking cows, heifers, dry cows, yearlings and calves at Massey No. 4 Dairy Farm. Except for a few animals with loose faeces, all animals were apparently healthy and showed no signs of enteritis or other illness.

Media, solutions and antibiotics used in this study were prepared as described in appendix I.

2.2.2 METHODS

2.2.2.1 EPIDEMIOLOGICAL INVESTIGATIONS

1. Initially, faecal samples were collected from 20 cows by convenience sampling from the milking herd, in order to confirm the presence of *C. jejuni* or *Campylobacter* spp. in the herd. It will be hoped within the 95% confident interval. This pilot sampling is to detect any presence of *Campylobacter*-positive animal in a sample from a population.

2. Subsequently, stratified sampling by age-group of animals was conducted. The sampling protocol involved:

a. Stratification of the entire herd into different age groups (calves, yearlings, heifers, and cows).

b. Convenience sampling of animals within groups, this method of sampling was selected because it is easy to carry out, although it may produce biased results.

c. Repeated sampling of selected milking cows at the end of lactation in the dry period, from June to August and again early in the following lactation.

d. Sampling of animals was conducted on nine occasions and included milking cows, heifers, yearlings and calves. Sampling of calves was done on three occasions, 20

samples being taken each time, for a total of 60 calves sampled. The timeline for each sampling size and time is indicated in Figure 4.

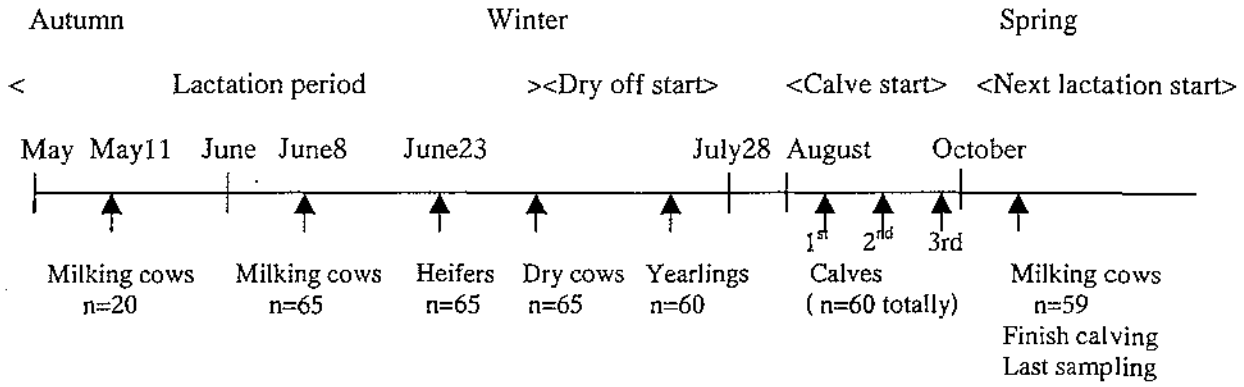

Figure 4. Timeline for nine samplings of animals at this study farm

3. Table 1 presents the results of the pilot study. A sample of twenty milking cows were tested. Thirteen were *Campylobacter* spp. positive, including nine positive *Campylobacter jejuni*. This result suggests a prevalence of 65% *Campylobacter* spp. positive milking cows and a prevalence of 45% *Campylobacter jejuni* positive.

From these estimates and with the aim to estimate prevalence with 95% or 90% confidence and a level of 10% accuracy expected prevalence is 50% (it implies that 50 % of cows may carry *Campylobacter*). Thus, with estimated prevalence of p = 0.45, 0.5, 0.65 we can determine the required sample sizes.

The required sample sizes can be calculated from the formula $n = z^2 * p\ (1 - p)/ L^2$
n: sample size, z: this values to be used in the formula are 1.65 and 1.96 for 90% and 95% confidence levels respectively, p: prevalence, (1 - p): probability of failure to detect positives, L: required precision or accuracy, it means how accurate estimate is supposed to be expressed in units of parameter of interest. (Martin *et al.*, 1987; Pfeiffer, 1999)

For example, if the expected prevalence is 50%, the required sample size will be
1) For 95% confidence level to estimate prevalence within 10% accuracy.
Sample size = $n = (1.96)^2 * 0.5 * 0.5 / (0.1)^2 = 96$

2) For 90% confidence level to estimate prevalence within 10% accuracy.

Sample size = n = $(1.65)^2 * 0.5 * 0.5 / (0.1)^2 = 68$

Considering the results from the pilot study, the required sample size will be

1) For 45% *Campylobacter jejuni* positive cows

n = $(1.96)^2 * 0.45 * 0.55 / (0.1)^2 = 95$ (For 95% confidence level within 10% accuracy).

n = $(1.65)^2 * 0.45 * 0.55 / (0.1)^2 = 67$ (For 90% confidence level within 10% accuracy).

2) For 65% *Campylobacter* spp. (including *C. jejuni*) positive cows

n = $(1.96)^2 * 0.65 * 0.35 / (0.1)^2 = 87$ (For 95% confidence level within 10% accuracy).

n = $(1.65)^2 * 0.65 * 0.35 / (0.1)^2 = 62$ (For 90% confidence level within 10% accuracy).

Required sample sizes can be obtained conveniently using specialised epidemiological computer software such as EpiInfo or Win-EpiScope. Win-EpiScope is used in this calculation of the selection of sample size. The procedures in Win-EpiScope are as follows: open "Samples", choose "Estimate Percentage", then enter "population size", enter "expected prevalence" (%), enter "accepted error" (%), enter "level of confidence" (%). The details as follow:

All the input of data are "population size" (400), "accepted error" (%) (10, if the precision or accuracy is 0.1), and

1) If "level of confidence" (%) is entered (95), the results will be:

a. For "expected prevalence" (%) is entered 65:

Sampling fraction (%) =17.93, sample size n = 87.40, adjusted sample size n (a) = 71.73, use value of n (a) = 72

b. For "expected prevalence" (%) is entered 50:

Sampling fraction (%) =19.36, sample size n = 96.04, adjusted sample size n (a) = 77.45, use value of n (a) = 78

c. For "expected prevalence" (%) is entered 45:

Sampling fraction (%) =19.21, sample size n = 95.08, adjusted sample size n (a) = 76.82, use value of n (a) = 77

2) If "level of confidence" (%) is entered (90), the results will be:

a. For "expected prevalence" (%) is entered 65

Sampling fraction (%) =13.34, sample size n = 61.55, adjusted sample size n (a) = 53.34, use value of n (a) = 54

b. For "expected prevalence" (%) is entered 50

Sampling fraction (%) =14.46, sample size n = 67.63, adjusted sample size n (a) = 57.85, use value of n (a) = 58

c. For "expected prevalence" (%) is entered 45

Sampling fraction (%) =14.34, sample size n = 66.96, adjusted sample size n (a) = 57.36, use value of n (a) = 58

A sample size of about 60 animals per sampling event was selected in order to estimate the prevalence of *Campylobacter* at the 90 % confidence level within 10% accuracy and expected prevalence 50%. The reason for this difference between formula result and computer result is that formula assumes infinite herd size and computer adjusts for herd size.

2.2.2.2 BACTERIOLOGICAL METHODS

2.2.2.2.1 SAMPLE COLLECTION

The project was undertaken at the Massey University section of Veterinary Microbiology and Public Health, with samples collected from Massey No.4 Dairy Farm. Faecal samples were collected from milking cows, heifers, yearlings and calves. For the initial of pilot study, 20 faecal samples were collected from milking cows on May 25. Subsequently, 65 faecal samples were collected from milking cows at the stage of "before drying off" on June 21. On June 23, 65 faecal samples were collected from heifers. On July 14, 65 faecal samples were collected again from the same group of milking cows during dry period. Faecal samples from 60 yearling heifers were collected on July 21. 20 faecal samples from calves less than 7 days were collected at each of three occasions in August in order to attain a total of 60 faecal samples. On October 5, 59 faecal samples were collected from the same group of milking cows at new lactation stage after calving. About 3g

faecal samples were collected in each plastic bottle with a gloved hand (one glove per cow).

2.2.2.2.2 CULTURE OF SAMPLES

From each faecal sample, 1g of faeces was inoculated into 9ml Preston enrichment broth within an hour and a half of collection and incubated in a microaerophilic environment at 42°C for 48 hours. An aliquot of each broth was plated on *Campylobacter* selective agar (MCCDA) and incubated at 42°C for 48 hours or further 72 hours. The plates were checked for spreading, grey, mucoid colonies characteristic of *Campylobacter* spp. (see Figure 6). Suspect colonies were examined and if the bacteria were found to be Gram negative curved rods they were also plated on sheep blood agar and incubated microaerophilically at 42°C for 48 hours. Subsequently, they were subcultured onto sheep blood agar at 42°C under microaerophilic conditions for a further 48 hours. Stocks of strains were preserved at -70°C in storage medium containing 10% glycerol (Newell, 1982). Positive control cultures were screened at each sampling.

2.2.2.2.3 IDENTIFICATION OF *CAMPYLOBACTER* SPP.

All presumptive *Campylobacter* isolates were identified using standard microbiological procedures (On, 1996). The flow diagram of examination procedures for *Campylobacter* spp. (including *C. jejuni*) is shown in Figure 5 (Hocking et al., 1997). These pure cultures were then identified by motility, catalase activity, oxidase, nitrate reduction, hippurate hydrolysis tests and sensitivity to Nalidixic acid and Cephalothin. *Campylobacter* spp. are all motile and oxidase positive. *C. jejuni* hydrolyses hippurate, and is sensitive to nalidixic acid and resistant to cephalothin. *C. coli* does not hydrolyse hippurate, is sensitive to nalidixic acid and resistant to cephalothin. *C. lari* does not hydrolyse hippurate, and is resistant to both nalidixic acid and cephalothin. A positive control and negative control were used at every stage using known strains of *C. jejuni* and *C. coli* for the hippurate hydrolysis test. Isolates confirmed as *Campylobacter* spp. were then spread onto blood agar plates, incubated for 48 hours and frozen in glycerol broth at -70°C for future characterisation.

1. Gram stain: The procedures are described in detail in appendix II. Gram negative is pink; Gram positive is purple; *Campylobacter* form S or gull-winged shapes (Figure 7).

2. Oxidase test: Single drop of oxidase reagent onto filter paper, wipe across colony and leave for ten. The development of a purple color indicates a positive oxidase reaction.

3. Catalase test: A drop of 3% hydrogen peroxide was applied to a discrete colony. The formation of bubbles indicated a positive reaction.

4. Antibiotic Sensitivity: Colonies were subcultured onto tryptric soy agar (TSA). A large loopful of growth was scraped off TSA and suspended in 2 ml heart infusion broth to turbidity. A sterile swab was used to streak the culture in three different directions over a TSA plate. A 30μg Nalidixic acid disc was placed on one side of the agar plate using sterile tweezers. A 30μg cephalothin disc was placed on the opposite side of the agar. The plate was incubated at 37°C for 48 hours under microaerophilic conditions. A zone of inhibited bacterial growth around the antibiotic disc indicated sensitivity of the isolate to the antibiotic whereas growth right up to the disc indicated of resistance (Figure 8).

5. Nitrate reduction test: A large loopful of growth was suspended in 4 ml of nitrate broth. The broth was incubated at 37°C for 48 hours under microaerophilic conditions. Three drops each of nitrate reagents A and B were added. The development of a red color indicates a positive reaction (Figure 9) because the isolate has reduced nitrates to nitrites.

6. Hippurate Hydrolysis: A 3.5% solution of ninhydrin was freshly prepared each time before testing by dissolving 0.35g of ninhydrin in a tube containing 10 ml of a 1:1 mixture of acetone-butanol. A large loopful of a fresh 48 hours culture growth from a blood agar plate was emulsified well into a tube of 0.4ml sterile H_2O with frequent mixing to form a smooth suspension. One BBL Taxo Hippurate Disc (Becton Dickinson, Auckland) was added into each tube and incubated at 37°C in a water bath for 2 hours. After incubation, 200μl of 3.5 % ninhydrin reagent was slowly added to each tube. Without shaking, the tubes were returned to the 37°C water bath for another 10 minutes then examined immediately, without shaking, for color development. A positive test was

indicated by a deep purple color, crystal violet-like, indicating the presence of glycine, which resulted from the hydrolysis of hippurate. A pale purple color or colorless tubes were considered negative for hippurate hydrolysis (Figure 10). (Lior, 1984)

2.2.2.2.4 SUBTYPING OF *CAMPYLOBACTER JEJUNI*

In this study, agarose-immobilized chromosomal DNA from *C. jejuni* strains isolated from cows were digested with the restriction endonuclease *Sma*I and analyzed by using PFGE. The results of Pulsed-Field Gel Electrophoresis are shown in Figure 11. Figure 12 shows a dendrogram determined by computer analysis of the PFGE bands.

Pure cultures of *Campylobacter* spp were stored frozen at -70°C before genetic analysis. Chromosomal DNA was isolated from *Campylobacter* isolates cultivated on blood agar. Lysis of harvested and washed bacteria was performed according to the methods described in Appendix III. After washing in Tris-EDTA, the lysed agarose blocks were equilibrated three times in restriction enzyme reaction buffer, and the consequent DNA digestion with *Sma*I was performed for 4 hours (Wassenaar *et al.*, 1998).

The detailed procedures are described in Appendix III. PFGE was performed using a BIO-RAD CHEF MAPPER (AIITECH ASSOCIATES INC, PO Box 100-352, NSMC Auckland).

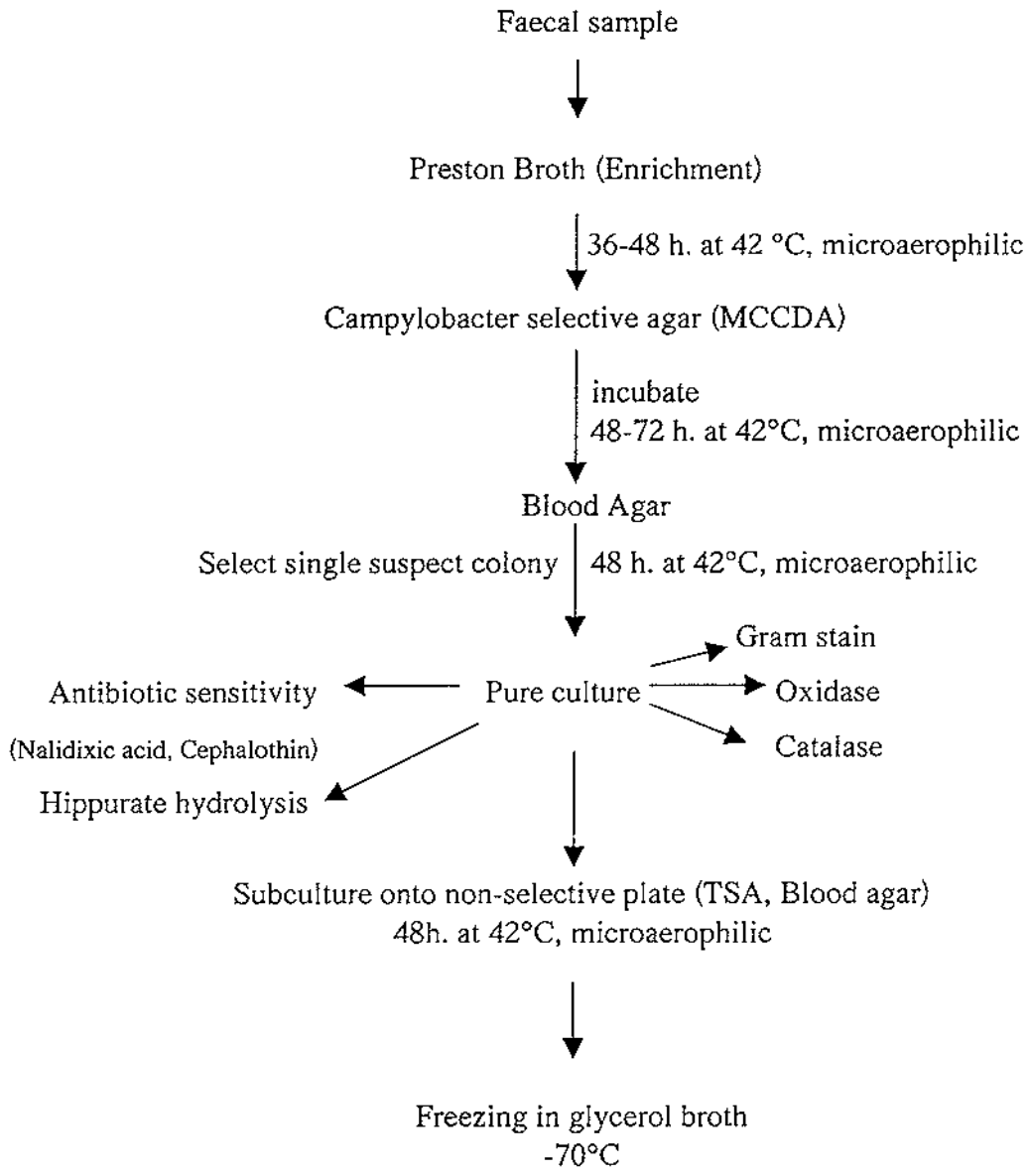

Figure 5. Flow diagram of examination procedures for *Campylobacter jejuni* and *Campylobacter* spp.

Figure 6: *Campylobacter* species grow as grey-white spreading colonies on MCCDA

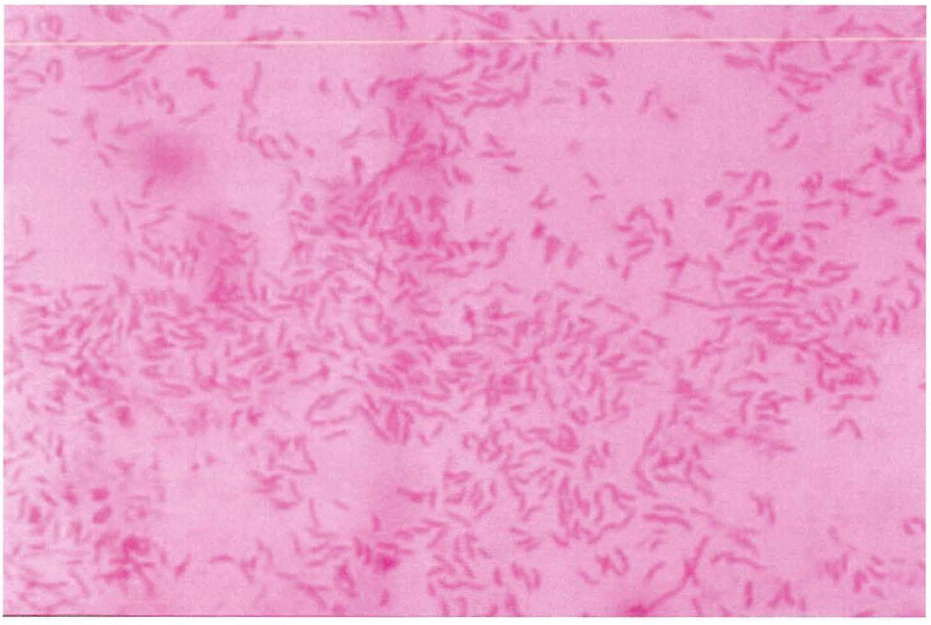

Figure 7: *Campylobacter jejuni* from a culture. Gram stain, x1000

Campylobacter jejuni are Gram negative and form S or gull-winged shapes.

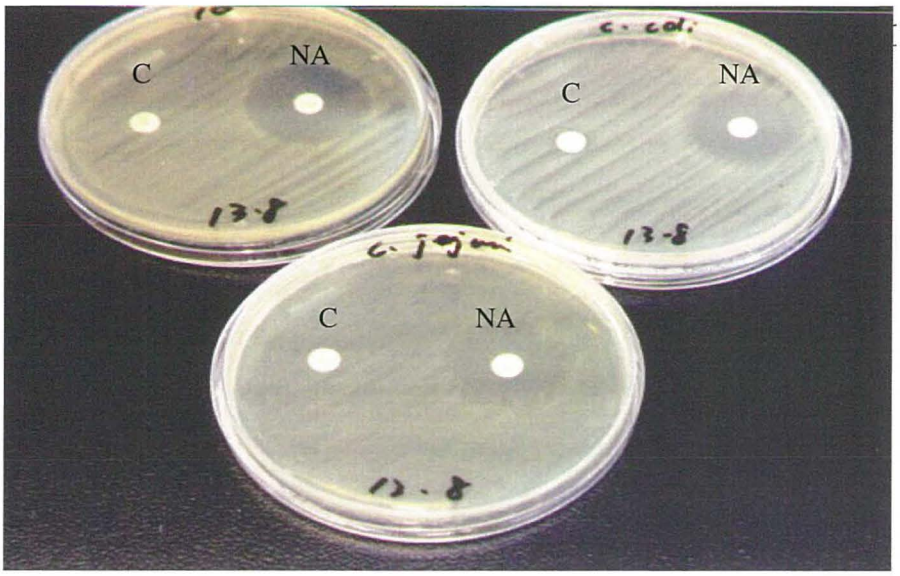

Figure 8. The left upper TSA plate shows a zone of inhibition of growth of *C. jejuni* around the antibiotic disc (Nalidixic acid, NA) indicating sensitivity of the isolate to this compound. Growth up to the disc (Cephalothin, C) on the left side of the medium is indicative of resistance. The right upper TSA plate contains is a control strain of *C. coli* and the TSA plate in the lower center is a control of *C.jejuni*.

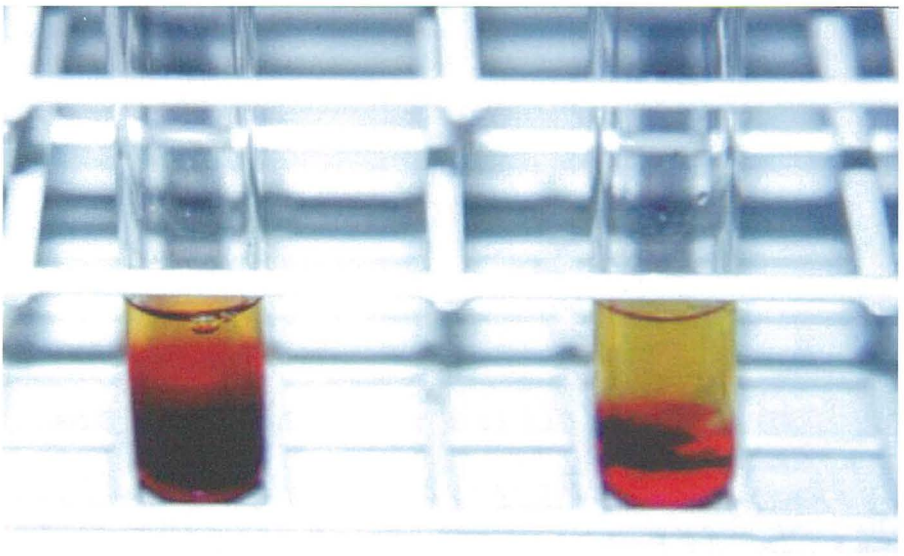

Figure 9. Nitrate test: a red colour indicated that the reaction is positive and that the isolate reduced nitrates to nitrites. The left tube is a control strain of *Campylobacter jejuni*; the right tube is a strain of *Campylobacter jejuni* isolated in this project.

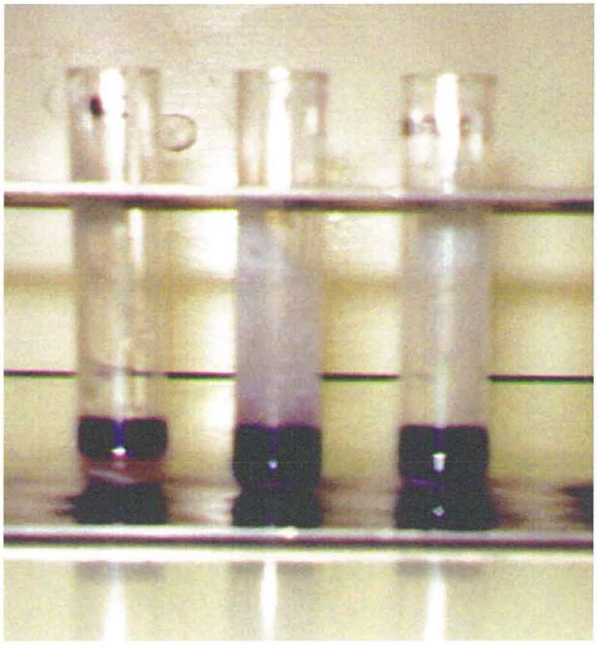

Figure 10. Hippurate test: a positive test was recorded as a deep purple color, crystal violet-like, indicating the presence of glycine, resulting from the hydrolysis of hippurate.

2.3 RESULTS

2.3.1 RESULTS OF *CAMPYLOBACTER* IDENTIFICATION

Campylobacter require specialised media and a modified atmosphere to be isolated from faecal samples. Depending on the use of an enrichment procedure, it takes from 2-4 days to obtain isolated colonies on a plate and then up to 3 more days before final confirmation (Figure 5). The results of identified *Campylobacter* strains in this project are listed in Table 1 to Table 8. The analysis of Pulsed-Field Gel Electrophoresis is relatively straightforward and some results are shown in Figure 11 and Figure 12.

RESULTS OF THE PILOT STUDY

Twenty animals were sampled to identify whether *Campylobacter* spp were present on the farm. *Campylobacter* spp. were isolated (65%) from 13 of the 20 animals sampled. Of these, nine were *C. jejuni* (45%), and the other isolates were *C. hyointestinalis* (10%) and *C. lari* (10%) (Table 1).

RESULTS OF THE ADULT CATTLE

Table 2, 4 and 7 show the results of testing of adult dairy cows of the same herd on three occasions (late laction, dry off and post-calving periods). Table 2 shows that eight (12%) of the 65 milking cows sampled during the late lactation period carried *C. jejuni* and no other *Campylobacter* species was found. Table 4 shows that 34 (52%) of the 65 milking cows sampled during the dry period had *Campylobacter* spp. isolated. Of these, 17 were *C. jejuni* (26%), eight were *C. hyointestinalis* (12%), five were *C. upsaliensis* (8%) and four were *C. lari* (6%). Table 7 shows that nine (15%) of the 59 milking cows sampled after calving carried *Campylobacter* spp. Of these, four (7%) were *C. jejuni*, and five (8%) were *C. hyointestinalis*. Both prevalence of *Campylobacter* shedding and the species isolated varied considerably over the course of this study.

RESULTS OF THE HEIFERS

Table 3 shows that 53 (82%) of the 65 heifers sampled were *Campylobacter* spp. positive. Of these, 25 (38%) were *C. jejuni*, 17 (26%) were *C. hyointestinalis* and 11 (17%) were *C. upsaliensis*. Strains of both *C. hyointestinalis* and *C. jejuni* were isolated from Cow number 514. *C. jejuni* had the highest prevalence among all isolated strains.

RESULTS OF THE YEARLINGS

Table 5 shows that 22 (37%) of the 60 yearlings sampled were carrying *Campylobacter* spp. Of these, only one (2%) was *C. jejuni*, seventeen were *C. hyointestinalis* (28%) and four were *C. upsaliensis* (7%). The prevalence of *C. jejuni* is very low whereas *C. hyointestinalis* had higher prevalence.

RESULTS OF THE CALVES

Table 6 shows that 13 (22%) of the 60 calves sampled were *Campylobacter* spp. positive. Only seven (12%) were *C. jejuni*, five were *C. hyointestinalis* (8%) and one (2%) was *C. upsaliensis*.

RESULTS OF OVERALL DISTRIBUTION OF *CAMPYLOBACTER* SPP.

Table 8 are about the numbers of cows positive for non-*Campylobacter* or non-*jejuni* or *Campylobacter* and *Campylobacter jejuni*, and the prevalences of *C. jejuni* and *Campylobacter* spp.

Table 1: *Campylobacter* **species isolated at the 1st sampling (n=20) on May 25, 2000 from milking cows at Massey No.4 Dairy Farm**

Cow ID	Species	MCCDA (42C)	Gram	Oxidase	3% hydrogen-peroxide	Antibiotic Sensitivity		Hippurate Hydrolysis
						Nalidixic acid (NA)	Cephalothin (C)	
4	*C. jejuni*	+	-	+	+	S	R	+
67	*C. hyointestinalis*	+	-	+	+	R	S	-
125	*C. jejuni*	+	-	+	+	S	R	+
149	*C. jejuni*	+	-	+	+	S	R	+
201	*C. jejuni*	+	-	+	+	S	R	+
211	*C. jejuni*	+	-	+	+	S	R	+
258	*C. lari*	+	-	+	+	R	R	-
281	*C. jejuni*	+	-	+	+	S	R	+
284	*C. hyointestinalis*	+	-	+	+	R	S	-
319	*C. lari*	+	-	+	+	R	R	-
356	*C. jejuni*	+	-	+	+	S	R	+
435	*C. jejuni*	+	-	+	+	S	R	+
490	*C. jejuni*	+	-	+	+	S	R	+

+, positive reaction; - , negative reaction; R, resistant; S, sensitive

Table 2: *Campylobacter* species isolated at the 2nd sampling (n=65) on June 21, 2000 from milking cows (before dry off) at Massey No.4 Dairy Farm

Cow ID	Species	MCCDA (42C)	Gram	Oxidase	Antibiotic Sensitivity		Hippurate Hydrolysis
					Nalidixic acid (NA)	Cephalothin (C)	
79	*C. jejuni*	+	-	+	S	R	+
83	*C. jejuni*	+	-	+	S	R	+
90	*C. jejuni*	+	-	+	S	R	+
101	*C. jejuni*	+	-	+	S	R	+
115	*C. jejuni*	+	-	+	S	R	+
207	*C. jejuni*	+	-	+	S	R	+
281	*C. jejuni*	+	-	+	S	R	+
398	*C. jejuni*	+	-	+	S	R	+

+, positive reaction; - , negative reaction; R, resistant; S, sensitive

Table 3: *Campylobacter* species isolated at the 3rd sampling (n=65) on June 23, 2000 from heifers at Massey No.4 Dairy Farm

Cow ID	species	MCCDA(42C)	Gram	Oxidase	Nitrate	NA	C	Hippurate
2	*C. jejuni*	+	-	+	+	S	R	+
6	*C. hyointestinalis*	+	-	+	+	R	S	-
29	*C. hyointestinalis*	+	-	+	+	R	S	-
33	*C. hyointestinalis*	+	-	+	+	R	S	-
58	*C. jejuni*	+	-	+	+	S	R	+
62	*C. jejuni*	+	-	+	+	S	R	+
84	*C. jejuni*	+	-	+	+	S	R	+
94	*C. upsaliensis*	+	-	+	+	S	S	-
95	*C. hyointestinalis*	+	-	+	+	R	S	-
106	*C. jejuni*	+	-	+	+	S	R	+
108	*C. jejuni*	+	-	+	+	S	R	+
135	*C. jejuni*	+	-	+	+	S	R	+
136	*C. jejuni*	+	-	+	+	S	R	+
197	*C. jejuni*	+	-	+	+	S	R	+
205	*C. hyointestinalis*	+	-	+	+	R	S	-
213	*C. upsaliensis*	+	-	+	+	S	S	-
220	*C. jejuni*	+	-	+	+	S	R	+
227	*C. jejuni*	+	-	+	+	S	R	+
237	*C. upsaliensis*	+	-	+	+	S	S	-
247	*C. hyointestinalis*	+	-	+	+	R	S	-
249	*C. hyointestinalis*	+	-	+	+	R	S	-
295	*C. upsaliensis.*	+	-	+	+	S	S	-
304	*C. hyointestinalis*	+	-	+	+	R	S	-
334	*C. upsaliensis.*	+	-	+	+	S	S	-
340	*C. hyointestinalis*	+	-	+	+	R	S	-
345	*C. jejuni*	+	-	+	+	S	R	+
372	*C. hyointestinalis*	+	-	+	+	R	S	-
375	*C. jejuni*	+	-	+	+	S	R	+
381	*C. jejuni*	+	-	+	+	S	R	+
389	*C. jejuni*	+	-	+	+	S	R	+
391	*C. jejuni*	+	-	+	+	S	R	+
396	*C. upsaliensis*	+	-	+	+	S	S	-
412	*C. hyointestinalis*	+	-	+	+	R	S	-
417	*C. jejuni*	+	-	+	+	S	R	+
420	*C. jejuni*	+	-	+	+	S	R	+
437	*C. hyointestinalis*	+	-	+	+	R	S	-
477	*C. jejuni*	+	-	+	+	S	R	+
480	*C. hyointestinalis*	+	-	+	+	R	S	-
483	*C. jejuni*	+	-	+	+	S	R	+
495	*C. jejuni*	+	-	+	+	S	R	+
499	*C. hyointestinalis*	+	-	+	+	R	S	-
500	*C. jejuni*	+	-	+	+	S	R	+
501	*C. hyointestinalis*	+	-	+	+	R	S	-
510	*C. hyointestinalis*	+	-	+	+	R	S	-
512	*C. jejuni*	+	-	+	+	S	R	+
513	*C. upsaliensis*	+	-	+	+	S	S	-
*514	*C. jejuni*	+	-	+	+	S	R	+
515	*C. upsaliensis.*	+	-	+	+	S	S	-
516	*C. upsaliensis*	+	-	+	+	S	S	-
517	*C. upsaliensis*	+	-	+	+	S	S	-
520	*C. upsaliensis*	+	-	+	+	S	S	-
521	*C. jejuni*	+	-	+	+	S	R	+
522	*C. hyointestinalis*	+	-	+	+	R	S	-

+, positive reaction; -, negative reaction; R, resistant; S, sensitive. *514cow also isolated *C. hyointestinalis*

Table 4: *Campylobacter* species isolated at the 4th sampling (n=65) on July 14, 2000 from dry cows at Massey No.4 Dairy Farm

Cow ID	Species	MCCDA (42C)	Gram	Oxidase	Nitrate	NA	C	Hippurate hydrolysis
20	*C. laris*	+	-	+	+	R	R	-
39	*C. hyointestinalis*	+	-	+	+	R	S	-
42	*C. hyointestinalis*	+	-	+	+	R	S	-
44	*C. upsaliensis*	+	-	+	+	S	S	-
80	*C. laris.*	+	-	+	+	R	R	-
83	*C. hyointestinalis*	+	-	+	+	R	S	-
105	*C. jejuni*	+	-	+	+	S	R	+
107	*C. jejuni*	+	-	+	+	S	R	+
115	*C. jejuni*	+	-	+	+	S	R	+
144	*C. laris*	+	-	+	+	R	R	-
153	*C. hyointestinalis*	+	-	+	+	R	S	-
193	*C. upsaliensis*	+	-	+	+	S	S	-
198	*C. jejuni*	+	-	+	+	S	R	+
209	*C. upsaliensis*	+	-	+	+	S	S	-
215	*C. hyointestinalis*	+	-	+	+	R	S	-
223	*C. jejuni*	+	-	+	+	S	R	+
231	*C. jejuni*	+	-	+	+	S	R	+
235	*C. jejuni*	+	-	+	+	S	R	+
248	*C. jejuni*	+	-	+	+	S	R	+
268	*C. laris*	+	-	+	+	R	R	-
281	*C. jejuni*	+	-	+	+	S	R	+
282	*C. jejuni*	+	-	+	+	S	R	+
285	*C. upsaliensis*	+	-	+	+	S	S	-
294	*C. jejuni*	+	-	+	+	S	R	+
307	*C. jejuni*	+	-	+	+	S	R	+
316	*C. hyointestinalis*	+	-	+	+	R	S	-
333	*C. jejuni*	+	-	+	+	S	R	+
350	*C. jejuni*	+	-	+	+	S	R	+
398	*C. jejuni*	+	-	+	+	S	R	+
431	*C. hyointestinalis*	+	-	+	+	R	S	-
449	*C. jejuni*	+	-	+	+	S	R	+
457	*C. hyointestinalis*	+	-	+	+	R	S	-
491	*C. upsaliensis*	+	-	+	-	S	S	-
496	*C. jejuni*	+	-	+	+	S	R	+

+, positive reaction; -, negative reaction; R, resistant; S, sensitive

Table 5: *Campylobacter* species isolated at the 5th sampling (n=60) on July 21, 2000 from yearlings at Massey No.4 Dairy Farm

cowID	species	MCCDA (42C)	Gram	Oxidase	Nitrate	NA	C	Hippurate hydrolysis
108	*C. hyointestinalis*	+	-	+	+	R	S	-
991	*C. hyointestinalis*	+	-	+	+	R	S	-
993	*C. upsaliensis*	+	-	+	+	S	S	-
995	*C. hyointestinalis*	+	-	+	+	R	S	-
9914	*C. hyointestinalis*	+	-	+	+	R	S	-
9921	*C. upsaliensis*	+	-	+	+	S	S	-
9924	*C. jejuni*	+	-	+	+	S	R	+
9942	*C. hyointestinalis*	+	-	+	+	R	S	-
9946	*C. hyointestinalis*	+	-	+	+	R	S	-
9947	*C. hyointestinalis*	+	-	+	+	R	S	-
9950	*C. hyointestinalis*	+	-	+	+	R	S	-
9956	*C. hyointestinalis*	+	-	+	+	R	S	-
9962	*C. hyointestinalis*	+	-	+	+	R	S	-
9966	*C. upsaliensis*	+	-	+	+	S	S	-
9970	*C. hyointestinalis*	+	-	+	+	R	S	-
9975	*C. upsaliensis*	+	-	+	+	S	S	-
9977	*C. hyointestinalis*	+	-	+	+	R	S	-
9979	*C. hyointestinalis*	+	-	+	+	R	S	-
9981	*C. hyointestinalis*	+	-	+	+	R	S	-
9998	*C. hyointestinalis*	+	-	+	+	R	S	-
99100	*C. hyointestinalis*	+	-	+	+	R	S	-
99122	*C. hyointestinalis*	+	-	+	+	R	S	-

+, positive reaction; -, negative reaction; R, resistant; S, sensitive

Table 6: *Campylobacter* species isolated at the respective 6th, 7th, 8th sampling (totally n=60) from calves at Massey No.4 dairy farm

Date	Cow ID	Species	MCCDA (42C)	Gram	Oxidase	Nitrate	NA	C	Hippurate hydrolysis
6th sampling collect (8/02/00) n=20	71	*C. hyointestinalis*	+	-	+	+	R	S	-
	343	*C. upsaliensis*	+	-	+	+	S	S	-
	344	*C. jejuni*	+	-	+	+	S	R	+
	345	*C. jejuni*	+	-	+	+	S	R	+
	346	*C. jejuni*	+	-	+	+	S	R	+
7th sampling collect (8/10/00) n=20	114	*C. hyointestinalis*	+	-	+	+	R	S	-
	152	*C. jejuni*	+	-	+	+	S	R	+
8th sampling collect (8/17/00) n=20	12	*C. hyointestinalis*	+	-	+	+	R	S	-
	183	*C. hyointestinalis*	+	-	+	+	R	S	-
	190	*C. jejuni*	+	-	+	+	S	R	+
	194	*C. jejuni*	+	-	+	+	S	R	+
	376	*C. jejuni*	+	-	+	+	S	R	+
	379	*C. hyointestinalis*	+	-	+	+	R	S	-

+, positive reaction; -, negative reaction; R, resistant; S, sensitive

Table7: ***Campylobacter*** **species isolated at the 9th sampling (n=59) on October 5, 2000 from milking cows (after calving) Massey No.4 dairy farm**

Cow ID	Species	MCCDA (42C)	Gram	Nitrate	Oxidase	3% hydrogen-peroxide	Antibiotic Sensitivity		Hippurate Hydrolysis
							Nalidixic acid	Cephalothin	
53	*C. jejuni*	+	-	+	+	+	S	R	+
105	*C. hyointestinalis*	+	-	+	+	+	R	S	-
144	*C. hyointestinalis*	+	-	+	+	+	R	S	-
153	*C. jejuni*	+	-	+	+	+	S	R	+
209	*C. hyointestinalis*	+	-	+	+	+	R	S	-
398	*C. hyointestinalis*	+	-	+	+	+	R	S	-
421	*C. jejuni*	+	-	+	+	+	S	R	+
449	*C. hyointestinalis*	+	-	+	+	+	R	S	-
491	*C. jejuni*	+	-	+	+	+	S	R	+

+, positive reaction; - , negative reaction; R, resistant; S, sensitive

Table 8: The data of animal ID and *Campylobacter* isolations in cows over the sampling period May-October, 2000.

	1st ,n=20 (11/5/00)	2nd sampling (8/6/00) n=65	3rd sampling (23/6/00) n=65	4th sampling (14/7/00)n=65	5th sampling (21/7/00)n=60	6th sampling (2/8/00)n=20	7th sampling (10/8/00)n=20	8th sampling (17/8/00)n=20	9th sampling (5/10/00) n=59
	Milking Cows ID	Milking Cows ID	Heifers ID	Dry Cows ID	Yearlings ID	Calves ID	Calves ID	Calves ID	Milking Cows ID
Not Campylo -bacter	37,39,86, 117,327, 459,468 (n=7)	15,20,39,42,44, 46,49,53,54,57, 73,80,100,105, 107,130,144,145, 152,153,162,193, 198,206,209,212, 215,216,223,231, 234,235,239,248, 268,282,285,292, 294,299,307,312, 316,317,333,335, 350,414,419,421, 424,430,431,449, 457,491,496 (n=57)	11,55,72,173,283, 318,358,361,447, 518,519,523 (n=12)	15,46,49,53,54, 57,73,79,90,100, 101,130,145,152, 162,206,207,212, 216,234,239,292, 299,312,317,335, 414,419,421,424, 430 (n=31)	992,997,999, 9911,9912,9913, 9915,9916,9917, 9918,9920,9922, 9923,9925,9928, 9929,9931,9932, 9933,9935,9937, 9938,9941,9943, 9944,9948,9954, 9957,9964,9965, 9967,9968,9972, 9978,9983,9995, 999102,999146 (n=38)	60,61,62,63,64, 65,66,67,68,69, 70,72,73,342, 347 (n=15)	103,111,120, 122,126,127, 129,130,131, 134,135,136, 139,141,144, 145,146,147 (n=18)	181,182,184, 185,186,187, 188,189,191, 192,193,196, 197,377 (n=14)	15,20,39,42,44,49, 54,57,73,79,80,90, 100,101,107,115, 130,145,152,193, 198,206,207,212, 215,216,223,231, 234,235,239,248, 281,282,285,292, 294,299,312,316, 317,333,335,350, 414,419,424,430, 431,457 (n=50)
C. spp. (exceptC .jejuni)	67,258, 284,319 (n=4)	ND	6,29,33,94,95,205, 213,237,247,249, 295,304,334,340, 372,396,412,437, 480,499,501,510, 513,515,516,517, 520,522 (n=28)	20,39,42,44,80, 83,144,153,193, 209,215,268,285, 316,431,457,491 (n=17)	108,991,993,995, 9914,9921,9942, 9946,9947,9950, 9956,9962,9966, 9970,9975,9977, 9979,9981,9998, 99100,99122(n=21)	71,343 (n=2)	114 (n=1)	12,183,379 (n=3)	105,144,209, 398,449 (n=5)
C.jejuni	4,125,149, 201,211, 281,356, 435,490 (n=9)	79,83,90,101,115, 207,281,398 (n=8)	2,58,62,84,106,108 ,135,136,197,220, 227,345,375,381, 389,391,417,420, 477,483,495,500, 512,514,521(n=25)	105,107,115,198, 223,231,235,248, 281,282,294,307, 333,350,398,449, 496 (n=17)	9924 (n=1)	344,345,346 (n=3)	152 (n=1)	190,194,376 (n=3)	53,153,421,491 (n=4)
Prevalen. *C .jejuni*	45%	12.31%	38.46%	26.15%	1.67%	15%	5%	15%	6.78%
Total Prevalen.	65%	12.31%	81.53%	52.30%	36.67%	25%	10%	30%	15.25%

RESULTS OF PULSE FIELD GEL ELECTROPHORESIS

Figure 11. *Sma*I PFGE restriction patterns of *C. jejuni* genomic DNA.

FIGURE LEGEND

Lane A, Lambda ladder PFG marker; Lane B, PY2; Lane C, PY6; Lane D, PY7; Lane E, PY8; Lane F, PY9; Lane G, PY10; Lane H, PY11; Lane I, PY12; Lane J, low mw marker; Lane K, PY14; Lane L, PY15; Lane M, PY20; Lane N, PY21; Lane O, PY23; Lane P, 74 (the isolated strain from the pond of the study farm); Lane Q, 75 (the isolated strain from the pond of the study farm); Lane R, Lambda ladder PFG marker. Genomic DNA from 15 *C. jejuni* was digested with the restriction endonuclease *Sma*I and subjected to PFGE. *Sma*I restriction patterns are shown in Figure 11.

RESULTS OF PULSOTYPES:

PY2, PY6, PY7, PY8, PY9, PY10, PY11, PY12, PY14 were isolated from 1st sampling specimens, and PY15, PY20, PY21, PY23 were isolated from 2nd sampling specimens. Strain 74 and strain 75 were sampled from the pond at the study farm.

C. jejuni PY2, PY7, PY10, PY9 and PY23 have indistinguishable restriction patterns. *C. jejuni* PY20 and PY21 have indistinguishable restriction patterns. *C. jejuni* PY11 and PY14 also have indistinguishable restriction patterns. *C. jejuni* strains 74 and 75 isolated from the pond display distinct restriction patterns and are different from the 15 *C. jejuni* strains from cattle. The degree of similarity of *C. jejuni* strains is shown in Figure12.

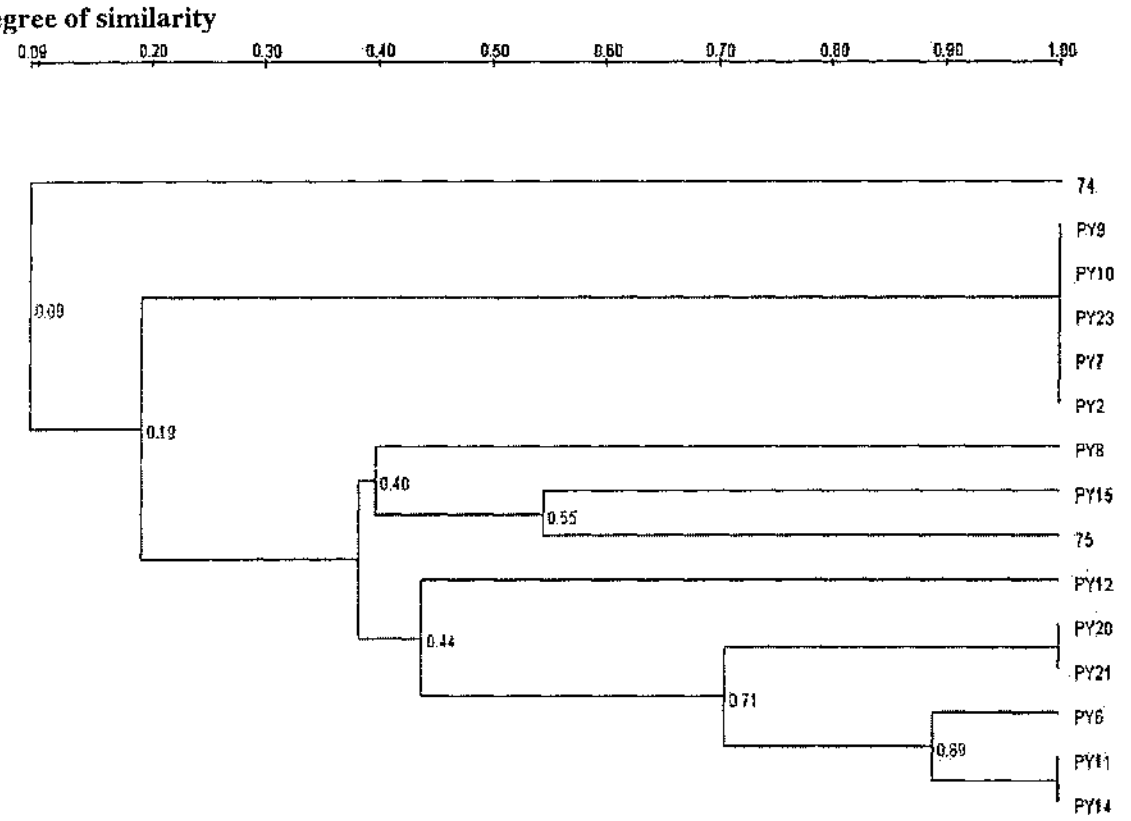

Figure 12: Dendrogram determined by UPGMA cluster analysis of PFGE bands shows the relationships of 15 *C. jejuni* strains. The strain numbers are listed along the y-axis. The x-axis is a measure of degree of similarity.

Figure 12 shows that the 15 strains were grouped in three broad clusters. In the first, there was only one of the water strains (9% related to all the others), the second cluster consisted of five indistinguishable strains, four strains from the first sampling, one from the second sampling. The third cluster was considerably heterogeneous and consisted of

nine strains grouped singly or in pairs with all but three showing less than 75% similarity. This cluster included the second isolated water strain.

2.3.2 DESCRIPTIVE STATISTICS STUDY

2.3.2.1 PREVALENCE

Table 9: Number and Prevalence of *Campylobacter* spp. and *C. jejuni* at each sampling period in the study

Group		Cows			Heifers	Yearlings	Calves
		Before calving	Dry cows	After calving			
Sampling Date/Size		8/6/00 n=65	14/7/00 n=65	5/10/00 n=59	23/6/00 n=65	21/7/00 n=60	2/8/00-17/8/00 n=60
Number of *Campylobacter negative* animals		57	31	50	12	38	47
Number of *Campylobacter* positive animals	*Campylobacter* spp. (except *C. jejuni*)	Not found	17	5	28	21	6
	C. jejuni	8	17	4	25	1	7
	Total number positive for *Campylobacter*	8	34	9	53	22	13
Prevalence percentage	Prevalence of non-*jejuni Campylobacter* spp.	0	26	8	43	35	10
	Prevalence of *C. jejuni*	12	26	7	38	2	12
	Total prevalence of *Campylobacter*	12	52	15	81	37	22

From Table 9, it can be seen that heifers had the highest prevalence of both *C. jejuni* and non-*jejuni Campylobacter* spp., followed by dry cows, yearlings, calves and milking cows. Milking cows had a relatively lower prevalence than other groups, both before and after calving. The prevalence of *Campylobacter* spp (except *C. jejuni*) in the milking cows before drying off was zero. The yearling group shows a prevalence of 35% for *Campylobacter* spp. (except *C. jejuni*) but 2% for *C. jejuni*.

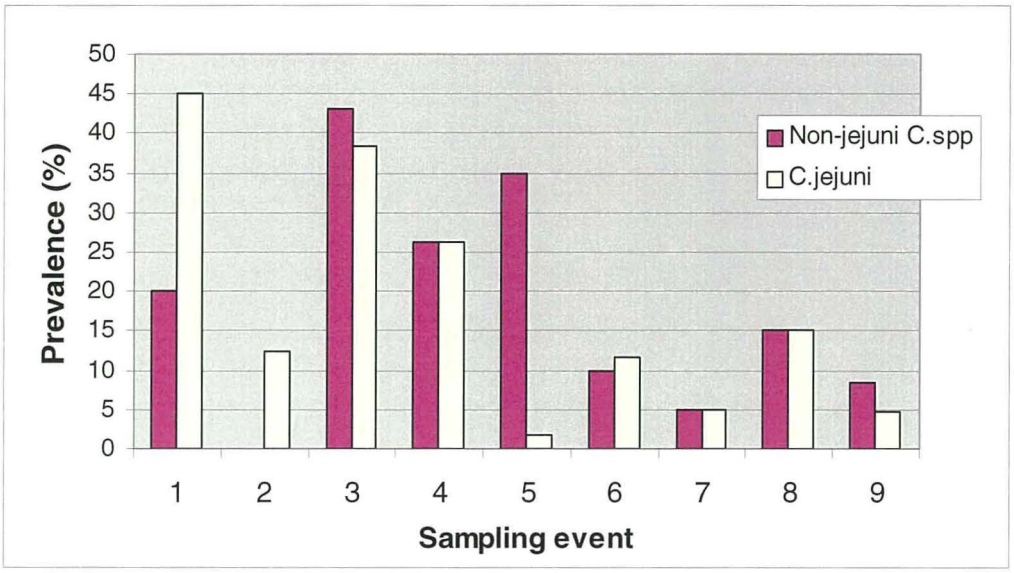

Figure13: The prevalence distribution of *Campylobacter jejuni* and non-*jejuni Campylobacter* spp. at various samplings (from 1st sampling to 9th sampling). Animal type: at 1st sampling pilot study, milking cows; 2nd sampling, milking cows (before dry time); 3rd sampling, heifers; 4th sampling, dry cows; 5th sampling, yearlings; 6th, 7th, 8th sampling, calves; 9th sampling, milking cows (after calving)

Figure 13, shows that heifers and dry cows had a higher prevalence than other animal groups although high prevalence was also found in the pilot study (1st sampling).
The prevalence of *C. jejuni* in yearling group is lower but it was zero in milking cows before dry off; on the contrary, the prevalence of *C. jejuni* in heifer group is highest. The other milking cows after calving time, calves and milking cows before dry time all have low *Campylobacter* prevalence.

Although the yearling group had the lowest prevalence of *C. jejuni*, it had much higher prevalence of other *Campylobacter* spp. The heifer group has highest prevalence of non-*jejuni Campylobacter* spp. No non-*jejuni Campylobacter* spp. were isolated from milking cows before dry off. Calves and milking cows after calving time have lower prevalences of *Campylobacter* spp. except *C. jejuni*.

2.3.2.2 CHANGE IN *CAMPYLOBACTER* STATUS OF ADULT CATTLE

Table10: *Campylobacter* status of individual cows sampled in late lactation (LL), during the dry period (DP), and after calving (AC).

	LL	DP Sampling	AC	Cow ID	No. of Case	Percentage
Status	—	—	—	15,49,54,57,73,100,130,145, 152,206,212,216,234,239,292, 299,312,317,335,414,419,424, 430	23	39%
	—	+spp	—	20,39,42,44,80,193,215,285, 316,431,457	11	19%
	—	+jejuni	—	107,198,223,231,235,248,282, 294,333,350	10	11%
	—	—	+ jejuni	53,421	2	3.4%
	+jejuni	—	—	79,90,101,207	4	6.8%
	—	+ spp	+ jejuni	153,491	2	3.4%
	—	+jejuni	+spp	105,449	2	3.4%
	—	+spp	+spp	144,209	2	3.4%
	+jejuni	+jejuni	—	115,281	2	3.4%
	+jejuni	+jejuni	+spp	398	1	1.7%
Missing Data	—	—		46,162	2	
	+jejuni	+spp		83	1	
	—	+spp		268	1	
	—	+jejuni		496,307	2	
Total					65	

+jejuni: *Campylobacter jejuni*-positive; + spp: Non-*jejuni Campylobacter species* positive; —: *Campylobacter-negative*

Comments on missing data:

Cow 46 was removed from the herd in September because of being empty at pregnancy test. Cow 83 was culled on 17 July because of abortion. Cow 162 because it died from milk fever on 10 August. Cow 268 was culled on 23 August because of negative pregnancy test. Cow 307 was in the mastitis mob when we went to get the samples. Cow 496 died on 11 July from unknown causes.

Table10 shows that after all three samplings, only 39% cases of cows remained *Campylobacter*-negative. The other 61% were positive for *Campylobacter* spp. at least once during the period of testing.

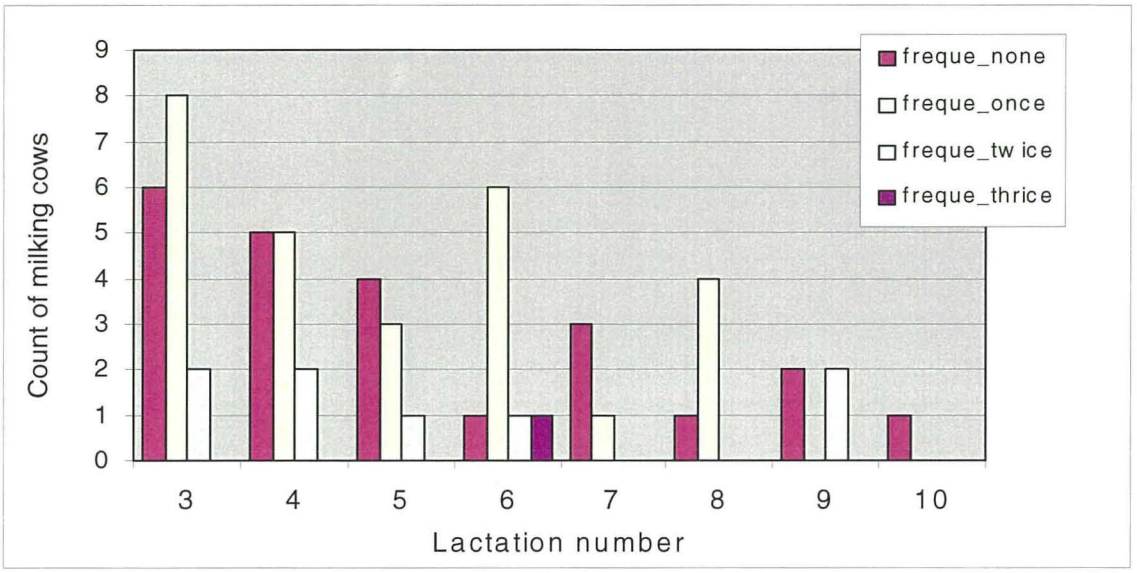

Figure 14: Count distribution of milking cows positive to *Campylobacter* species compared with their lactation number and the frequency number of *Campylobacter* species isolated positive from the same milking cows (n=59) after 2nd, 4th, 9th samplings from 8 June to 5 October, 2000.

freque_none means *Campylobacter* spp.were not isolated from the animals.
freque_once means *Campylobacter* spp.was isolated on only one occasion.
freque_twice means *Campylobacter* spp.was isolated on two occasions.
freque_thrice means *Campylobacter* spp.was isolated on three occasions.

Figure 14, shows that the ages of cows are mainly within lactation numbers (in June) from three to six. The frequency of *Campylobacter* species occuring positive of milking cows mostly also concentrates within lactation numbers from three to six, also with higher prevalence if compared with Table11.

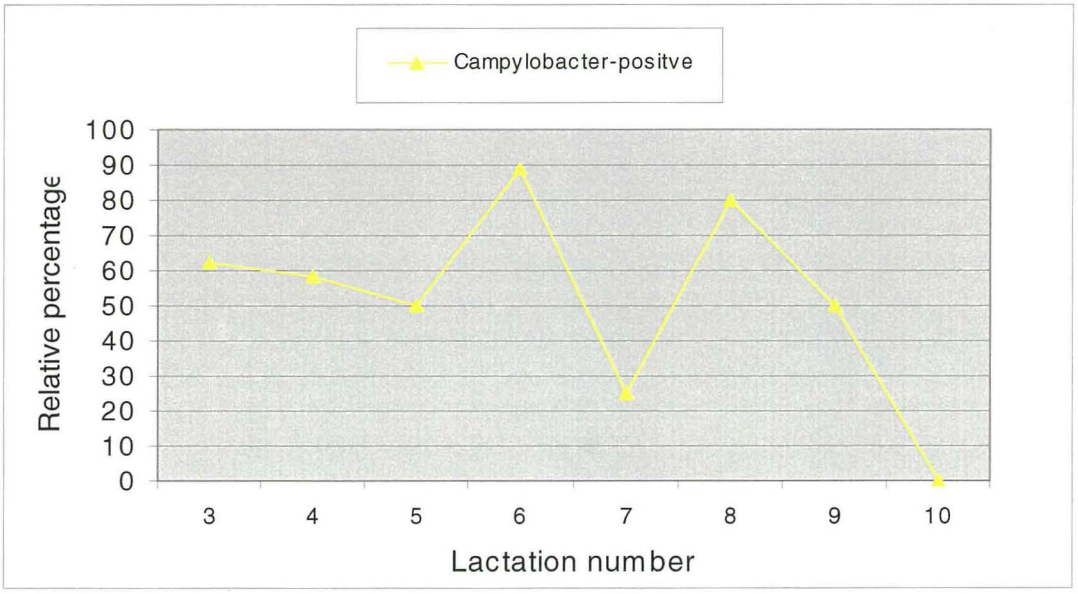

Figure15: The variation of percentage positive about their total count of *Campylobacte*-positive cows relative to their respective total count of cows under their respective lactation number condition. They are compared within the same milking herd (n=59, except 6 missing cows) from 8 June to 5 October 2000.

Figure15 shows that the percentage of *Campyloabcter*-positive cows (yellow line) seemed to decline with increasing age.

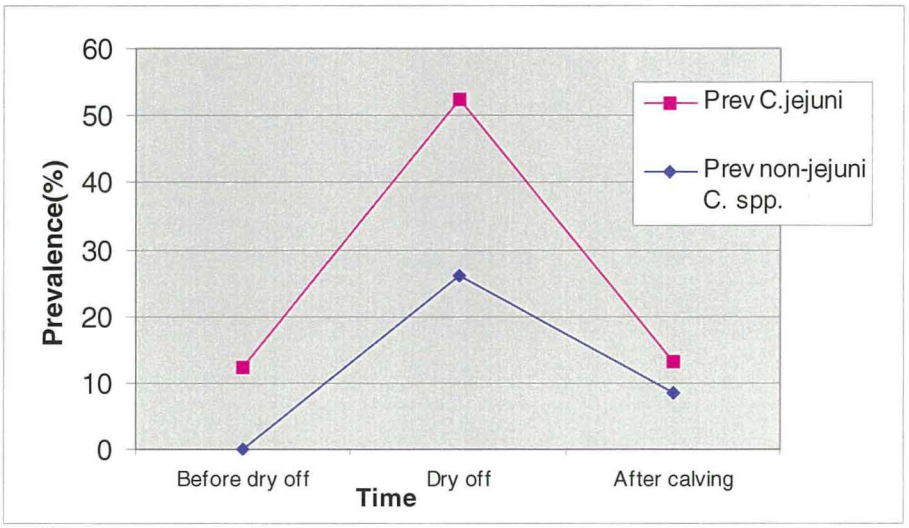

Figure16: The prevalence distribution of *Campylobacter jejuni* and other *Campylobacter* spp. within the milking herd before dry off, during dry off and after calving.

From Figure16, it is apparent that the prevalence of both *Campylobacter jejuni* and other *Campylobacter* spp. during dry off was higher than before dry off and after calving. The *C. jejuni* prevalence of before drying off is very similar to that after calving within the same milking cows sampled. The prevalence of non-*jejuni Campylobacter* species before dry off is lower than after calving. The prevalence of *C. jejuni* is higher at any stage than that of non-*jejuni Campylobacter*. Moreover, according to the statistic analysis of p values from McNemars test in SPSS Chi-square tests, *C. jejuni* positive for milking cows during dry off compared with before dry off were significantly different because their p value (0.07) was less than 0.1. Also during dry off as compared with after calving were significantly different because their p value (0.01) were less than 0.1.

2.3.2.3 CONFIDENCE INTERVALS

Table11: Calculating 95% confidence intervals for proportions (prevalence) in different populations.

Source Type	Milking Cows (before dry)	Heifers	Dry Cows	Yearlings	Calves	Milking Cows (after calving)
Total number of animals in population	389	143	389	120	124	384 (Five cows are removed)
Sample size	65	65	65	60	60	59
Total number of samples positive	8	53	34	22	13	9
Total measured prevalence for *Campylobacter* species	12.3%	81.5%	52.3%	36.7%	21.7%	15.3%
Variance	0.001661	0.002317	0.003838	0.003871	0.002829	0.002191
SEp	0.04075	0.04814	0.06195	0.06221	0.05319	0.04680
95% Confidence Interval for prevalence (p±1.96 SEp)	0.123± 0.080	0.815 ± 0.094	0.523± 0.121	0.367±0.122	0.217±0.104	0.153± 0.092
Upper confidence limit	28.3%	91.0%	64.4%	48.9%	32.1%	24.4%
Lower confidence limit	4.3%	72.1%	40.2%	24.5%	11.2%	6.1%

In sampling, parameters of interest are calculated from a subset of the population. The sample mean is then used as an estimate of the population mean. Many samples can be drawn to form a binomial distribution graph. If one should repeat this process of taking a random sample from the population a hundred times then the sampling mean would be within the assessed interval (Noordhuizen *et al.*, 1997). In other words, if repeated samples of the same number of individual n were selected, the calculated rate p would vary from sample to sample (Martin *et al.*, 1987). The extent of this variability is described by the standard error (SE) of the mean and is estimated from the sample to be:

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

p: proportion of sample test positive, e.g. measured prevalence, $p = X/n$, X is a binomial count.

Variance $= p\ (1 - p)/n$

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

Where z = 2.567 for 99%, 1.96 for 95% and 1.65 for 90%.

A 95% confidence interval may be constructed to allow for a 2.5% chance that the population proportion is higher than the upper confidence limit and a 2.5% chance that the population proportion is lower than the lower confidence limit (Noordhuizen *et al.*, 1997; Pfeiffer, 1999; Martin *et al.*, 1987).

For example, at the 4^{th} sampling from the population of heifers in this study:

Number of animal tested is 65

Number of animals that were positive is 53 (= 28 + 25)

Measured total prevalence for *Campylobacter* (p) = (28 + 25)/65 = 81.5% = 0.815

Variance = [0.815 (1 - 0.815)] ÷65 = 0.002317 =2.317×10^{-3}

$SEp = (2.317 \times 10^{-3})^{1/2}$ =0.04814

Confidence Interval = $0.815 \pm 1.96 \times 0.04814 = 0.815 \pm 0.094$

Hence, at 95% confidence interval, the prevalence of *Campylobacter* carriage by the population of heifers ranges between 72.1% and 91.0%.

As a result, Table 11 will be obtained.

From Table11, it means as follows below:

All at 95% confidence interval, the prevalence of *Campylobacter* carriage in the population of milking cows before dry time ranges between 4.3% and 28.3%. The prevalence of *Campylobacter* carriage in the population of milking cows at dry time ranges between 40.2% and 64.4%. The prevalence of *Campylobacter* carriage in the population of milking cows after calving ranges between 6.1% and 24.4%. The prevalence of *Campylobacter* carriage in the population of heifers ranges between 72.1% and 91.0%. The prevalence of *Campylobacter* carriage in the population of yearlings ranges between 24.5% and 48.9%. The prevalence of *Campylobacter* carriage in the population of calves ranges between 11.2% and 32.1%.

2.4 DISCUSSION

The overall observations in this study indicate considerable variablity occurs in the prevalence and types of *Campylobacter* shed in the faeces of dairy cattle, both between age groups on a farm and within the same groups of cows over time.

Some methodological observations made during these studies suggested that extending the incubation time from 48 to 72 hours led to a higher isolation rate of *Campylobacter* species. We believe that the optimal incubation time using selective media depends to some extent on the experience of operator. Skillful microbiologists can recognize small *Campylobacter* colonies present after 48-hour culture on a selective medium, but others may need another 24 hours of growth to be able to recognize these colonies. There was also some subjectivity in interpretation of the hippurate test. A positive test was recorded as a deep purple color (crystal violet-like), indicating the presence of glycine, which resulted from the hydrolysis of hippurate and a pale purple color or colorless tubes were considered negative for hippurate hydrolysis. However, if it appeared within 10 minutes, a purple color was considered positive; if it appeared between 10 and 20 minutes, it was considered weakly positive (On and Holmes, 1992). Thus, strains of *Campylobacter jejuni*, which show intermediate blue color intensities, should be retested. In addition, mixing or shaking the tubes after the addition of ninhydrin reagent could result in false positive readings, and should be avoided. *C. jejuni* reference strain (positive control) and *C. coli* reference strain (negative control) are essential to every test.

For the purpose of this pilot study (to describe the distribution and types of *Campylobacter* shedding among groups and over time in a herd), we considered that estimation of prevalence within 10% precision at the 90% confidence level was adequate. The initial sampling of 20 cows indicated that it was appropriate to use an expected prevalence of 50% for this purpose (being the worst-case scenario). That is, with this approach prevalence would be measured with at least this level of precision and confidence.

Campylobacter species can be isolated from the faeces of dairy cows and in a study of different age groups at Massey No.4 Dairy Farm, all groups were found to be *Campylobacter*-positive. The prevalence of positive animals ranged from 12% to 82% among groups, with a mean value of 42%. The calves at less than a week of age in the shed only had access to chlorinated water from Palmerston North Municipal Water Distribution Centre, which may affect risk of infection. In contrast, milking cows had access to either ponds or streams when grazing. In this study, heifers and dry cows had higher prevalences than other groups (Figure 13). It is speculated that because *C. jejuni* was also isolated from ponds (Figure11 and Figure 12) and dry cows and heifers could conveniently drink from ponds or streams this might affect their risk of infection.

It is plausible that there might be a possible association between *Campylobacter* prevalence and feeding of supplements from the troughs, where the tops have been pecked by many wild birds. Appropriate precautions should be taken against this potential risk and to prevent *Campylobacter* infection. At Massey No.4 Dairy Farm, all milking cows will be fed supply of supplements from the troughs near the milking shed before they milk. Wild birds may be important reservoirs of *C. jejuni* infection of cows. The yearling group is grazed off the farm, and had relatively low prevalence of *C. jejuni* infection but the prevalence of other *Campylobacter* species was 35% (Table8). As age and location are confounded, it is not possible to explain this observation.

With respect to the isolates from effluent, *C. jejuni* 74 and 75 (from the pond at this study farm) displayed distinct restriction patterns from the other 15 *C. jejuni* strains from cattle. Strain 74 only had 9 % similarity with any one of PY2, PY7, PY9, PY10 and PY23 strains. Strain 75 has 55% similarity with PY15 strain, but they both have 40 % similarity with PY8 strain. To determine whether this reflects differential adaption of strains or is due to sampling error would require more comprehensive studies. Although some identical strains occurred across the 1st and 2nd samplings, variation within and between sampling events was evident. Together these limited observations on sub-types indicate a very complex ecology of *Campylobacter* on the farm. If future workers wish to explore

the ecology of the organism more fully, consideration will need to be given to the diversity of strains that are likely to co-exist on a farm.

Failure to isolate the organism may not imply its absence as it may be present only in localized areas of the intestinal mucosa and may be shed intermittently in faeces. Also, bacterilogical culture has imperfect sensitivity. Serum antibody determination is only of value if the strain to which the animal was exposed is used as an antigen (Taylor, 1983). Table 10, shows the changes in *Campylobacter* status of individual animals within different stages of sampling. The percentage of animals positive at all three samplings was only 1.7%. Most cows remained either negative at all three samplings (38.98%) or negative at one to two samplings (39.34% and 13.56% respectively). Such intermittent recovery of bacteria from faecal samples is not unusual.

The most striking pattern was the significant change in prevalence in dry cows compared with the prevalence in the same cows before drying off and after the subsequent calving. The magnitude of this change was considerable and warrants replication and further investigation. Physiological, nutritional and management factors could be involved.

2.5 CONCLUSIONS

This study confirms that dairy cattle may be a significant reservoir for *Campylobacter* and a possible source for human infection via milk, meat and particularly environmental contamination. Animals shedding *Campylobacter* in their faeces may also assist in the transmission of the microorganism between herds. In addition, domestic and wild animals are also known to be asymptomatic carriers of thermophilic *Campylobacter* spp. All populations of different age groups in this study farm have been detected positive for *Campylobacter* infections including calves and yearlings, and heifers had highest prevalence. A water sample from the pond was also incidentally included in this study. As far as we are aware, all waterborne outbreaks of campylobacteriosis reported so far have involved the consumption of unchlorinated or insufficient chlorinated water (Melby *et al.*, 1991). This study used molecular typing for *C. jejuni* isolated either from bovine faecal samples or from the pond used as drinking water source for cows during the pasture season. Because effluent from the dairy shed is washed away after each milking to a series of settling and oxidation ponds, then water flowing out of the last pond enters the stream. There are also a number of small streams on the farm, which provide an alternative source of water when cows are in those paddocks, as does surface water after heavy rain. It might imply that the cycle of cattle-surface water-cattle was important in the transmission of the infection from one animal to one other. Also some cows can persistently excrete one *C. jejuni* strain. This study showed those molecular typing patterns of *C. jejuni* originating from animals or water were discriminative and important in epidemiological studies. The marked variability found in prevalence and strains in this pilot study need to be considered in the design of future ecological studies of livestock reservoirs for *Campylobacter*.

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APPENDIX I

Media

Campylobacter Blood Free Selective Medium (Modified CCDA-Improved)

Formula	g/litre
Peptone blend	25
Bacteriological Charcoal	4
Sodium chloride	3
Sodium desoxycholate	1
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar No. 2	12

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 X112 supplement, mix well and pour into petri dishes. Continuously mix whilst pouring to prevent the charcoal settling.

Sheep's Blood Agar

Composition per litre:	
Pancreatic digest of casein (Oxoid)	14.0 g
Agar (Gibco BRL)	12.5 g
NaCl	5.0 g
Bacto-peptone (Gibc BRL)	4.5 g
Yeast extract (Gibco BRL)	4.5 g
Sheep blood, defibrinated	70.0 ml

1 L of distilled/deionized 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-

50°C before the addition of 70 mL sterile, defibrinated sheep's blood and media poured into sterile Petri dishes.

Preston Enrichment Broth

Ingredients	g/475 ml
Nutrient broth No.2 (Oxoid)	12.5
Distilled water	475 ml

Dissolve the nutrient broth in the distilled water. Sterilize at 121°C for 15 minutes. Cool to 50°C. Add 25 ml of sterile defibrinated sheep blood.

Add one vial of reconstituted Preston *Campylobacter* Selective Supplement and one vial of *Campylobacter* Growth Supplement to 500 ml (475 of basal medium + 25 ml of blood) of tempered medium. Mix well. Aseptically pipette 5ml volumes into sterile bijou bottles. Minimal space should remain above the liquid to prevent oxidation of the broth. The prepared broth may be stored at 2 to 8°C for one week.

Commercial preparations:

Nutrient Broth No.2 (Oxoid)
Preston *Campylobacter* Selective Supplement (Oxoid SR 117)
Campylobacter Growth Supplement (Oxoid SR 84)

Ninhydrin Reagent

Ingredients	
N-Ninhydrin	3.5g
Acetone	50 ml

Butanol	50 ml

50 ml Acetone and 50 ml Butanol mix well, then 3.5 g N-Ninhydrin powder is added and dissolve to this solution to become 3.5% solution of Ninhydrin.

Nitrite Test Reagents

Solution A:	
Glacial acetic acid	30 ml
Distilled water	120 ml
Sulfanilic acid	0.5 gm
Prepare as below	

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

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, B in refrigerator.

APPENDIX II

Gram Stain

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

Freezing Procedure

1. Need to have a plate (usually blood agar) with 1 colony of the isolate streaked out to check for purity.
2. Label the cryogenic vial with a waterproof marker with the freezer number and the date it is frozen.
3. Use sterile swab to harvest all of the growth from the plate and transfer it into 3 ml of 15% glycerol broth.
4. Transfer approximately 1.5 ml of the glycerol broth to the cryogenic vial using a sterile pipette.
5. Place the vial into a freezer box that is labeled with the type of isolates.

APPENDIX III

PFGE of *Campylobacter* spp.

DAY ONE PLUG PREPARATION

1. Harvest the growth off a 48 hour Blood plate into BHI broth.
2. Measure the Optical density of cells at 610nm and adjust the cells to an OD of 1.4 .

DAY TWO PLUG PREPARATION

3. Measure the Optical density of the broth at 610nm and adjust the cells to an OD of 1.4 .
4. Place 200µl of cells in an eppendorf tube and centrifuge at 13000rpm for 5 minutes.
5. Remove supernatant and resuspend cells in 150µl of cold PETT IV buffer.
6. Centrifuge for 5 minutes at 13000rpm.
7. Remove supernatant and resuspend pellet in 50µl of PETT IV buffer.
8. Prepare 1% Low Melt Agarose as follows:

Add 4ml of PETT IV buffer to 40mg of Low Melt agarose heat in a boiling waterbath until the agarose has dissolved. Allow agarose to cool.

Add 100µl of molten agarose to the cell suspension and pipette up and down to mix.

9. Dispense 100µl of cells/agarose suspension into plug molds.
10. Cool plugs on ice for an hour.
11. Prepare Lysis buffer as follows:

50mM EDTA		**2ml of 0.5M per 20ml buffer**
50mM Tris-HCl		**1ml of 1M 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

12. Place plugs into 1ml of Lysis buffer in eppendorf tubes.
13. Incubate overnight at 56°C.

DAY THREE **PLUG WASHING**

1. Transfer the plugs into plastic universals containing 10ml TE buffer each.
2. Place universals on ice and incubate for 1 hour on the rocking machine.
3. Drain TE buffer off and replace with another 10 ml of TE buffer.
4. Place universals on ice and incubate on rocker for 1 hour.
5. Repeat wash step another 3 times.
6. Store plugs in 1ml TE buffer in an eppendorf tube at 4°C until required.

RESTRICTION DIGEST

7. Prepare restriction buffer (*Sma*I)
8. **For each plug add the following and mix**
   12µl of restriction buffer (10X NE Buffer 4)
   88µl sterile MQ water

   Flame a glass slide which has been dipped in ethanol.
9. Carefully remove the plug from the eppendorf tube and place onto the glass slide. Slice off one third of the plug, using a flamed scalpel.
10. Place plug slice into 100µl of restriction buffer and equilibrate on ice for 45 minutes.
11. Place the remaining two-thirds of the plug into 1ml TE in an eppendorf and store at 4°C until required.
12. Prepare cutting buffer as follows:

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

13. Remove restriction buffer and replace with 100µl of cutting buffer and equilibrate on ice for 45 minutes.
14. Incubate plugs at 37°C for 12-24 hours.

DAY FOUR GEL RUNNING

1. Prepare 1% PFC Agarose as follows:

Prepare 80ml agarose for standard mold and 140ml for wide/long mold.
e.g. for 80ml mold
0.8g agarose
80ml 0.5X TBE buffer
Heat for around 3 minutes in microwave until agarose is dissolved. Place agarose at ~ 50°C to cool for ~ 5 minutes.

2. Prepare gel mold and comb.
3. Pour into gel mold, popping any bubbles that come up at the corners.
4. Allow gel to solidify for 1 hour and then carefully remove the comb.
5. Pre-electroporese the gel at 6V/Cm, 5-5 sec., for 1.5 hours.
6. Remove the gel from the chamber and pipette the remaining buffer out of the wells.
7. Tip plugs out of eppendorf tubes onto flamed glass slides, then slide plugs into wells. Push plugs to the bottom front of the well using a 'hockey stick' (load marker plugs also).
8. Fill wells with molten 1% agarose and allow to set.
9. Place gel in electrophoresis chamber and run at the following conditions:

6V/Cm
22 hours
Initial switch = 3 seconds
Final switch = 35 seconds

DAY FIVE **STAINING AND PHOTOGRAPHING GEL**

1. Add 80µl of Ethidium bromide to 800ml MQ water in a plastic container.
2. Remove gel from chamber and slip it off the black plate into the Ethidium bromide solution.
3. Stain for 10 to 20 minutes.
4. Transfer gel to a plastic container containing MQ water and rinse briefly.
5. Photograph gel as follows:
   - i. Open 'Quantity One' or 'Diversity database' on the computer
   - ii. From the File menu select 'GelDoc'
   - iii. Place gel on transilluminator
   - iv. Turn ' Epi-Light' on
   - v. Click on 'Live Focus'
   - vi. Position gel
   - vii. Turn off 'Epi-Light' and turn 'Transilluminator' on
   - viii. Turn 'UV preparative' on
   - ix. Select 'Auto Expose' or 'Manual Expose' and adjust time
   - x. Click freeze and adjust 'High', 'Low' and 'Gamma' controls
   - xi. Click 'Video Print' to print photo
   - xii. Don't forget to save your image
   - xiii. Clean transilluminator with ethanol after use