TYPING OF *CAMPYLOBACTER* ISOLATES FROM HUMANS AND ANIMALS IN NEW ZEALAND

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ABSTRACT

Campylobacteriosis is currently the most commonly notified communicable disease in New Zealand. The sources of *Campylobacter* infections are not known, although the consumption of incompletly cooked poultry, untreated water, unpasteurised milk and contact with animals are associated with an increased risk of infection.

The aim of this study was to establish a simple and reliable method for typing *Campylobacter* isolates in order to investigate the sources of *Campylobacter* infections in humans in New Zealand.

Campylobacter isolates from humans and animals were identified to the species and sub-species level with a series of biochemical tests. The isolates were then examined by three genotypic typing methods: restriction fragment length polymorphism (RFLP) analysis of chromosomal DNA, randomly amplified polymorphic DNA (RAPD) typing using the polymerase chain reaction and RFLP analysis of the flagellin genes.

The flagellin gene, *fla*B, was examined by PCR amplification followed by digestion with the restriction endonucleases *Pst*I and *Hin*dIII. This method was the most reproducible of the three and provided a high level of discrimination, a total of 26 *Pst*I/*Hin*dIII groups were found among 140 human *Campylobacter* isolates. Over 98% of *C. jejuni* and *C. coli* isolates could be typed using this method. The results of this study indicated that sheep, cows and calves may be important sources of *Campylobacter* infection in humans.

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LIST OF ABBREVIATIONS

bp	Base pair(s)
BRL	Bethesda research laboratories
CNSM	Charcoal non-selective medium
CSM	Charcoal selective medium
CTAB	Hexadecyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra-acetate
kb	Kilobase
mins	Minutes
MEE	Multilocus enzyme electrophoresis
NCTC	National collection of type cultures
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
ppm	Parts per million
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
S_{AB}	Similarity value
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris EDTA
TNE	Tris NaCl EDTA
Tris	Tris-(hydroxymethyl) aminomethane
U	Unit(s)
UV	Ultra Violet
V	Volts
w/	With
(w/v)	Weight: volume ratio
X	Times concentrated

CHAPTER 1

INTRODUCTION

1.1 TAXONOMY AND HISTORICAL BACKGROUND

Bacteria belonging to the genus *Campylobacter* were originally referred to as "microaerophilic vibrios" and were first described in 1913 by McFadyean and Stockman (Véron and Chatelain, 1973; Karmali and Skirrow, 1984). They implicated these organisms as agents of abortion in sheep. Several years later in 1918 Smith isolated similar organisms from aborted bovine fetuses (Karmali and Skirrow, 1984). These organisms were named *Vibrio fetus* by Smith and Taylor in 1919 (Karmali and Skirrow, 1984). Sebald and Véron proposed the genus name *Campylobacter* (Greek for "curved rod") in 1963 to reflect the fundamental differences between these organisms and other Vibrios (Véron and Chatelain, 1973; Karmali and Skirrow, 1984).

"Microaerophilic vibrios" were first associated with human disease in the 1940's. In 1946, Levy described a large institutional outbreak of gastroenteritis. "Microaerophilic vibrios" were isolated from blood cultures of 13 patients. Levy suggested that these organisms were identical to *Vibrio jejuni* described by Jones and collegues in 1931 (Karmali and Skirrow, 1984). It was not until the 1970's that Butzler and co-workers isolated *Campylobacter* from the feces of children with acute enterocolitis, thus establishing a definite link between these organisms and diarrhoeal disease (Goossens and Butzler, 1992).

The taxonomy of the genus *Campylobacter* has been extensively revised, particularly over the past decade. Table 1.1 shows the 15 *Campylobacter* species and 6 subspecies which are currently recognised. Early reviews of *Campylobacter* history and taxonomy were contributed by Karmali and Skirrow (1984), and Penner (1988) and an extensive review of *Campylobacter* taxonomy was published by On in 1996.

1.2 *CAMPYLOBACTER* BIOLOGY

Campylobacteria are small (1.5-6.0μM long and 0.2-0.5μM wide) curved or S-shaped Gram negative bacteria (Penner, 1988; Ketley, 1997). They generally have a single polar

flagellum or a flagellum at each end (Penner, 1988). *Campylobacter jejuni* and *Campylobacter coli* are microaerophilic, requiring 3-15% oxygen and 3-5% carbon dioxide, and are thermophilic, growing best at 42°C (Ketley, 1997).

Table 1.1 Taxonomic position of species of the genus *Campylobacter*[a]

Taxon	Former Taxon
Campylobacter fetus subsp. *fetus*	*Campylobacter fetus* subsp. *intestinalis*
Campylobacter fetus subsp. *venerealis*	*Campylobacter fetus* subsp. *fetus*
Campylobacter hyointestinalis subsp. *hyointestinalis*	—
Campylobacter hyointestinalis subsp. *lawsonii*	—
Campylobacter concisus	—
Campylobacter mucosalis	*Campylobacter sputorum* subsp. *mucosalis*
Campylobacter sputorum bv. sputorum	incorporating biovar bubulus
Campylobacter sputorum bv. fecalis	*Campylobacter fecalis*
Campylobacter curvus	*Wolinella curva*
Campylobacter rectus	*Wolinella rectus*
Campylobacter showae	*Wolinella curva* subsp. *intermedius*
Campylobacter gracilis	*Bacteroides gracilis*
Campylobacter upsaliensis	Catalase negative or weak *Campylobacter*
Campylobacter helveticus	—
Campylobacter hyoilei	—
Campylobacter coli	*Vibrio coli*
Campylobacter jejuni subsp. *jejuni*	*Vibrio jejuni*
Campylobacter jejuni subsp. *doylei*	—
Campylobacter lari	*Campylobacter laridis*

[a] Data was obtained from On (1996).

1.3 CAMPYLOBACTERIOSIS

Campylobacteriosis is the most common cause of bacterial diarrhoea in humans in the world (Taylor, 1992). *Campylobacter jejuni* subsp. *jejuni* (referred to as *C. jejuni*) is the main pathogen, accounting for over 95% of infections (Lane *et al.*, 1993). *Campylobacter coli* accounts for less than 5% of infections in developed countries (Taylor, 1992). The proportion of infections due to *C. coli* can be much higher in developing than developed countries (Taylor, 1992).

Other species associated with diarrhoea in humans include *Campylobacter lari*, *Campylobacter concisus*, *Campylobacter hyointestinalis*, *Campylobacter fetus* subsp. *fetus* (referred to as *C. fetus*) , *Campylobacter upsaliensis*, *Campylobacter curvus* and *Campylobacter sputorm* biovar sputorum (On, 1996).

1.3.1 Epidemiology of Campylobacteriosis

Campylobacteriosis is currently the most commonly notified communicable disease in New Zealand. A total of 7,710 cases were reported in 1996 and this corresponds to a rate of 229 cases per 100,000 population (Anonymous, 1997). Since Campylobacteriosis became a notifiable disease in New Zealand in 1980, the number of cases reported has increased steadily until 1993 when a record rate of 240 cases/100,000 was reported (Eberhart-Phillips *et al.*, 1996). The rate of notifications far exceeds that of other developed countries but the causes for New Zealands high incidence rates are not known.

The incidence of Campylobacteriosis in New Zealand has a bimodal age distribution with peaks in the 0-4 year age group and 20-29 year age group (Lane *et al.*, 1993; Eberhart-Phillips *et al.*, 1996). Isolation rates are slightly higher for males than for females (Lane *et al.*, 1993). These patterns are seen in most developed countries. In the developing world the peak isolation rate occurs in children less than 2 years of age (Skirrow and Blaser, 1992). Notifications show a seasonal increase over the Spring and Summer months from September to March (Lane *et al.*, 1993; Eberhart-Phillips *et al.*, 1996). Similar seasonal variation is reported in other countries in temperate regions (Skirrow and Blaser, 1992). However, seasonal trends are less evident in tropical and subtropical countries. In the developed world the majority of cases are sporadic (Lane *et al.*, 1993).

However, outbreaks have been associated with the consumption of unpasteurised milk and untreated water (Lane *et al.*, 1993; Eberhart-Phillips *et al.*, 1996). *Campylobacter* infections are endemic in developing countries and outbreaks are rarely reported (Skirrow and Blaser, 1992).

1.3.2 Sources of Infection and Transmission

Although the sources of *Campylobacter* infections are not known, epidemiological evidence has linked Campylobacteriosis to the consumption of incompletly cooked poultry, unpasteurised milk, untreated water and direct contact with infected animals (Tauxe, 1992). Campylobacters are part of the natural intestinal flora of a wide variety of wild and domestic birds and animals and *C. jejuni* frequently contaminates foods derived from animals, as a result of fecal contact during processing (Ketley, 1997). A 1992/1993 New Zealand study revealed that 51.2% of raw poultry and poultry products were found to be positive for *C. jejuni* (Gilbert, 1993). However, eating chicken carries a risk only if it is undercooked, as campylobacters are killed by modest heat (Skirrow and Blaser, 1992). Cross-contamination from raw chicken to other foods during storage and preparation could also be an important source of infection.

Unpasteurised milk is likely to become contaminated through faecal contamination and also bovine mastitis caused by *C. jejuni* (Tauxe, 1992). Waterborne outbreaks of Campylobacteriosis have been associated with unchlorinated water and with unhygenic water supplies which may have been contaminated with bird faeces (Skirrow and Blaser, 1992; Tauxe, 1992).

Domestic pets, particularly puppies with diarrhoea, have been implicated in the transmission of *C. jejuni* to humans (Stern, 1992). Direct contact with domestic farm animals and their faeces may also be important in transmitting infection. *Campylobacter* enteritis is also a cause of travellers' diarrhoea (Taylor, 1992).

ESR- Health conducted a large case-control study from June 1994 to February 1995 to identify the contributions of major risk factors for *Campylobacter* infection in New Zealand. The study found that the consumption of incompletely cooked poultry, unpasteurised milk, untreated water and contact with animals were associated with an increased risk of infection (Eberhart-Phillips *et al.*, 1996).

1.3.3 Clinical Illness

C. jejuni has a low infectious dose with as few as 500 cells sufficient to cause illness in an adult male (Humphrey, 1995). The incubation period of acute disease is usually 3-4 days but may be as long as 10 days (Lane *et al.*, 1993). The predominant symptoms of Campylobacteriosis are diarrhoea, which is often bloody, abdominal pain, malaise, and fever lasting 2-5 days in mild cases (Lane *et al.*, 1993; Humphrey, 1995). The illness is usually self-limiting although infection can result in a prolonged or severe illness (Lane *et al.*, 1993). Complications are uncommon but about 1% of patients suffer from reactive arthritis (Skirrow and Blaser, 1992; Humphrey, 1995). A smaller percentage of cases develop Guillain-Barré syndrome (Skirrow and Blaser, 1992).

Campylobacter infection is milder in developing than developed countries (Taylor, 1992). Asymptomatic infections are common in developing countries (Skirrow and Blaser, 1992) and the usual clinical manifestation of symptomatic infection is profuse watery diarrhoea (Tauxe, 1992). The differences in the age distribution and disease expression in developing countries is likely to be a result of the early acquisition of immunity by children exposed to endemic *Campylobacter* infection (Skirrow and Blaser, 1992).

1.3.4 Pathogenesis

Campylobacteriosis in humans is primarily associated with the colonisation of the lower intestine (Humphrey, 1995). A combination of the flagellum and cell shape allow the organisms to penetrate the thick mucous blanket covering the intestinal epithelium. Following colonisation of the mucous the campylobacter cells adhere to the epithelial cell surface. There are several mechanisms by which the organisms may damage epithelial cells which leads to diarrhoea (Ketley, 1997). The presence of blood in stools suggests that campylobacter invade epithelial cells, and in doing so cause damage (Humphrey, 1995). The production of toxins may contribute to the disease. Campylobacters have been reported to produce a cholera-like toxin and several cytotoxins, although this finding is controversial. Intestinal inflammation following tissue damage may also indirectly lead to fluid loss. These mechanisms are not mutually exclusive and any combination may have a role in infection (Ketley, 1997).

1.3.5 Diagnosis and Treatment

Campylobacter infection of humans is usually diagnosed by the microbiological examination of faecal specimens (Humphrey, 1995). This is achieved by direct plating onto *Campylobacter*-selective agar with incubation in a microaerophilic atmosphere for 2-3 days at 37-42°C (Humphrey, 1995). Selective media such as Skirrow medium, Campy-BAP and Karmali charcoal selective medium have been used for this purpose (Goossens and Butzler, 1992).

Campylobacteriosis is usually self-limiting and the majority of cases do not require treatment (Doyle and Jones, 1992). However, if symptoms are prolonged treatment with erythromycin is recommended. Treatment with erythromycin will eradicate the organisms from stools within 2-3 days (Lane *et al.*, 1993).

1.3.6 Control and Prevention

The basic measures that need to be taken for the prevention of Campylobacteriosis are the provision of treated water supplies, pasteurisation of any milk sold to the public and the hygenic disposal of sewage (Skirrow and Blaser, 1992). The public needs to be educated on the importance of the thorough cooking of meats and poultry, washing hands after contact with animals and raw foods, and the avoidance of unpasteurised dairy products (Eberhart-Phillips *et al.*, 1996).

A number of studies have been conducted to try and reduce campylobacter infection of poultry. Several studies have examined the use of competitive exclusion as a means of preventing *C. jejuni* colonisation of newly hatched chickens. The selective breeding of broiler chickens on the basis of their resistance to *C. jejuni* colonisation may also be an advantage (Stern, 1992).

Data from a number of countries have demonstrated that broiler chickens are not *Campylobacter* positive upon hatching but acquire campylobacters during the second or third week of life. There is some debate over the sources of *Campylobacter* infection in broiler flocks. One potential source for these organisms is drinking water. Untreated wells are frequently used as a source of water for broiler flocks. *Campylobacter*

contamination of the well reservoir could initiate flock contamination (Stern and Robach, 1997). The use of untreated water is common among broiler farms in New Zealand (*Campylobacter* Working Group, personal communication). A recent study by Stern and Robach (1997) examined the effects that chlorination of drinking water had on the frequency of campylobacter colonisation of broiler flocks. Campylobacters are susceptible to 2ppm of chlorine however, drinking water levels of 2-5ppm did not reduce the frequency or level of contamination of the birds. Incidently, the Palmerston North municipal water supply contains only 0.5ppm of chlorine (Toka, personal communication). A study in Switzerland indicated that water is not the principle source of *Campylobacter* contamination of broiler flocks (Jemmi *et al.*, 1997). Other sources of infection, such as insects, may be responsible for broiler flock contamination.

1.4 TYPING METHODS

Typing methods that can discriminate between strains are important in understanding the epidemiology of *Campylobacter* infections. Such typing methods would be useful in determining the sources of campylobacter infections in humans as well as poultry and other animals. Numerous typing schemes have been developed including both phenotypic and genotypic methods although many of these are not suitable for routine use.

1.4.1 Phenotypic Methods

1.4.1.1 Biochemical Identification

Campylobacter species can be identified through a series of phenotypic tests, such as tests for: tolerance to antimicrobial compounds; growth temperature and biochemical characteristics. Differentiation of *C. jejuni* from *C. coli* relies on one test for hippurate hydrolysis and atypical reactions in this test may result in incorrect identification (Goossens and Butzler, 1992). DNA-hybridisation studies have demonstrated the existence of hippuricase negative *C. jejuni* (Roop *et al.*, 1984).

Skirrow and Benjamin developed a biotyping scheme to distinuish *C. jejuni*, *C. coli* and *C. lari* (Patton and Wachsmuth, 1992). The scheme includes tests for hippurate hydrolysis, resistance to naladixic acid and rapid H_2S production (Patton and Wachsmuth, 1992).

The Preston biotyping scheme, developed by Bolton and collegues employs 12 tests, including growth at 28°C, hippurate hydrolysis and 10 resistotyping tests. The resistotyping tests determine the susceptibility of *Campylobacter* species to a selection of antibiotics and chemicals. A four-figure biotype code is generated for each isolate which indicates both species and biotypes (Bolton *et al.*, 1984).

The most commonly used biotyping scheme is that of Lior and co-workers which differentiates *C. jejuni* into 4 biotypes and *C. coli* and *C. lari* into two biotypes each. The scheme involves tests for hippurate hydrolysis, H_2S production and DNase production (Lior *et al.*, 1984).

Considerable concern (Goossens and Butzler, 1992; On and Holmes, 1991a, 1991b, and 1992) has been expressed over the lack of standardisation of phenotypic tests. Most workers perform the tests by methodologies unique to their own laboratories (On and Holmes, 1991a). The reproducibility of certain tests is dependant upon both the inoculum size and the composition of the basal medium (On and Holmes, 1991b). Biotyping is a logical starting point for determining *Campylobacter* species. However, it is of little use as an epidemiological tool and is recommended for use with other typing methods (Patton *et al.*, 1991).

1.4.1.2 Phage Typing

In 1985, Grajewski and coworkers developed a phage typing scheme for *C. jejuni* and *C. coli* using a set of 14 lytic bacteriophages isolated from poultry faeces (Patton and Wachsmuth, 1992; Wareing *et al.*, 1996). Subsequently Lior and co-workers extended the scheme to incorporate an additional 11 new phages (Patton and Wachsmuth, 1992). Salama and collegues developed the Preston phage typing scheme in 1990 (Wareing *et al.*, 1996). This scheme utilises 6 phages of the Grajewski scheme and incorporates 10 new phages which were isolated in the United Kingdom. Phage typing is a reasonably simple and reproducible means of typing *Campylobacter* isolates, and is recommended for use with other typing methods (Patton and Wachsmuth, 1992).

1.4.1.3 Serotyping

Serotyping is the most commonly used method of distinguishing between *Campylobacter* strains. The serotyping scheme of Penner and Hennessey (1980) detects the O (formerly heat stable) lipopolysaccharide antigens. This method identifies 66 *Campylobacter* serotypes through passive hemagglutination (Lastovica, 1996). The heat-labile (HL) serotyping scheme of Lior and co-workers (1982) identifies 108 serotypes through slide agglutination (Patton and Wachsmuth,1992). The flagellum was originally thought to be the serodeterminant of the heat-labile system. However, Alm and co-workers (1992) have recently showed that while flagellum can be the serodeterminant, in most isolates non-flagellar antigens were the serodeterminants.

Serotyping is relatively simple and rapid to perform and provides a high level of discrimination between strains (Patton *et al.*, 1991). However, a significant number of strains are non-typable by either scheme and some isolates react with two or more antisera (Patton and Wachsmuth, 1992). On subculture, some serotypeable isolates tend to become non-typeable and *vice versa*. This may be due variations in the expression or presentation of antigens (Mazurier *et al.*, 1992).

The main disadvantage of serotyping is that the antisera are not available commercially and are expensive and time-consuming to produce (Patton *et al.*, 1991). Therefore, only a few reference laboratories can provide serotyping data.

1.4.2 Genotyping

Genotypic typing methods have an important advantage over phenotypic methods. They measure relatively stable chromosomal differences whereas phenotyping methods measure characteristics such as enzymes and anitgens which may not be stably expressed (Patton *et al.*, 1991).

1.4.2.1 Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis (MEE) characterises isolates by the relative mobility of a range of cellular enzymes (Selander *et al.*, 1986; Patton and Wachsmuth, 1992)

Enzyme mobility differences are assumed to relate directly to allelic variation in the structural gene locus for each enzyme. MEE provides an advantage over other genotypic methods in that data can be analysed to determine the genetic relatedness among the isolates (Selander *et al.*, 1986). MEE provides a high level of discrimination. However, this method is complex and time consuming to perform and is therefore not practical for routine use (Patton and Wachsmuth, 1992).

1.4.2.2 Restriction Endonuclease Analysis

Several studies have investigated the use of restriction endonucleases to distinguish between *Campylobacter* strains (Collins and Ross, 1984; Kakoyiannis *et al.*, 1984; Owen *et al.*, 1989; Fox *et al.*, 1989; Patton *et al.*, 1991; Lind *et al.*, 1996). The restriction patterns observed with enzymes such as *Hae*III, *Hin*dIII, *Bst*EII, *Xho*I and *Bgl*I appear to be highly stable and provide a high level of discrimination. One disadvantage of this method is that the restriction patterns contain a large number of fragments, making them difficult to compare (Patton *et al.*, 1991). Another disadvantage is that this method requires a large amount of intact chromosomal DNA, which can be difficult to produce.

1.4.2.3 Ribotyping

Ribotyping is based on the detection of restriction fragment length polymorphisms containing ribosomal RNA (rRNA) genes (Owen *et al.*, 1993b). In this method restriction endonuclease digests are Southern blotted onto nitrocellulose and hybridised with a biotinylated probe. The level of discrimination is dependant upon the choice of both the enzyme and the probe for hybridisation (Patton and Wachsmuth, 1992). Restriction endonucleases such as *Hin*dIII, *Hae*III, *Xho*I, *Bgl*I, *Pvu*II and *Pst*I have been used in ribotyping studies . Two of the probes that have been used for this purpose include an *E. coli* 16S + 23S rRNA probe and a *C. jejuni* 16S rRNA probe (Owen *et al.*, 1993b; Moreau *et al.*, 1989; Owen *et al.*, 1990; Patton *et al.*, 1991; Gibson *et al.*, 1995; Owen and Hernandez, 1996). The advantage of this technique over restriction endonuclease analysis is that the number of bands in the patterns are greatly reduced making them much easier to interpret. However, ribotyping is complex and time consuming to perform (Owen and Hernandez, 1993).

1.4.2.5 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) involves the digestion of chromosomal DNA with an appropriate restriction endonuclease such as *Sma*I or *Kpn*I. The resulting large molecular weight fragments are separated on a contour-clamped homogeneous electric field (CHEF) system (Yan *et al.*, 1991; Salama *et al.*, 1992; Rennie *et al.*, 1994; Fujita *et al.*, 1995; Gibson *et al.*, 1995; Owen *et al.*, 1995). PFGE analysis of *Campylobacter* isolates results in relatively simple restriction profiles providing a high level of discrimination (Yan *et al.*, 1991; Gibson *et al.*, 1995). However, this method is also time-consuming and complex to perform.

1.4.2.5 RAPD PCR

Mazurier and collegues (1992) described a PCR-based method of typing *Campylobacter* isolates. Randomly amplified polymorphic DNA (RAPD) profiles were generated using a set of randomly designed 10-mer primers. Subsequent studies by Fayos and coworkers (1993) and Hernandez and coworkers (1995) have demonstrated that RAPD profiles provide a high level of discrimination between strains and are easy to compare visually. However, Hernandez and collegues (1995) described a lack of interlaboratory reproducibility with this method.

1.4.2.6 RFLP Analysis of *Campylobacter* Flagellin Genes

The *Campylobacter* flagellum is an important virulence determinant. The flagellum imparts high motility on the bacterium and plays an important role in the colonisation of the viscous mucous lining of the gastrointestinal tract (Guerry *et al.*, 1990).

The flagellin genes have been cloned and characterised for several strains (Logan *et al.*, 1989; Nuijten *et al.*, 1989; Fischer and Nachamkin, 1991). The *C. coli* VC167 chromosome contains two tandemly orientated flagellin genes termed *fla*A and *fla*B. The genes share 93% sequence homology and are thought to have arisen as a result of gene duplication. The genes are under the control of separate and distinct promoters (Guerry *et al.*, 1990). The flagellar filament contains both FlaA and FlaB proteins, although the FlaA protein is produced in far greater amounts than FlaB (Guerry *et al.*, 1991). Mutants

that can only express the *fla*A gene product produce a flagellar filament that is visually indistinguishable from the wild-type filament. Mutants that can express only the *fla*B gene produce a severly truncated filament that imparts only partial motility to the cell (Alm *et al.*, 1992). The flagellin genes of *C. jejuni* 81116 and IN1 show similar organisation. However, the *fla*B gene product is not expressed in *C. jejuni* (Nuijten *et al.*, 1989; Fischer and Nachamkin, 1991).

Tandem gene duplications are usually rapidly eliminated in bacteria by intragenic recombination. The conservation of the tandem arrangement in *Campylobacter* suggests that this property must endow the bacterium with a strategic advantage (Alm *et al.*, 1993a).

Several studies have examined the use of restriction fragment length polymorphism (RFLP) analysis of the flagellin genes as a method of discriminating between *Campylobacter* isolates (Alm *et al.*, 1993a; Nachamkin *et al.*, 1993; Owen *et al.*, 1993; Owen *et al.*, 1994; Mohran *et al.*, 1996; Nachamkin *et al.*, 1996; Santesteban *et al.*, 1996). The outer regions of the genes are conserved whereas the internal regions are variable (Alm *et al.*, 1993a). Primers designed to the conserved regions can be used to PCR-amplify the genes. The amplicons can then be digested with restriction endonucleases to generate RFLP's. The majority of studies have examined polymorphisms within the *fla*A gene. Studies by Alm's group (1993b) and Mohran and coworkers (1996) involved RFLP analysis of both the *fla*A and *fla*B genes. The *fla*B gene was present in all isolates tested and in most cases produced identical polymorphic patterns to those found with the *fla*A gene. RFLP analysis of the flagellin genes is a relatively quick and simple and provides a high level of discrimination between strains.

This study aimed to establish a simple and reliable method for typing *Campylobacter* isolates in order to investigate the sources of *Campylobacter* infections in humans in New Zealand.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

All chemicals were supplied by BDH Chemicals Ltd. unless stated otherwise.

2.1.1 Charcoal Selective Medium (CSM) (Karmali *et al.*, 1986)

1) Mixture A:

Columbia Agar (Oxoid)	22.0g
Activated charcoal	2.0g
0.32% Hematin stock solution	5.0ml
Distilled water	485ml

2) Mixture B:

Sodium Pyruvate (Prolabo)	50mg
Vancomycin (DBL)	10mg
Cefoperozone (Sigma)	16mg
Cyclohexamide (Sigma)	50mg
Distilled water	10ml

Mixture A was autoclaved at 121°C for 15 mins and allowed to cool to 45°C; mixture B (filter sterilized) was then added to A and the pH was adjusted to 7.4.

2.1.2 Hematin Stock Solution

0.32g hematin was dissolved in 10ml 0.1M NaOH. Distilled water was added to 100ml.

2.1.3 Charcoal Non-Selective Medium (CNSM)

1) Mixture A:

Columbia Agar (Oxoid)	22.0g
Activated charcoal	2.0g
0.32% Hematin stock solution	5.0ml

	Distilled water	485ml

2) Mixture B:

Sodium Pyruvate (Prolabo)	50mg
Distilled water	10ml

Mixture A was autoclaved at 121°C for 15 mins and allowed to cool to 45°C; mixture B (filter sterilized) was then added to A and the pH was adjusted to 7.4.

2.1.4 Glycerol Broth

Brain heart infusion broth (Difco)	323g
Glycerol	150ml
Distilled water to	1000.0ml

Dispensed into 2ml aliquots and autoclaved at 121°C for 15mins.

2.1.5 Catalase Production Test

1) 3% Hydrogen Peroxide

2.1.6 Antibiotic Sensitivity Test

1) Heart Infusion Broth

Heart Infusion Broth (Difco)	25.0g
Distilled water	1000.0ml

Dispensed into 2ml aliquots and autoclaved at 121°C for 15mins.

2) 30µg Cephalothin Discs (Oxoid)

3) 30µg Naladixic Acid Discs (Oxoid)

4) Sterile Swabs

5) CNSM Agar

2.1.7 Nitrate Reduction Test

1) *Campylobacter* Nitrate Broth

Heart Infusion Broth (Difco)	25.0g
Potassium nitrate (May and Baker)	2.0g
Distilled water	1000.0ml

Dispensed into 4ml aliquots and autoclaved at 121°C for 15mins.

2) Nitrate Reagent A

Sulfanilic acid (Acros)	8.0g
5N acetic acid	1000.0ml

3) Nitrate Reagent B

dimethyl-alpha-naphthylamine (Aldrich)	6ml
5N acetic acid	1000.0ml

2.1.8 Hippurate Hydrolysis Test

1) Sodium Hippurate 1% (w/v)

2) Ninhydrin Reagent

3.5% ninhydrin dissolved in a 1:1 mixture of actone:butanol.

2.1.9 H_2S Production Test

1) Triple Sugar Iron Slopes

Triple Sugar Iron Agar (Difco)	65.0g
Distilled water	1000.0ml

Dispensed 10ml aliquots into 20/150mm tubes. Autoclaved at 121°C for 10mins and placed on a slant while cooling.

2.1.10 Indoxyl Acetate Hydrolysis Test

1) Indoxyl Acetate Solution

10% (w/v) indoxyl acetate (Sigma) in acetone.

2) Sterile Blank Concentration Discs (Difco)

2.1.11 DNase Production Test

1) DNase test agar with methyl green

DNase test agar w/ methyl green (Difco)	42g
Distilled water	100.0ml

Autoclaved at 121°C for 15mins.

2.1.12 DNA Extractions

1) TE Buffer

1M Tris-HCl (pH 8.0)	10ml
0.2M EDTA (pH 8.0)	5ml
Distilled water to	1000.0ml

2) 0.1M NaOH

3) 0.1M KH_2PO_4

4) Proteinase K 20 mg/ml (Boehringer Mannheim)

Dissolved in buffer containing 50mM Tris-HCl, 62.5mM EDTA.

5) Sodium Dodecyl Sulphate (SDS) 10% (w/v)

6) 5M Sodium Perchlorate

7) Phenol/Chloroform/Iso-amylalcohol

A 25:24:1 ratio respectively, stored in the dark at 4°C (phenol, equillibrated and ultrapure, USB).

8) Chloroform/Iso-amylalcohol

A 24:1 ratio respectively.

9) 5M NaCl

10) Absolute Ethanol

Stored at -20°C

11) Phosphate Buffered Saline (pH 7.3)

NaCl	8.0g
K_2HPO_4	1.21g
KH_2PO_4	0.34g
Distilled water to	1000.0ml

12) Lysozyme 40 mg/ml (Sigma)

Dissolved in buffer containing 25mM Tris-HCl, 50mM glucose, 10mM EDTA (pH 8.0).

13) Tween 20 Lysis solution (pH 8.0)

2% Tween 20 (Serva)

50mM Tris-HCl

62.5mM EDTA

14) Sodium Dodecyl Sulphate (SDS) 20% (w/v)

15) Isopropanol

16) 70% Ethanol

17) Hexadecyltrimethyl ammonium bromide (CTAB) /NaCl

10% CTAB

0.7M NaCl

4.1g of NaCl was dissolved in 80ml of water. CTAB was added slowly while heating and stirring. The final volume was adjusted to 100ml.

2.1.13 Determination of DNA Concentration

1) 10X TNE Buffer

Tris Base (Gibco BRL)	12.1g
EDTA (disodium) (Univar)	3.7g
NaCl	58.4g
Distilled water to	1000.0ml

The pH was adjusted to 7.4 with HCl. The buffer was filtered and stored at 4°C.

2) Hoescht Stock Solution

Hoescht 33258	10.0mg
Distilled water	10.0ml

Stored at 4°C protected from light.

3) Working Dye Solution

Hoescht stock solution	10.0µl
Distilled water	10.0ml

Stored at room temperature. Prepared fresh daily.

4) Calf thymus DNA stock solution 1mg/ml

As supplied by the manufacturer (Hoescht).

2.1.14 Restriction Endonuclease Digestion of DNA

1) Restriction Endonucleases 10U/µl

*Hae*III

*Cla*I

*Pst*I

*Hin*dIII

As supplied by the manufacturer (Gibco BRL).

2) 10X Restriction Buffers

As supplied by the manufacturer (Gibco BRL).

3) Sterile Distilled Water

4) 10X Bromophenol Blue Dye

10% Glycerol

62.5mM Tris

0.1% Bromophenol Blue

The pH was adjusted to 7.4.

2.1.15 Agarose Gel Electrophoresis

1) 10X TAE Buffer

Tris Base	72.6g
EDTA (Univar)	11.2g
Glacial Acetic Acid	17.1ml
Distilled water to	1000.0ml

The pH was adjusted to 8.5. The buffer was diluted 1/10 for electrophoresis.

2) 10X E-Buffer

Tris Base	48.44g
EDTA (disodium) (Univar)	3.72g
Sodium Acetate (Univar)	4.1g

The pH was adjusted to 7.8 with glacial acetic acid and distilled water was added to a final volume of 1000.0ml. The buffer was diluted 1/10 with distilled water for electrophoresis.

3) Agarose Ultrapure DNA grade (Biorad).

Agarose gels were prepared according to the method of Sambrook *et al* (1989).

4) λ *Hin*dIII Ladder

5) 1kb Ladder (Gibco BRL) 0.1µg/µl

6) 10bp Ladder (Gibco BRL) 0.1µg/µl

7) Ethidium Bromide 5µg/ml

2.1.16 RAPD PCR

1) 10X PCR Buffer

As supplied by the manufacturer (Gibco BRL).

2) $MgCl_2$ 50mM

As supplied by the manufacturer (Gibco BRL).

3) dNTP's 1.25mM (Promega)

4) Oligonucleotide Primer

The oligonucleotide primer OPA11 was synthesised commercially (Gibco BRL).

OPA11 Sequence: 5' CAA TCG CCG T 3'

5) *Taq* DNA Polymerase 5U/µl

As supplied by the manufacturer (Gibco BRL).

6) Sterile Distilled Water

7) Lp1 DNA

DNA extracted from *Epichloe typhina-N.lolli* hybrid from *Lolium perenne*.

2.1.17 Flagellin Gene PCR

1) 10X PCR Buffer

Contains 15mM $MgCl_2$. As supplied by the manufacturer (Qiagen).

2) $MgCl_2$ 25mM

As supplied by the manufacturer (Qiagen).

3) dNTP's 1.25mM (Promega)

4) Oligonuleotide Primers 10µM

The following primers were commercially synthesised (Gibco BRL).

pg50	5' ATG GGA TTT CGT ATT AAC 3'
RAA19	5' GCA CC(CT) TTA AG(AT) GT(AG) GTT ACA CCT GC 3'
RAA9	5' AAG GAT TTA AAA TGG GTT TTA GAA TAA ACA CC 3'

5) C9 DNA

DNA extracted from sample C9.

6) *Taq* DNA Polymerase 5U/µl

As supplied by the manufacturer (Qiagen).

2.2 METHODS

2.2.1 Cultural Conditions

Campylobacter isolates were grown on charcoal selective medium (CSM). The plates were incubated at 37°C for 48 hours in BBL Gaspak Jars in a microaerophilic atmosphere containing 5% oxygen, 5% carbon dioxide, 10% hydrogen and 80% nitrogen. These conditions were achieved by evacuating the air from the jars and replacing it with a mixture of carbon dioxide, hydrogen and nitrogen. 5% oxygen was added to the jar using a syringe.

2.2.2 Preservation of Cultures

A single colony was plated out onto two CNSM plates. The plates were incubated at 37°C for 48 hours under microaerophilic conditions. The cells were scraped off the plates and were resuspended in 2ml of glycerol broth. 0.5ml aliquots of the cell suspension were transfered into glass vials. The vials were stored at -70°C.

2.2.3 Catalase Production Test

A drop of 3% hydrogen peroxide was applied to a *Campylobacter* colony. The formation of bubbles indicated a positive reaction (Skirrow and Benjamin, 1980).

2.2.4 Antibiotic Sensitivity Tests

A large loopful of growth was scraped off a CNSM plate and suspended in 2ml heart infusion broth to a turbidity matching a McFarlane no. 1 standard. A sterile swab was

used to streak the culture in three different directions over a CNSM plate. A 30µg naladixic 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 the sensitivity of the isolate to the antibiotic whereas growth right up to the disc was indicative of resistance (Karmali *et al,* 1980).

2.2.5 Nitrate Reduction Test

A large loopful of growth was suspended in 4ml of nitrate broth. The broth was incubated at 37°C for 72 hours under microaerophilic conditions. Three drops each of nitrate reagents A and B were added. The development of a red colour indicated that the isolate had reduced nitrates to nitrites (MacFaddin, 1980).

2.2.6 Hippurate Hydrolysis Test

A large loopful of growth was suspended in 0.5ml of sodium hippurate solution (1%). The suspension was incubated in a waterbath at 37°C for 2 hours. 0.2ml of ninhydrin reagent was added and the tube was returned to the waterbath for a further 10 minutes. The development of a deep purple colour indicated that hippurate hydrolysis had occured. A faint purple colour or no colour change indicated a negative result (Hwang and Ederer, 1975).

2.2.7 H_2S Production Test

A straight sterile wire was used to streak the slope and stab the butt of a triple sugar iron slope. The slope was incubated at 37°C under microaerophilic conditions for 7 days. The slope was examined daily for blackening of the butt, which indicated the production of H_2S (Barrett and Clark, 1987).

2.2.8 Test for Growth at 25°C and 42°C

A 48 hour culture of *Campylobacter* growth was streaked out onto two CNSM plates. One of the plates was incubated at 25°C, and the other at 42°C under microaerophilic conditions for 48 hours. The plates were examined for the presence of growth.

2.2.9 Indoxyl Acetate Hydrolysis Test

A 50µl aliquot of indoxyl acetate solution (10%) was placed onto a blank concentration disc. After the disc was allowed to dry at room temperature, it was inoculated with a large loopful of growth. A dark blue colour change within 5 -10 minutes indicated the hydrolysis of indoxyl acetate (Mills and Gherna, 1987).

2.2.10 DNase Production Test

Two circular areas of a DNase (methyl green) agar plate were inoculated with a large loopful of growth. The plates were incubated at 37°C and were examined daily for 5 days. Clear colourless zones around the inoculum indicated a positive result (Lior and Patel, 1987).

2.2.11 DNA Extraction Method #1

This method was based on that of Wilson and co-workers (1987). A *Campylobacter* isolate was plated out onto two CNSM plates and incubated for 48 hours at 37°C under microaerophilic conditions. The *Campylobacter* cells were scraped off the plates and resuspended in 1.5ml of PBS in an eppendorf tube. The tube was centrifuged for 5 minutes at 3000rpm to pellet the cells. After centrifugation, the supernatant was discarded and the pellet was resuspended in 567µl of TE buffer. 60µl of proteinase K (20mg/ml) and 30µl of SDS (10%) were added and the solution was mixed by inversion. The tubes were incubated for 90 minutes at 37°C. 100µl of NaCl and 80µl of CTAB/NaCl were added and the solution was mixed and incubated at 65°C for 10 minutes.

An equal volume of chloroform/iso-amylalcohol (24:1) was added and gently mixed by inversion. The solution was centrifuged for 5 minutes at 3000rpm. The upper aqueous

phase was transfered to a fresh eppendorf tube. An equal volume of phenol/chloroform/iso-amylalcohol (25:24:1) was added and gently mixed. The solution was centrifuged for 10 minutes at 3000rpm. The upper aqeous phase was transfered to another eppendorf tube and the DNA was precipitated with 0.6 volumes of isopropanol. The tube was centrifuged for 10 minutes at 3000rpm to pellet the DNA. The pellet was washed with 70% ethanol and centrifuged for another 10 minutes. The ethanol was removed and the pellet was left at room temperatue to dry. The dry pellet was resuspended in 100µl of TE buffer and placed at 65°C to dissolve.

2.2.12 DNA Extraction Method #2

This method was based on that of Fox and collegues (1989). *Campylobacter* cells were harvested off two CNSM plates as previously described. The cells were resuspended in 1ml PBS in an eppendorf tube. The tube was centrifuged for 5 minutes at 3000rpm to pellet the cells. The pellet was resuspended in 500µl of lysozyme (40mg/ml) mixed, and left on ice for 15 minutes. 500µl of Tween 20 lysing mix was added followed by two 100µl aliquots of SDS (20%). 100µl of proteinase K (20mg/ml) was added and the solution was incubated on ice for two hours.The solution was then incubated overnight at 50°C.

Half of the lysate was transfered to a fresh eppendorf tube. Both tubes were extracted with chloroform/iso-amylalcohol (24:1) and phenol/chloroform/iso-amylalcohol (25:24:1) as previosly described. The supernatants from the final extraction were combined into a single eppendorf tube. The DNA was precipitated by adding 0.6 volumes of isopropanol. The tube was gently rocked back and forth until a stringy white precipate became visible. The tube was centrifuged at 3000rpm for 10 minutes to pellet the DNA. The pellet was washed in cold 70% ethanol and centrifuged for 5 minutes. The ethanol was removed and the pellet was left at room temperature to dry. The DNA was resuspended in 100µl of TE buffer and placed at 65°C to dissolve.

2.2.13 DNA Extraction Method #3 (Ionas, personal communication)

Campylobacter cells were harvested off two CNSM plates as previously described. The cells were resuspended in 1ml of TE buffer in an eppendorf tube. The tube was centrifuged for 6 minutes at 3000rpm to pellet the cells. The supernatant was discarded

and the tube was flicked to dislodge the pellet. The pellet was frozen at -70°C for 15 minutes and then thawed at 56°C.

200µl of 0.1M NaOH was added and mixed by inversion. 400µl of KH_2PO_4 was added and mixed. 100µl of proteinase K (20mg/ml) and 60µl of SDS (10%) were added, the tube was mixed and incubated at 56°C overnight.

175µl of 5M sodium perchlorate was added and the solution was incubated at 56°C for one hour. The solution was extracted twice with phenol/chloroform/iso-amylalcohol (25:24:1) as previously described. The supernatant was extracted once with chloroform/iso-amylalcohol (24:1). The supernatant was transfered to a fresh eppendorf tube and the DNA was precipitated with 1/20 volume of cold absolute ethanol (-20°C). The tube was rocked back and forth until a stringy white precipitate became visible; the tube was left at -20°C overnight.

The tube was centrifuged for 5 minutes at 3000rpm to pellet the DNA, 1ml of 70% ethanol was added and the tube was centrifuged for a further 5 minutes. The pellet was washed with 70% ethanol and left at room temperature to dry. The dry pellet was resuspended in 100µl TE buffer and placed at 60°C to dissolve.

2.2.14 Determination of DNA Concentration

The concentration of DNA in each extract was determined using a TKO 100 mini-fluorometer. This was done according to the manufacturers instructions using Calf thymus DNA as a standard.

2.2.15 Restriction Endonuclease Analysis

2.2.15.1 Restriction Endonuclease Digestion of Genomic DNA

1.5µg of DNA was added to an eppendorf tube containing 0.5µl (5 Units) of restriction endonuclease (*Hae*III or *Cla*I), 2.5µl of 10X restriction buffer plus enough sterile distilled water to make the final volume up to 25µl. The tube was briefly flicked and then centrifuged for a 6 second pulse in a microfuge. The tube was incubated at 37°C for one

hour. At the completion of the incubation 10µl of bromophenol blue dye was added to stop the reaction.

2.2.15.2 Agarose Gel Electrophoresis of Restriction Endonuclease Digests

The digested DNA fragments were size fractionated through a 1% agarose gel in 1X TAE buffer for 2 hours at 100V. 10µl of λHindIII ladder was added to the first well, 15µl of the DNA digests were added to the remaining wells. The gels were stained in ethidium bromide and visualised under UV light.

2.2.16 RAPD PCR TYPING

2.2.16.1 RAPD PCR

This procedure was based on a method described by Hernandez and co-workers (1995). The standard PCR reaction mixture contained 1X PCR buffer, 2.5mM $MgCl_2$, 100µM each of dATP, dGTP, dCTP and dTTP, 0.2µM of the primer OPA11, 2ng of template DNA, 0.8U of *Taq* DNA polymerase. The volume was made up to 20µl by the addition of sterile distilled water. A positive control containing Lp1 DNA was included. A negative control in which DNA was replaced with sterile distilled water was also included. The solutions were cycled through the following temperatures. 1 cycle of: 95°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes; 45 cycles of: 94°C for 1 minute, 36°C for 1 minute, 72°C for 2 minutes; 1 cycle of 94°C for 1 minute, 36°C for 1 minute, and 72°C for 5 minutes. The amplification was performed in a Corbett FTS-960 Thermal Sequencer.

2.2.16.2 Agarose Gel Electrophoresis

The PCR products were size fractionated through a 1.6% agarose gel in 1X TAE buffer at 80V for 2 hours. The gels were stained with ethidium bromide and visualised under UV light.

2.2.17 Flagellin Gene PCR Typing

2.2.17.1 PCR Amplification of *Campylobacter* Flagellin Genes

This protocol was based on a method described by Alm and collegues (1993a). The standard PCR reaction mixture contained 1X PCR buffer, 3.5mM $MgCl_2$, 200µM of each dNTP, 0.25µM of each primer, 1.6 Units of *Taq* DNA polymerase. The volume was made up to 40µl by the addition of sterile distilled water. Using a 5µl loop a small colony of *Campylobacter* growth was resuspended in the reaction mixture. A positive control containing 40ng of C9 DNA was included. A negative control in which bacterial growth was replaced with sterile distilled water was also included. Amplification was performed in a Corbett FTS-960 Thermal Sequencer with the following temperatures: 1 cycle of: 94°C for 5 minutes; 30 cycles of: 94°C for 45 sec, 60°C for 45 seconds, 72°C for 2 minutes and 30 seconds; 1 cycle of: 72°C for 5 minutes.

The primers pg50 and RAA19 were used to amplify the *flaA* gene; RAA9 and RAA19 were used to amplify *flaB*.

2.2.17.2 Agarose Gel Electrophoresis of the Amplicon

3µl of the completed PCR reaction was mixed with 2µl of 5X bromophenol blue dye and was size fractionated through a 1.6% agarose gel in 1X E-buffer at 100V for 1 hour. The gel was stained with ethidium bromide and the presence of the amplicon was confirmed under UV light.

2.2.17.3 Restriction Endonuclease Digestion of PCR Product

15µl of the PCR product was transfered to an eppendorf tube containing 1.5µl of 10X restriction buffer. 0.5µl of restriction endonuclease (*Pst*I or *Hin*dIII) was added and the mixture was incubated at 37°C for 90 minutes. At the end of the incubation period, 5µl of 5X bromophenol blue dye was added to stop the reaction.

2.2.17.4 Agarose Gel Electrophoresis of Digested Amplicon

The digested product was size fractionated through a 3% agarose gel in 1X E-buffer at 100V for 90 minutes. 7µl of BRL 1kb ladder marker was added to the first well ; 7µl of BRL 10bp ladder marker was added to the last well of the gel. 10µl of the digested amplicon was added to the remaining wells. The gel was stained with ethidium bromide and visualised under UV light.

2.2.17.5 Estimation of Fragment Sizes

Agarose gels were visualised and photographed using the Alphalmager 2000 digital imaging system. BRL 1kb and 10bp ladder markers were included on each gel. The sizes of DNA fragments were calculated using the molecular weight determination function of the Alphalmager 2000 system.

2.2.17.6 Analysis of RFLP Patterns

A dendogram was constructed according to the method of Schmid and collegues (1990), using the Dendron programme. Dendron constructs dendograms by the unweighted pair group method. A similarity value (S_{AB}) was determined to assess the similarity between RFLP patterns of two isolates A and B, using the following formula:

$$S_{AB} = \frac{\sum_{i=1}^{k} (a_i + b_i - | a_i - b_i |)}{\sum_{i=1}^{k} (a_i + b_i)}$$

where a_i and b_i are the intensities of bands i in patterns A and B respectively, and k is the number of bands. Band intensities were scored in arbitrary units: 0U, absence of band; 1U, presence of band; 2U, presence of extra bright band. If the RFLP patterns for isolates A and B are identical the S_{AB} value is 1.0. If the RFLP patterns for isolates A and B are completly nonidentical the S_{AB} value is 0.0.

2.2.17.7 Estimation of the Number of Generations Derived from a Single Bacterial Cell

If we assume that there are 10^7 bacteria per colony, or that one colony contains 2^{23} bacteria: then ~24 generations are derived from one bacterial cell within one colony. The isolates were sub-cultured every two days for thirty days.

15 subcultures = 15 x 24 generations
= ~360 generations

2.2.17.8 Desription of the Havelock North Campylobacteriosis Outbreak

Fourteen of the isolates included in this study were from an outbreak of Campylobacteriosis which occured in Havelock North in June, 1997.

Thirty-three cases of Campylobacteriosis were notified to the Public Health unit of Health Care Hawkes Bay over the period of one week, June 11-18th 1997. Twenty-eight of the cases (24 Havelock North cases, 3 Hastings cases and 1 Napier case) were interviewed by phone using a detailed questionaire. However, no single food or event was identified as the source. *Campylobacter* isolates from 15 of the cases were serotyped, 14 of the isolates were serotype O 23,36 (McElnay, 1997). It is these isolates that were included in the present study.

CHAPTER 3

RESULTS

3.1 BIOTYPING

Table 3.1 shows the differential reactions and characteristics for selected *Campylobacter* species. Tests for catalase production, nitrate reduction, susceptibility to naladixic acid and cephalothin, hippurate hydrolysis, growth at 25°C and 42°C, indoxyl acetate hydrolysis and H_2S production were included in this study.

3.2 SOURCE OF ISOLATES

The origins of the *Campylobacter* isolates used in this study; and the species, as determined by biotyping are listed in Table 3.2.

3.3 DNA EXTRACTIONS

Three methods were used to extract genomic DNA from *Campylobacter* cells. The DNA yields varied greatly between isolates; yields of less than 200ng/µl were considered insufficient for direct analysis with restriction endonucleases. All of the methods consisted of three basic steps: cell lysis, removal of proteins and cellular debris and precipitation of the DNA.

The cell lysis step was accomplished through the use of detergents (methods #1 and #2) or alkaline lysis (method #3). Lysozyme was included in method #2 to produce holes in the cell wall peptidoglycan layer to allow the detergents to enter. Proteinase K was included in all DNA extraction methods to aid the removal of proteins. DNA extraction method #1 used CTAB/NaCl to remove polysaccharides. Proteins were removed from the solutions through a series of phenol/chloroform/iso-amyl alcohol extractions. The DNA was precipitated by the addition of isopropanol (methods #1 and #2) or ethanol (method #3).

TABLE 3.1 Differential reactions and characteristics of *Campylobacter* species[a].

Species	Reactions and characteristics[b]								
	Catalase	Nitrate reduction	Susceptibility		Hippurate hydrolysis	Growth		Indoxyl acetate hydrolysis	H_2S production
			Naladixic acid	Cephalothin		25°C	42°C		
C. jejuni subsp. *jejuni*	+	+	S	R	+	−	+	+	−
C. jejuni subsp. *doylei*	d	−	S	R	+	−	(+)	+	−
C. coli	+	+	S	R	−	−	+	+	−
C. lari	+	+	R	R	−	−	+	−	−
C. upsaliensis	(−)	+	S	S	−	−	+	+	−
C. fetus subsp. *fetus*	+	+	R	S	−	+	(−)	−	−
C. fetus subsp.*venerealis*	+	+	R	S	−	+	−	−	−
C. hyointestinalis	+	+	R	S	−	(+)	+	−	+

[a] Data were obtained from Penner (1988), Penner (1991), Goossens and Butzler (1992) and Humphrey (1995).

[b] +, Positive reaction ; − , negative reaction ; (+), most strains positive but a low percentage negative ; (−), most strains negative but a low percentage positive ; d, 10 to 90% positive ; R, resistant ; S, sensitive.

Footnoes to Table 3.2

[x] Abbreviations: VPH, Faculty of Veterinary Pathology and Public Health, Massey University; MAF, Ministry of Agriculture and Fisheries, Batchelor Animal Health Laboratories.

[b] 140 *Campylobacter* isolates from 121 patients were obtained from the local community laboratory. The ages of the patients ranged from 8 months to 83 years old. Thirty-six samples were recieved from 17 patients who had been resampled two or three times. The isolates were designated a number from C1 to C200.

[c] 20 *Campylobacter* isolates from sheep carcasses from a North Island freezing works were provided by S.Fenwick, Faculty of Veterinary Pathology and Public Health, Massey University.

[d] 22 *Campylobacter* isolates were provided by C. Nicol, Institute of Environmental Science and Research. 14 of these isolates were from an outbreak of Campylobacteriosis which occured in Havelock North in June, 1997.

[e] Type strains provided by P. Short, Institute of Environmental Science and Research, Keneperu Science Centre.

TABLE 3.2 The source and identification of *Campylobacter* isolates used in this study

Strain designation	Place of isolation[x]	Host	Species
C1 - C200	Palmerston North[b]	Human	89% *C. jejuni* 11% *C. coli*
S71- S90	Freezing works[c]	Sheep	*C . jejuni*
D1	VPH	Dog	*C. jejuni*
D2	MAF	Dog	*C. upsaliensis*
D3	MAF	Dog	*C. upsaliensis*
F1	MAF	Calf	*C. jejuni*
F2	MAF	Calf	*C. jejuni*
F5	MAF	Calf	*C. fetus*
F6	MAF	Calf	*C. fetus*
B1	VPH	Cow	*C. fetus*
B3	VPH	Cow	*C. fetus*
B4	VPH	Cow	*C. hyointestinalis*
B6	VPH	Cow	*C. hyointestinalis*
B7	VPH	Cow	*C. coli*
B8	MAF	Cow	*C. fetus*
B11	MAF	Cow	*C. fetus*
B12	MAF	Cow	*C. jejuni*
B13	MAF	Cow	*C. fetus*
B14	MAF	Cow	*C. fetus*
B15	MAF	Cow	*C. fetus*
E1	Wellington[d]	Human	*C. jejuni*
E2	Wellington[d]	Human	*C. jejuni*
E3	Wellington[d]	Human	*C. jejuni*
E4	Wellington[d]	Human	*C. jejuni*
E5	Waikato[d]	Human	*C. jejuni*
E6	Northland[d]	Human	*C. jejuni*
E7	Wellington[d]	Human	*C. jejuni*
E8	Wellington[d]	Human	*C. jejuni*
E9 - E14	Havelock North[d]	Human	*C. jejuni*
E1a - E8a	Havelock North[d]	Human	*C. jejuni*
NCTC 11351[e]	—	—	*C. jejuni* subsp. *jejuni*
NCTC 11951[e]	—	—	*C. jejuni* subsp. *doylei*
NCTC 11366[e]	—	—	*C. coli*
NCTC 11352[e]	—	—	*C. lari*
NCTC 10842[e]	—	—	*C. fetus* subsp. *fetus*

DNA extraction methods #1 and #2 produced sheared DNA from many isolates and only 47% of isolates yielded sufficient DNA. Several variations of these methods were examined: the proteinase K and SDS concentrations were varied. Cells were also held at 4°C for 48 hours prior to DNA extraction to aid lysis, but overall these modifications did not improve yields. DNA extraction method #3 (which was based on alkaline lysis) yielded DNA from 70% of isolates, but the DNA obtained was also frequently sheared.

3.4 RESTRICTION ENDONUCLEASE ANALYSIS

Campylobacter genomic DNA (prepared using DNA extraction method #3) was digested with the restriction endonucleases *Hae*III and *Cla*I. Some samples required more than the standard two phenol/chloroform/iso-amyl alcohol extractions before they could be digested. Figure 3.1 shows DNA from 8 different *Campylobacter* isolates digested with *Hae*III. Isolates C64, C47, C67, C68 and C85 (lanes 2-6) have a similar restriction pattern; C97, C100 and B13 (lanes 7-9) have different patterns. Figure 3.2 shows *Cla*I digests of DNA from the same 8 isolates. Isolates C64, C47, C67, C68 and C85 (lanes2-6) have similar restriction patterns; C97 and C100 (lanes 7 and 8) share a similar pattern; whereas B13 has a different pattern.

Over 100 *Campylobacter* isolates were analysed by this method. The restriction patterns were hard to interpret due to the large number of bands. Many isolates produced sheared DNA, which gave rise to blurry restriction patterns. It was difficult to distinguish between *Campylobacter* strains using this method.

3.5 RAPD PCR TYPING

3.5.1 Optimisation of PCR Reaction

The PCR reaction was optimised by varying the $MgCl_2$ and DNA template concentrations. Standard PCR conditions (Section 2.2.16.1) were used to titrate the DNA of isolate C47 over a range of 10ng to 200ng (Figure 3.3). It was concluded that 20ng of DNA was the optimal amount because at this concentration the high molecular weight and the lower molecular weight bands were bright and clear.

Figure 3.1 *Hae*III digestion of *Campylobacter* genomic DNA.
Lane 1, λ HindIII marker; lane 2, C47; lane 3, C64; lane 4, C67; lane 5, C68; lane 6, C85; lane 7, C97; lane 8, C100; lane 9, B13.

Isolates C64, C47, C67, C68 and C85 (lanes 2-6) have a similar restriction pattern; C97, C100 and B13 (lanes 7-9) have different patterns.

Figure 3.2 *Cla*I digestion of *Campylobacter* genomic DNA.
Lane 1, λ HindIII marker; lane 2, C47; lane 3, C64; lane 4, C67; lane 5, C68; lane 6, C85; lane 7, C97; lane 8, C100; lane 9, B13.

Isolates C64, C47, C67, C68 and C85 (lanes 2-6) have similar restriction patterns; C97 and C100 (lanes 7 and 8) share a similar pattern; whereas B13 has a distinct pattern.

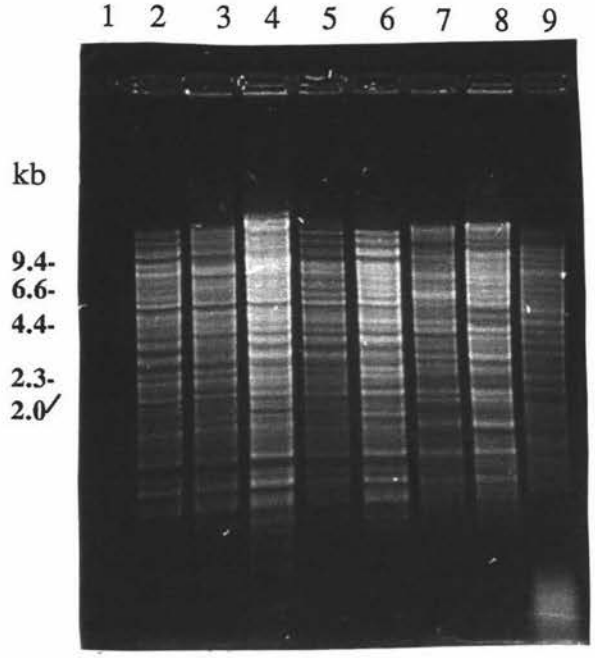


Figure 3.1

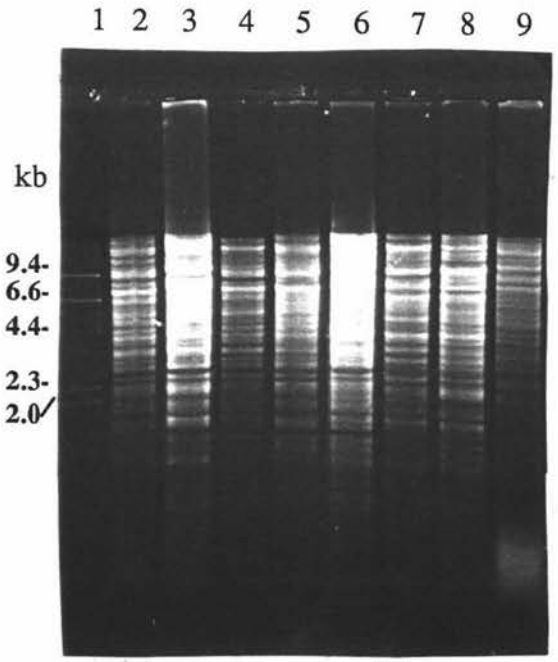


Figure 3.2

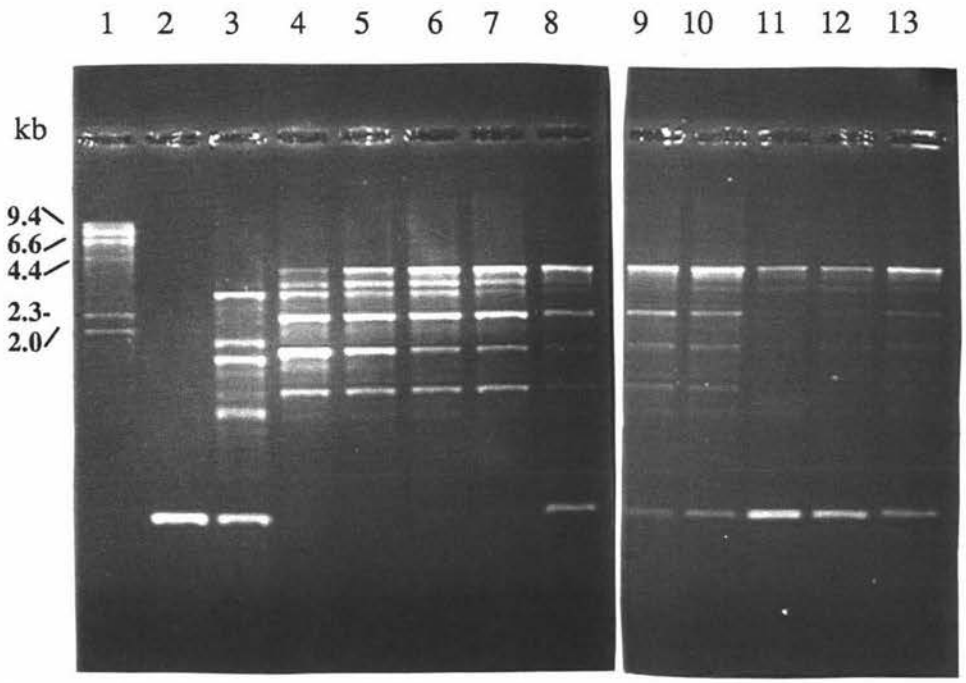


Figure 3.3 Optimisation of DNA template concentration.

Lane1, λ HindIII marker; lane 2, negative control; lane 3, positive control; lane 4, 10ng DNA; lane 5, 20ng DNA; lane 6, 40ng DNA; lane 7, 60ng DNA; lane 8, 80ng DNA; lane 9 100ng DNA; lane 10, 120ng DNA; lane 11, 160ng DNA; lane 12, 180ng DNA; lane 13, 200ng DNA.

20ng of DNA per PCR reaction was considered the optimal amount, because at this concentration both the high molecular weight and the lower molecular weight bands were bright and clear.

The $MgCl_2$ concentration was titrated over a range of 1.5mM to 4.0mM. It was concluded that 2.5mM $MgCl_2$ was the optimal concentration because both the higher molecular weight and lower molecular weight bands were bright and clear.

3.5.2 RAPD Analysis of *Campylobacter* Isolates

The standard PCR reaction was used to amplify the DNA of several *Campylobacter* isolates. However, having standardised conditions it was difficult to get reproducible results.

3.6 FLAGELLIN GENE RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) TYPING

3.6.1 PCR Amplification of the *fla*B Gene

PCR primers RAA9 and RAA19 were used to amplify a 1.46kb fragment of the *fla*B gene. The primers, designed by Alm and co-workers (1993a), were based on known sequences of the flagellin genes of *C. coli* VC167. The *fla*B-specific forward primer RAA9 is located on the plus strand and begins 11bp upstream of the translated start site; the reverse primer RAA19 binds 274bp from the 3′ end of the gene (Alm *et al* 1993a).

3.6.1.2 Optimisation of the PCR Reaction

The PCR reaction was optimised by varying the annealing temperature; dNTP, $MgCl_2$ and DNA template concentrations. The concentration of dNTP's in the reaction was varied over a range of 20μM to 200μM. The *fla*B gene did not amplify at dNTP concentrations below 50μM. Of the range tested a concentration of 200μM gave the brightest PCR product of the expected size. The $MgCl_2$ concentration was titrated over a range of 2.5mM to 4.0 mM and it was concluded that 3.5mM $MgCl_2$ was the optimal concentration. The DNA template concentration was varied over a range of 0.02ng to 20ng. The gene amplified at DNA concentrations as low as 0.04ng. However, a template concentration of 20ng was chosen for subsequent reactions. Two annealing temperatures were tested. At 55°C extra faint bands were present, whereas at 60°C only the expected PCR product was present. The presence of a single PCR product of constant size was confirmed by visualisation on an agarose gel (Figure 3.4). For most isolates the *fla*B

gene was amplified directly from bacterial colonies without purification of the template DNA. Some isolates did not produce a PCR product and many of these were later shown to produce DNases (Section 2.2.10). For DNase producing strains, genomic DNA was extracted and purified using DNA extraction method #3 (alkaline lysis) prior to PCR amplification.

3.6.2 Restriction Endonuclease Digestion of the Amplicon

The amplicon was digested with the restriction endonucleases *Pst*I and *Hin*dIII to detect restriction fragment length polymorphisms. The RFLP patterns were compared on the basis of the sizes of the constituent fragments (Table 3.3). The variation in fragment size of any given pattern, from gel to gel was less then 5%. The total size of the PCR product deduced from the summation of the constituent fragments also varied between the different RFLP patterns.

TABLE 3.3 Fragment sizes of *fla*B *Pst*I and *Hin*dIII patterns from human and animal isolates

*Pst*I Types	Constituent fragments (kb)[a]	*Hin*dIII Types	Constituent fragments (kb)
P1	0.73, 0.53, 0.08	H1	0.98, 0.54
P2	1.46 (uncut)	H2	0.98, 0.27, 0.14
P3	1.22, 0.41	H3	0.98, 0.18, 0.14, 0.08
P4	0.76, 0.25, 0.21, 0.09	H4	0.54, 0.49, 0.26
P5	0.79, 0.62, 0.08	H5	0.50, 0.30, 0.28, 0.14
P6	1.54, 0.18	H6	0.52, 0.30, 0.25, 0.16
P7	1.27, 0.27	H7	0.49, 0.25, 0.20, 0.15, 0.08
P8	1.78,0.09	H8	1.5, 0.22
P9	0.75 *	H9	0.98, 0.61
P10	0.95, 0.34, 0.17	H10	0.98, 0.35, 0.22
P11	0.79, 0.25, 0.12, 0.09, 0.08	H11	0.49, 0.24, 0.19, 0.14, 0.08
P12	0.78, 0.32, 0.24	H12	1.46 kb (uncut)
P13	1.33, 0.21, 0.09	H13	1.08, 0.38, 0.26
P14	0.96, 0.80		

[a] Some of the lower molecular weight bands did not photograph well but were present on the original gels.

* This band was brighter than others and was assumed to be a doublet band.

3.6.3 *fla*B Gene RFLP Typing of Human Isolates

One hundred and forty human isolates from a local community laboratory were analysed using this method. Twelve isolates, which did not produce a PCR product using whole bacteria, tested positive for DNase production. DNA was extracted and purified from DNase positive isolates prior to PCR amplification.

Thirteen distinct RFLP patterns were generated when the amplicons were digested with *Pst*I. The patterns, containing 1 to 5 bands, were assigned labels from P1 to P13. Eight distinct RFLP patterns, containing 2 to 5 bands, were produced when the amplicon was digested with *Hin*dIII. The *Hin*dIII patterns were assigned labels from H1 to H8. Examples of the *Pst*I and *Hin*dIII RFLP patterns are shown in Figures 3.5 and 3.6 respectively.

The results of the two digests were combined to give 26 *Pst*I/*Hin*dIII combined types. The P4/H2 type and the P4/H1 type were the most common (16 isolates; 13%) followed by P5/H1 (15 isolates; 12%), and P6/H1 (11 isolates; 9%). Table 3.4 shows the distribution of RFLP types among the isolates, and the distribution of *C. jejuni* and *C. coli* among RFLP types. Two RFLP types, P3/H2 and P8/H4, contained *C. coli* exclusively and five RFLP types contained both *C. jejuni* and *C. coli*.

The *Pst*I and *Hin*dIII patterns of seven DNase-producing isolates are shown in figures 3.7 and 3.8 respectively. A diverse range of RFLP types were found among the twelve DNase producing isolates (Table 3.5). All *Pst*I and *Hin*dIII patterns had been found previously among DNase negative isolates. However, three isolates C13, C55 and C62, only had unique RFLP combined types, P12/H2, P2/H1 and P2/H6 respectively.

3.6.3.1 *fla*B Gene RFLP typing of Outbreak Isolates

Twenty two *Campylobacter* isolates of serotype O23 and/or O36 were provided by C. Nicol, Institute of Environmental Science and Research (ESR); fourteen of the isolates were from an outbreak of Campylobacteriosis which occured in Havelock North in June, 1997; four isolates were cultured from humans in the Wellington region, isolated in

1997; and four were isolated in 1996 from Wellington, Waikato and Northland (Table 3.6).

TABLE 3.4 Distribution of RFLP types among human *Campylobacter* isolates[a]

RFLP Type	Number of isolates	Percentage of each type	Number of *C. jejuni* isolates	Number of *C. coli* isolates
P4/H2	16	13	16	0
P1/H1	16	13	14	2
P5/H1	15	12	11	4
P6/H1	11	9	11	0
P8/H2	7	6	7	0
P12/H1	7	6	7	0
P11/H1	7	6	7	0
P10/H7	6	5	4	2
P6/H6	6	5	3	3
P4/H1	4	3	4	0
P6/H8	5	4	5	0
P9/H2	3	2	2	1
P11/H2	3	2	3	0
P2/H4	3	2	3	0
P3/H3	1	<1	1	0
P2/H1	1	<1	1	0
P2/H2	1	<1	1	0
P2/H6	1	<1	1	0
P3/H2	1	<1	0	1
P5/H2	1	<1	1	0
P6/H7	1	<1	1	0
P7/H6	1	<1	1	0
P8/H4	1	<1	0	1
P10/H5	1	<1	1	0
P12/H2	1	<1	1	0
P13/H2	1	<1	1	0

[a]Only one isolate from each patient was included in these results.

Table 3.5 RFLP types found among DNase producing isolates

Strain Designation	RFLP Type
C7	P6/H8
C13	P12/H2
C52	P6/H8
C55	P2/H1
C57	P6/H8
C62	P2/H6
C88	P6/H1
C99	P2/H4
C122	P11/H1
C132	P11/H1
C154	P4/H1
C164	P6/H1

Table 3.6 Distribution of RFLP types among ESR isolates

Strain Designation	Serotype[a]	RFLP Type
E1	O 23, 36	P6/H9
E2	O 23, 36	P6/H9
E3	O 23, 36	P6/H9
E4	O 23, 36	P4/H10
E5	O 23, 36	P6/H9
E6	O 23, 36	P6/H9
E7	O 23	P6/H9
E8	O 36	P4/H10
E9	O 23, 36	P6/H9
E10	O 23, 36	P6/H9
E11	O 23, 36	P6/H9
E12	O 23, 36	P6/H9
E13	O 23, 36	P6/H9
E14	O 23, 36	P6/H9
E1a	O 23, 36	P6/H9
E2a	O 23, 36	P6/H9
E3a	O 23, 36	P6/H9
E4a	O 23, 36	P6/H9
E5a	O 23, 36	P6/H9
E6a	O 23, 36	P6/H9
E7a	O 23, 36	P6/H9
E8a	O 23, 36	P6/H9

[a] Serotyping of the heat stable (O) antigens was performed by C. Nicol, Institute of Environmental Science and Research.

The ESR isolates were tested blind to prevent any bias in the interpretation of the results. Two distinct *Pst*I patterns, P4 and P6 (Figure 3.9); and two *Hin*dIII patterns, H9 and H10 (Figure 3.10) were found among the ESR isolates. The patterns H9 and H10 were similar but not identical to (variation was greater than 5%) patterns H1 and H2 respectively. The isolates which gave these patterns (H9 and H10) were examined twice and had the same patterns both times. The results of the two digests were combined to give two *Pst*I/*Hin*dIII types. Table 3.6 shows the distribution of RFLP types and serotypes among the ESR isolates. Two isolates (E4 and E8) had the P4/H10 type, and twenty isolates had the P6/H9 type. All fourteen outbreak isolates had the P6/H9 pattern.

3.6.4 *fla*B Gene RFLP Typing of Animal Isolates

Twenty sheep isolates were analysed using this method. Four isolates failed to amplify directly from bacterial growth and the isolates were shown to produce DNases. Six distinct RFLP patterns were generated when the amplicons were digested with *Pst*I (Figure 3.11). *Hin*dIII digestion of the amplicon also produced six RFLP patterns (Figure 3.12). Two isolates, S71 and S80, produced a *Hin*dIII pattern which was not found among human isolates, H11, which was similar but not identical to H7.

Fifteen *Campylobacter* isolates from bovines were analysed by *fla*B RFLP typing. The *fla*B gene amplified in only 3 isolates; B12, F1 and F2 which were all *C. jejuni*. The remaining 12 isolates were *C. fetus* (9), *C. hyointestinalis* (2) or *C. coli* (1); all were DNase negative. Two *Pst*I patterns (P4 and P5), and two *Hin*dIII patterns (H1 and H2) were found among the bovine isolates (Figures 3.13 and 3.14). The results of the digests were combined to give two *Pst*I/*Hin*dIII types: P4/H2 and P5/H1.

*fla*B RFLP typing was used to examine three *Campylobacter* isolates from dogs. One isolate (D1) was *C. jejuni* and two were *C. upsaliensis* (D2 and D3). The *C.jejuni* isolate did not produce a PCR product and this isolate was shown to produce DNases. After the DNA was extracted and purified from D1 using the alkaline lysis method, the *fla*B gene still did not amplify. The *fla*B gene was amplified in the *C. upsaliensis* isolates from dogs and the amplicons were digested with *Pst*I and *Hin*dIII. Two *Pst*I patterns were

produced, P2 and P14; P14 was a new pattern which had not previously been seen among the human isolates. The amplicons did not cut with *Hin*dIII, this pattern was designated H12. The pattern H12 had not been seen among the human isolates. The distribution of
RFLP types among the animal isolates are listed in Table 3.7.

Table 3.7 Distribution of RFLP types among animal isolates

RFLP Type	Number of isolates	Percentage of each type
P2/H12	1 (dog)	4%
P4/H2	8 (6 sheep, 2 bovine)	32%
P5/H1	6 (5 sheep, 1 bovine)	24%
P6/H1	3 (sheep)	12%
P8/H4	1 (sheep)	4%
P10/H11	2 (sheep)	8%
P11/H1	3 (sheep)	12%
P14/H12	1 (dog)	4%

3.6.5 RFLP Typing of Reference Strains.

Five reference strains were included in this study; *C. jejuni* subsp. *jejuni* NCTC 11351, *C. jejuni* subsp. *doylei* NCTC 11951, *C. coli* NCTC 11366, *C. fetus* subsp. *fetus* NCTC 10842, and *C. lari* NCTC 11352. The primers RAA9 and RAA19 were used to amplify the *fla*B gene in each strain. Initially *C. jejuni* subsp. *jejuni* was the only strain to produce a PCR product. The isolates which didn't amplify were tested for DNase production. *C. coli* was the only isolate shown to produce DNases. The DNA was extracted and purified from the isolates which failed to amplify using whole cells by DNA extraction method # 3 (alkaline lysis). Further attempts to amplify the *fla*B gene were sucessful with *C. coli*, but DNA from the remaining reference strains did not produce a PCR product with these primers. The amplicons were digested with *Pst*I and *Hin*dIII to generate RFLP's (Figure 3.15). *C. jejuni* subsp. *jejuni* had the P11/H13 type and *C. coli* had the P2/H2 type. The *Hin*dIII pattern H13, found in *C. jejuni* subsp. *jejuni* was not seen in any other isolates examined.

3.6.6 Stability of RFLP Patterns *in vitro*

The stability of the restriction fragment length polymorphism patterns was assesed over approximately 360 generations. Two isolates (C9 and C10) were grown for 30 days, (sub-cultured on CNSM agar every 2 days) at 37°C under microaerophilic conditions. The *fla*B gene was amplified from the isolates every 10 days (approximately 120 generations), and was digested with *Pst*I and *Hin*dIII to generate RFLP's. The patterns that were produced are shown in Figures 3.16 and 3.17. The RFLP patterns of the two strains remained stable over 30 days (approximately 360 generations).

3.6.7 Stability of RFLP Patterns *in vivo*

Six of the isolates included in this study were recieved from two patients who had been resampled three times each. For both patients, all three isolates had identical RFLP patterns.

Thirty of the isolates were recieved from 15 patients who had been resampled twice. For 13 of the patients, both isolates had identical RFLP patterns. However, the repeated samples from two patients had different *Pst*I patterns from the original isolates. Isolates C137 and C139 were from the same patient and had the *Pst*I patterns P5 and P4 respectively. Isolates C148 and C149 were from the same patient and had the *Pst*I patterns P5 and P12 respectively.

3.6.8 Analysis of RFLP Patterns

A dendogram was constructed for the 168 isolates from which RFLP patterns were obtained (Figure 3.18). The dendogram shows seventeen distinct clusters of isolates each with similarity values of 1.0. These clusters represent isolates with idetnical *Pst*I/*Hin*dIII patterns. Four clusters, III, IV, VII and XI, contained both human and animal isolates. Cluster VII was the largest and contained 24 isolates, eight of which were animal isolates. In addition to the isolates showing identical patterns there were several sets which were closely related: isolate C3 (human) and cluster VII had an S_{AB} value of 0.92. Isolates C160 (human) and S78 (sheep) had S_{AB} values of 0.90. Cluster XIV and

C116 (human) had an S_{AB} value of 0.88. Cluster X and C55 (human) had an SAB value of 0.86. Cluster XV and C159 (human) and clusters II and III, and IV and V had S_{AB} values of 0.80. All other isolates had S_{AB} values of less than 0.80. Two *C. upsaliensis* isolates, D2 and D3, had S_{AB} values of 0.40. This indicates that these isolates are distantly related.

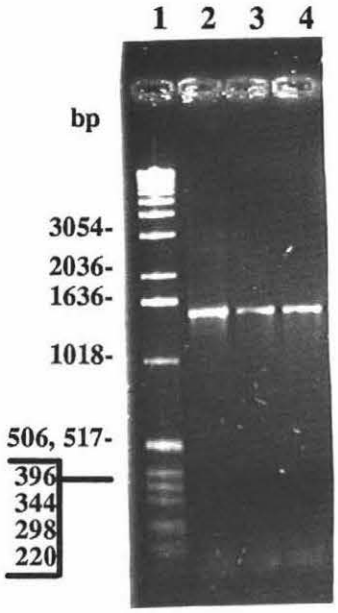


Figure 3.4 PCR amplification of the *fla*B gene with primers RAA9 and RAA19.

Lane1, BRL 1kb ladder; lane 2, C9; lane 3, C10; lane 4, E4.

All samples show a single PCR product of a constant size ~ 1.46 kb.

Figure 3.5 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*B genes of representative human *Campylobacter* isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), P3; lane 3, C131, P1; lane 4, C127, P2; lane 5, C8, P3; lane 6, C194, P4; lane 7, C40, P5; lane 8, C189, P6; lane 9, C179, P7; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, C160, P8; lane 3, C156, P9; lane 4, C159, P10; lane 5, C190, P11; lane 6, C193, P12; lane 7, C111, P13; lane 8, E4; lane 9, BRL 10bp ladder.

Thirteen distinct RFLP patterns were found among the human isolates when the amplicons were digested with *Pst*I. The patterns, containing 1-6 bands were assigned labels from P1 to P13. Isolate E4 (Figure 3.5B, lane 8) had a *Pst*I pattern which was initially thought to be distinct; however, when fresh restriction endonuclease was used, the 1.6kb band disappeared and the pattern matched that of P4.

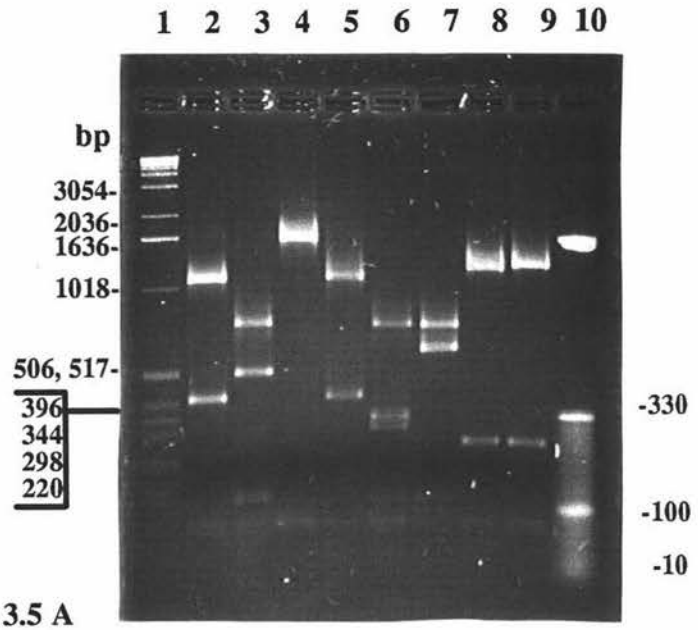


Figure 3.5 A

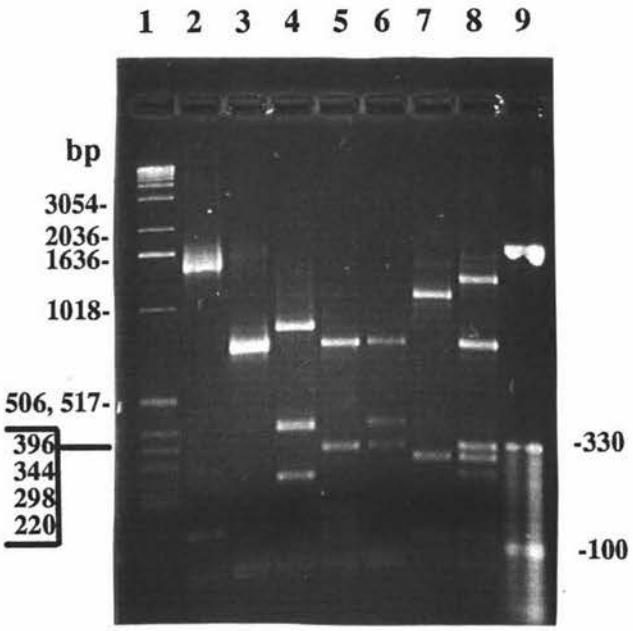


Figure 3.5 B

Figure 3.6 Restriction fragment length polmorphism patterns produced by *Hin*dIII digestion of the *fla*B genes of representative human *Campylobacter* isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), H3; lane 3, C131, H1; lane 4, C194, H2; lane 5, C8, H3; lane 6, C127, H4; lane 7, C159, H5; lane 8, C176, H6; lane 9, C189, H7; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, C192, H8.

Eight distinct RFLP patterns were found among the human isolates when the amplicons were digested with *Hin*dIII. The patterns, containing 2-5 bands were assigned labels from H1 to H8.

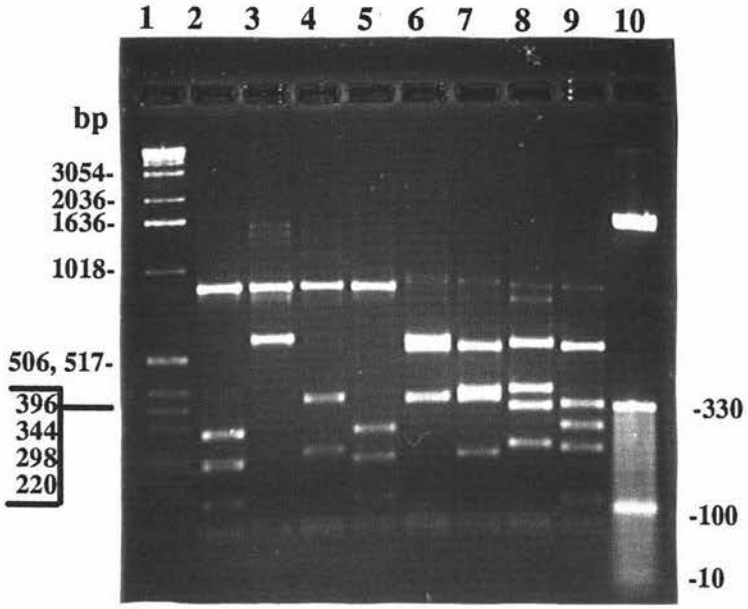


Figure 3.6 A

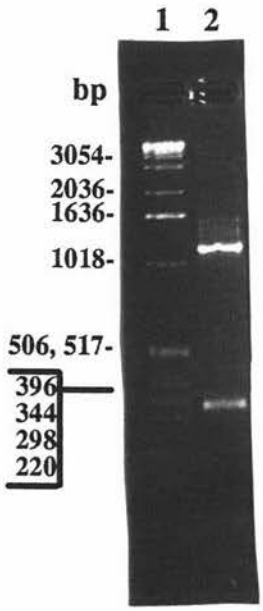


Figure 3.6 B

Figure 3.7 Restriction fragment length polymorphism patterns produced by *Pst*I digestion of the *fla*B gene of the following DNase producing isolates.

Lane 1, BRL 1kb ladder; lane 2, positive control (C9) P3; lane 3, C52, P6; lane 4, C55, P2; lane 5, C57, P6; lane 6, C62, P2; lane 7, C88, P6; lane 8, C99, P2; lane 9, C132, P11; lane 10, BRL 10bp ladder.

Three *Pst*I patterns were produced (P2, P6 and P11). All had been found previously among DNase negative human isolates.

Figure 3.8 Restriction fragment length polymorphism patterns produced by *Hin*dIII digestion of the *fla*B gene of the following DNase producing isolates.

Lane 1, BRL 1kb ladder; lane 2, positive control (C9) H3; lane 3, C52, H8; lane 4, C55, H1; lane 5, C57, H8; lane 6, C62, H6; lane 7, C88, H1; lane 8, C99, H4; lane 9, C132, H1; lane 10, BRL 10bp ladder.

Four *Hin*dIII patterns were produced (H1, H4, H6, and H8). All had been found previously among DNase negative human isolates.

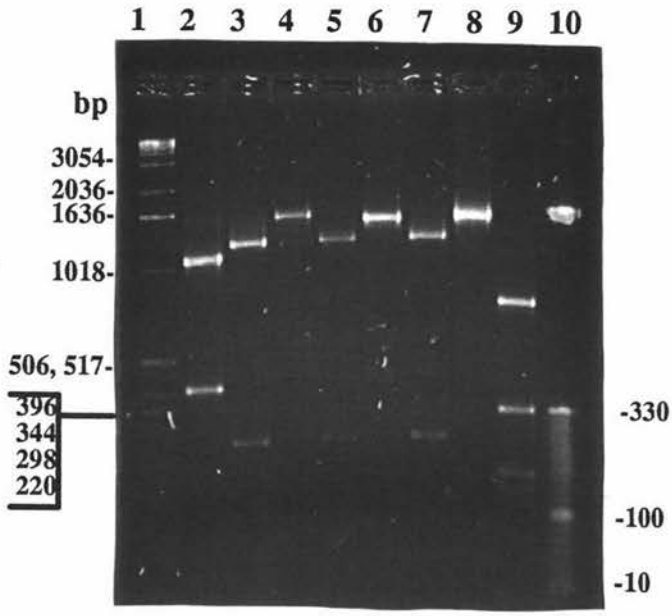


Figure 3.7

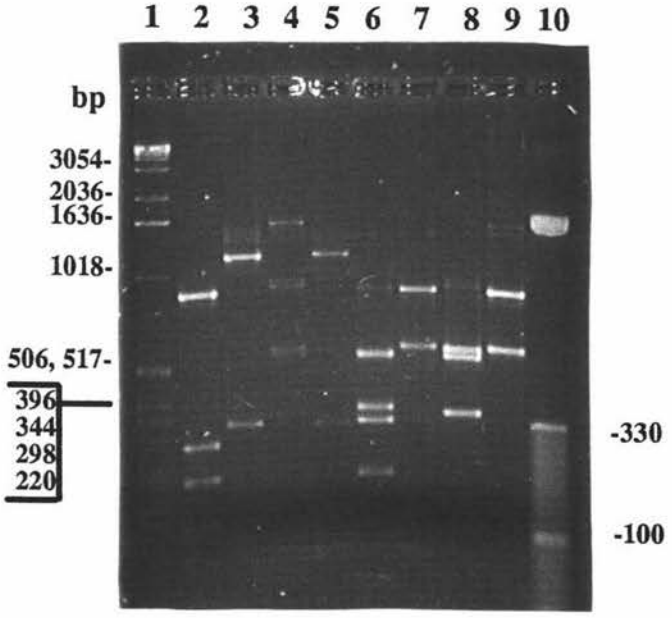


Figure 3.8

Figure 3.9 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*B genes of the ESR isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), P3; lane 3, E1, P6; lane 4, E2, P6; lane 5, E3, P6; lane 6, E4, P4; lane 7, E5, P6; lane 8, E6, P6; lane 9, E7, P6; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, E8, P4; lane 3, E9, P6; lane 4, E10, P6; lane 5, E11, P6; lane 6, E12, P6; lane 7, E13, P6; lane 8, E14, P6; lane 9, E1a, P6; lane 10, BRL 10bp ladder.
(C) Lane 1, BRL 1kb ladder; lane 2, C189, P6; lane 3, E2a, P6; lane 4, E3a, P6; lane 5, E4a, P6; lane 6, E5a, P6; lane 7, E6a, P6; lane 8, E7a, P6; lane 9, E8a, P6; lane 10, BRL 10bp ladder.

Two *Pst*I patterns were found among the ESR isolates, E4 and E8 had the P4 pattern and the remaining isolates had the P6 pattern. All outbreak isolates had P6 pattern. A human isolate (C189), representing the pattern P6, was included to compare with the ESR patterns.

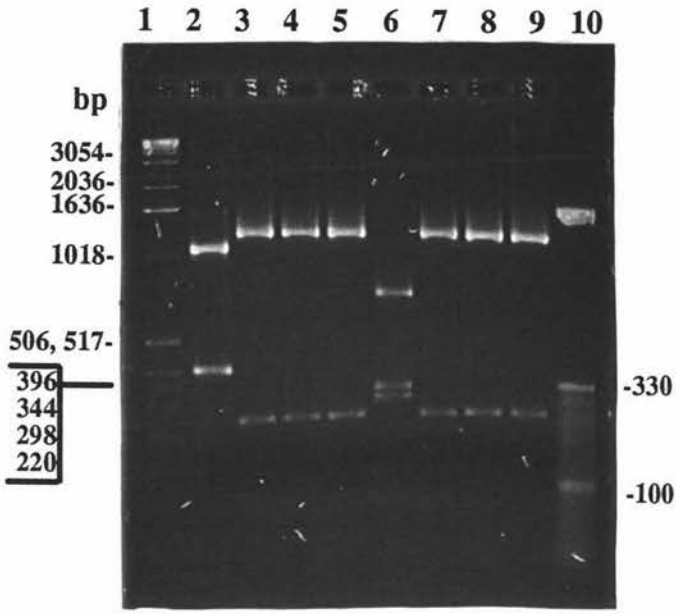


Figure 3.9 A

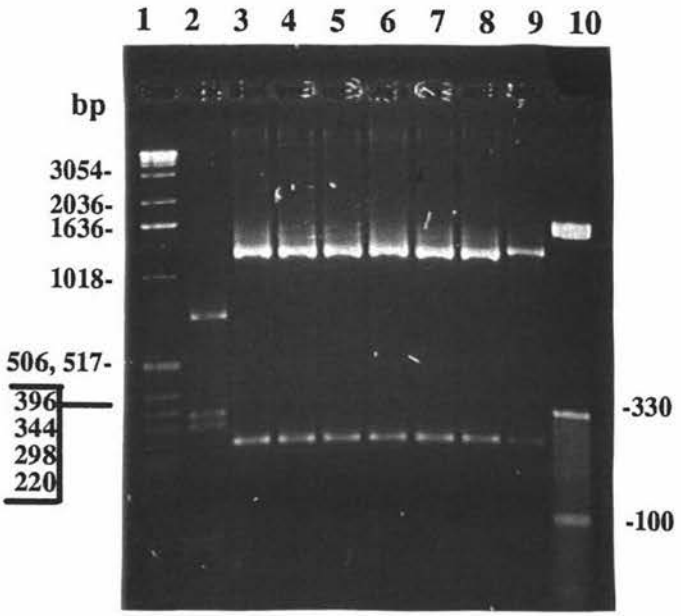


Figure 3.9 B

Figure 3.9 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*B genes of the ESR isolates.

(C) Lane 1, BRL 1kb ladder; lane 2, C189, P6; lane 3, E2a, P6; lane 4, E3a, P6; lane 5, E4a, P6; lane 6, E5a, P6; lane 7, E6a, P6; lane 8, E7a, P6; lane 9, E8a, P6; lane 10, BRL 10bp ladder.

Figure 3.10 Restriction fragment length polmorphism patterns produced by *Hin*dIII digestion of the *fla*B genes of the ESR isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), P3; lane 3, E1, H9; lane 4, E2, H9; lane 5, E3, H9; lane 6, E4, H10; lane 7, E5, H9; lane 8, E6, H9; lane 9, E7, H9.
(B) Lane 1, BRL 1kb ladder; lane 2, E8, H10; lane 3, E9, H9; lane 4, E10, H9; lane 5, E11, H9; lane 6, E12, H9; lane 7, E13, H9; lane 8, E14, H9; lane 9, E1a, H9.
(C) Lane 1, BRL 1kb ladder; lane 2, E2a, H9; lane 3, E3a, H9; lane 4, E4a, H9; lane 5, E5a, H9; lane 6, E6a, H9; lane 7, E7a, H9; lane 8, E8a, H9; lane 9, C194, H2.

Two *Hin*dIII patterns were found among the ESR isolates, E4 and E8 had the H10 pattern and the remaining isolates had the H9 pattern. All outbreak isolates had the H9 pattern. A previously isolated human isolate (C194) was included to compare with the ESR patterns.

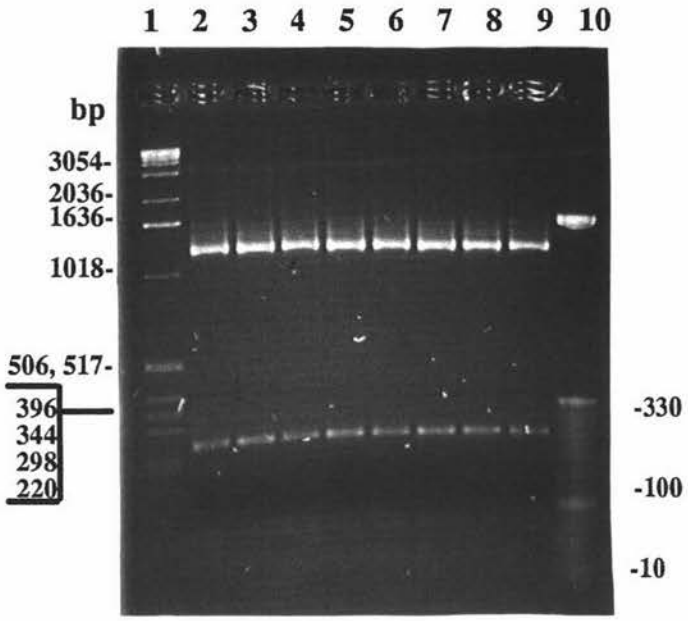


Figure 3.9 C

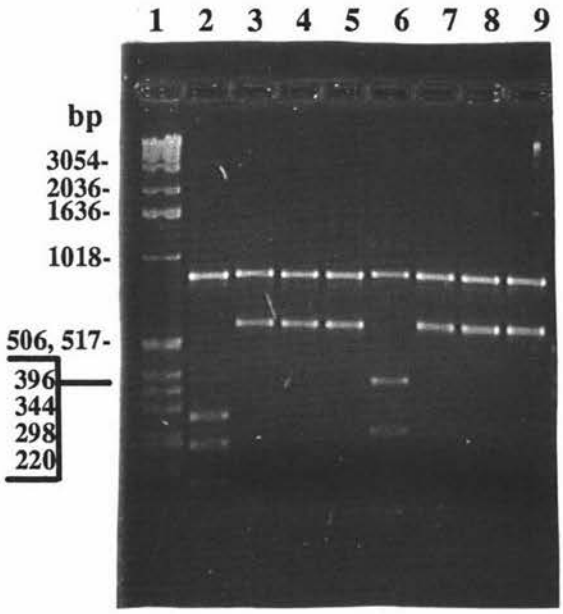


Figure 3.10 A

Figure 3.10 Restriction fragment length polmorphism patterns produced by *Hin*dIII digestion of the *fla*B genes of the ESR isolates.

(B) Lane 1, BRL 1kb ladder; lane 2, E8, H10; lane 3, E9, H9; lane 4, E10, H9; lane 5, E11, H9; lane 6, E12, H9; lane 7, E13, H9; lane 8, E14, H9; lane 9, E1a, H9.
(C) Lane 1, BRL 1kb ladder; lane 2, E2a, H9; lane 3, E3a, H9; lane 4, E4a, H9; lane 5, E5a, H9; lane 6, E6a, H9; lane 7, E7a, H9; lane 8, E8a, H9; lane 9, C194, H2.

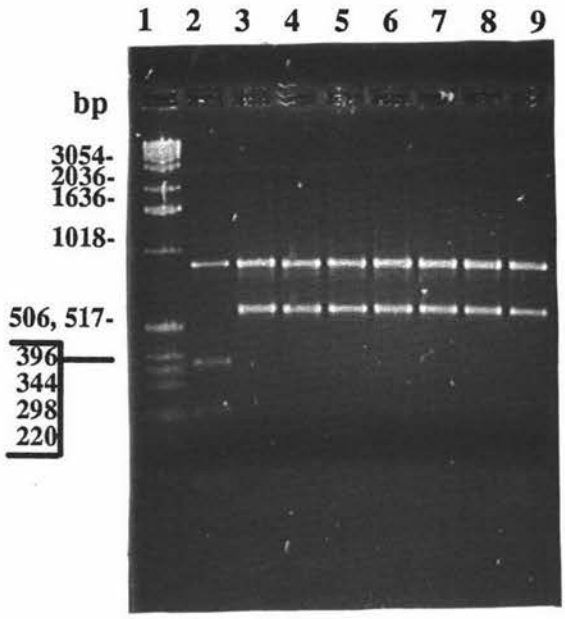


Figure 3.10 B

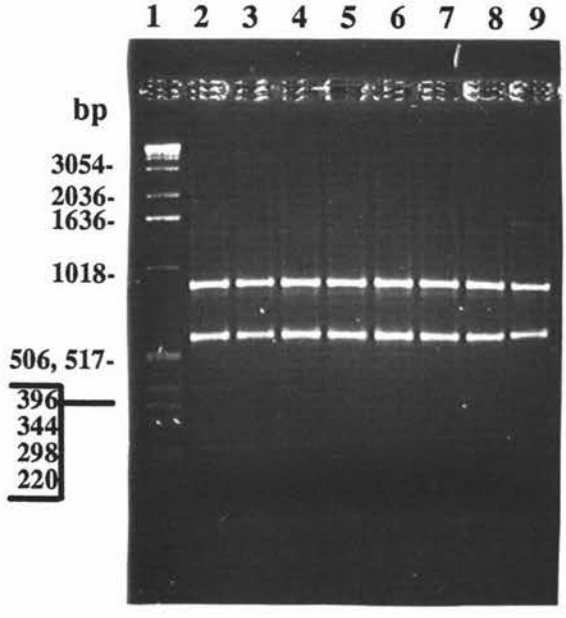


Figure 3.10 C

Figure 3.11 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*B genes of 20 sheep isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), P3; lane 3, S72, P4; lane 4, S73, P5; lane 5, S75, P4; lane 6, S76, P4; lane 7, S78, P8; lane 8, S77, P5; lane 9, S79, P11; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, S80, P10; lane 3, S82, P5; lane 4, S83, P11; lane 5, S84, P4; lane 6, S86, P5; lane 7, S87, P5; lane 8, S88, P6; lane 9, S89, P6.
(C) Lane 1, BRL 1kb ladder; lane 2, S90, P6; lane 3, S71, P10; lane 4, S74, P4; lane 5, S81, P11; lane 6, S85, P4.

Six *Pst*I patterns were found among the sheep isolates, P4, P5, P6, P8, P10 and P11. Four isolates (S71, S74, S81 and S85) failed to amplify directly from bacterial growth and these isolates were shown to produce DNases.

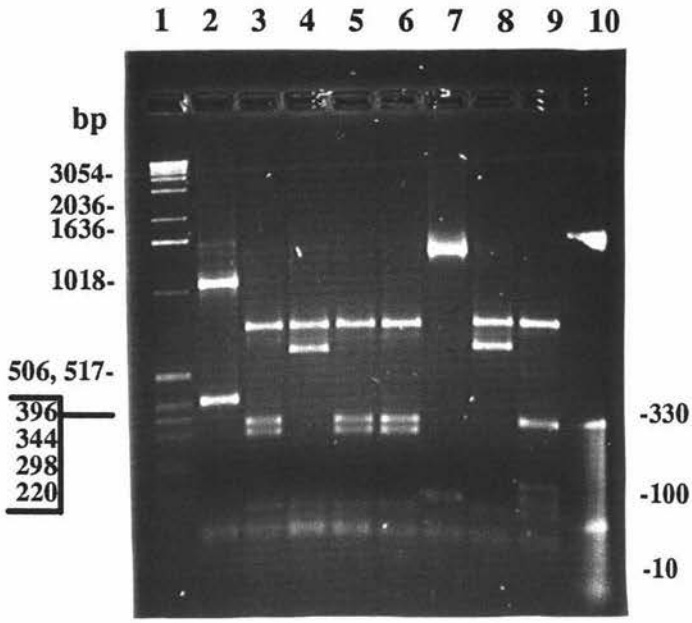


Figure 3.11 A

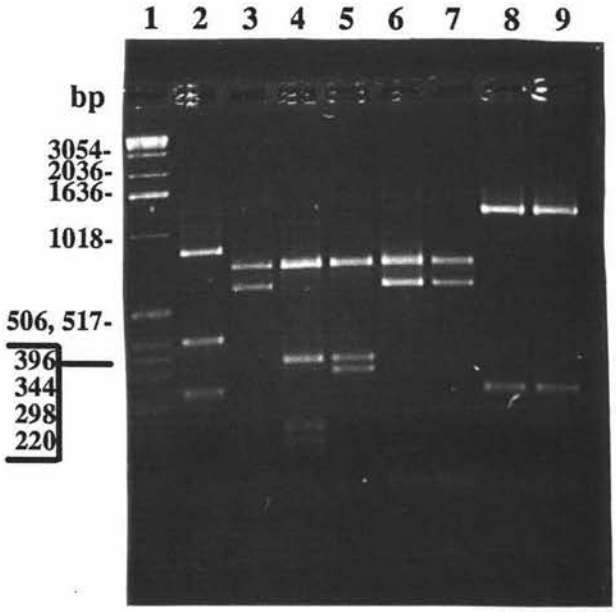


Figure 3.11 B

Figure 3.11 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*B genes of 20 sheep isolates.

(C) Lane 1, BRL 1kb ladder; lane 2, S90, P6; lane 3, S71, P10; lane 4, S74, P4; lane 5, S81, P11; lane 6, S85, P4.

Figure 3.12 Restriction fragment length polmorphism patterns produced by *Hin*dIII digestion of the *fla*B genes of 20 sheep isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, S72, H2; lane 3, S73, H1; lane 4, S75, H2; lane 5, S76, H2; lane 6, S77, H1; lane 7, S78, H4; lane 8, S79, H1; lane 9, positive control (C9), P3; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, C189, H7; lane 3, S80, H11; lane 4, S82, H1; lane 5, S83, H1; lane 6, S84, H2; lane 7, Empty; lane 8, S86, H1; lane 9, S87, H1; lane 10, BRL 10bp ladder.
(C) Lane 1, BRL 1kb ladder; lane 2, S88, H1; lane 3, S89, H1; lane 4, S90, H1; lane 5, S71, H11; lane 6, S74, H2; lane 7, S81, H1; lane 8, S85, H2.

Four *Hin*dIII patterns were found among the sheep isolates, H1, H2, H4 and H11. Four isolates (S71, S74, S81 and S85) failed to amplify directly from bacterial growth, these isolates were shown to produce DNases. Two isolates, S71 and S80 had a pattern which was similar but not identical to H7. These isolates were assigned the label H11. One human isolate (Figure 3.12B, lane 2) was included to compare pattern H7 with those of isolates S80 (Figure 3.12B, lane 3) and S71(Figure 3.12C, lane5).

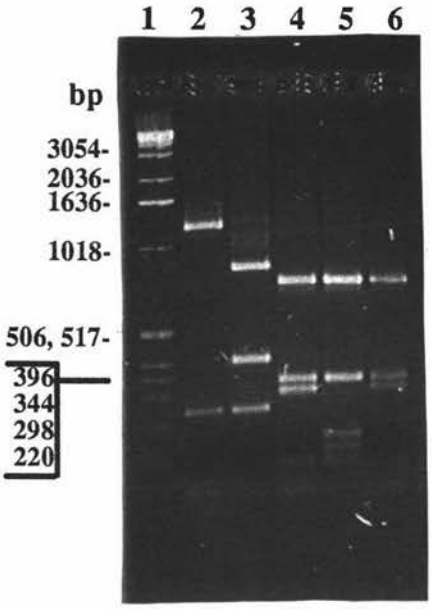


Figure 3.11 C

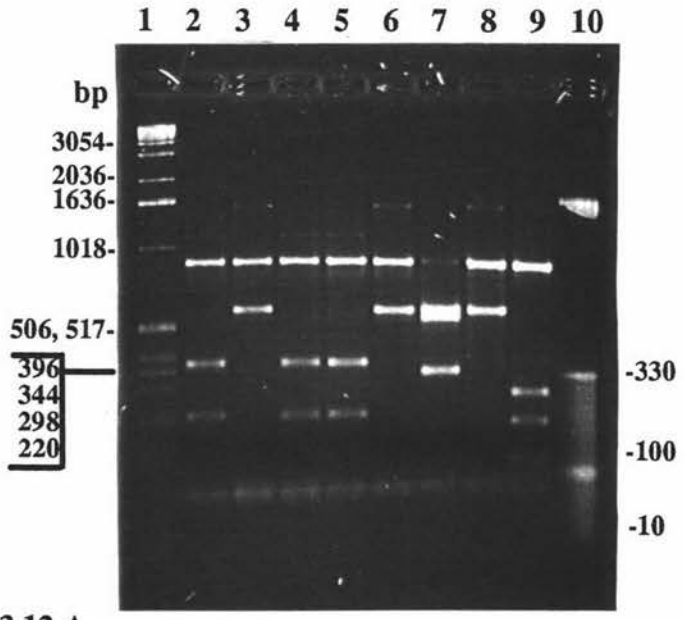


Figure 3.12 A

Figure 3.12 Restriction fragment length polmorphism patterns produced by *Hind*III digestion of the *fla*B genes of 20 sheep isolates.

(B) Lane 1, BRL 1kb ladder; lane 2, C189, H7; lane 3, S80, H11; lane 4, S82, H1; lane 5, S83, H1; lane 6, S84, H2; lane 7, Empty; lane 8, S86, H1; lane 9, S87, H1; lane 10, BRL 10bp ladder.
(C) Lane 1, BRL 1kb ladder; lane 2, S88, H1; lane 3, S89, H1; lane 4, S90, H1; lane 5, S71, H11; lane 6, S74, H2; lane 7, S81, H1; lane 8, S85, H2.

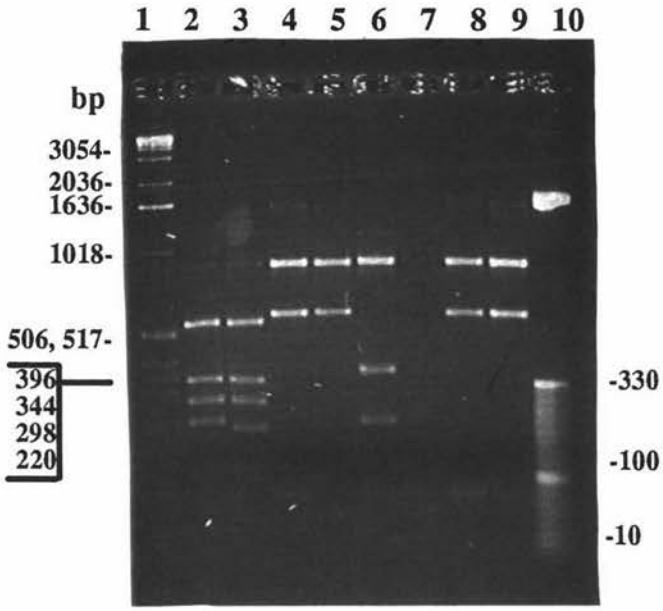


Figure 3.12 B

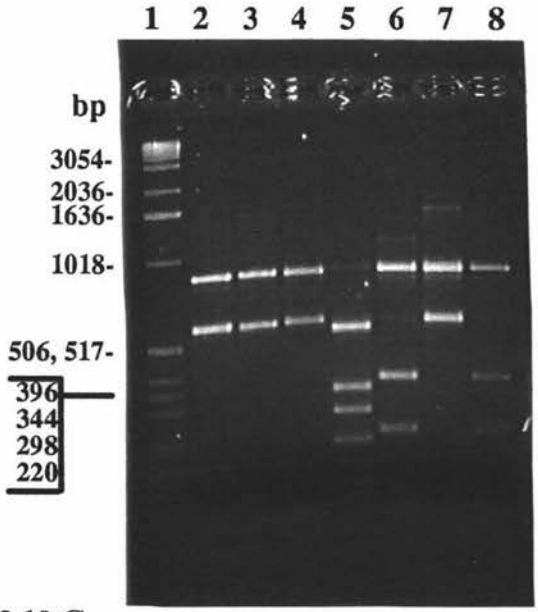


Figure 3.12 C

Figure 3.13 Restriction fragment length polymorphism patterns produced by *Pst*I digestion of the *fla*B gene of the following animal isolates.

Lane 1, BRL 1kb ladder; lane 2, positive control (C9) P3; lane 3, B12, P4; lane 4, F1, P4; lane 5, F2, P5; lane 6, D2, P2; lane 7, D3, P14; lane 8, C194, P4; lane 9, C40, P5.

Four *Pst*I patterns were found among the animal isolates (P2, P4, P5 and P14). D2 and D3 were *C. upsaliensis* and had the patterns P2 and P14 respectively. The P14 pattern had not been seen previously among humans. Two human isolates, C194 and C40, were included to compare patterns P4 and P5 with the patterns of the animal isolates.

Figure 3.14 Restriction fragment length polymorphism patterns produced by *Hin*dIII digestion of the *fla*B gene of animal isolates.

Lane 1, BRL 1kb ladder; lane 2, positive control (C9) H3; lane 3, B12, H2; lane 4, F1, H2; lane 5, F2, H1; lane 6, D2, H12; lane 7, D3, H12; lane 8, C131, H2; lane 9, C194, H1.

Three *Hin*dIII patterns were found among the animal isolates (H1, H2 and H12). D2 and D3 were *C. upsaliensis* and did not digest with *Hin*dIII. These isolates were assigned the label H12 which had not been seen previously among humans. Two human isolates, C131 and C194, were included to compare the patterns H1 and H2 with the patterns of the animal isolates.

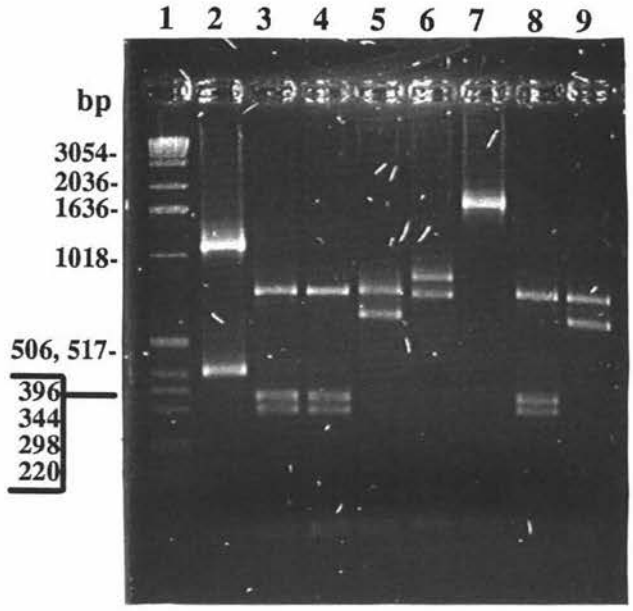


Figure 3.13

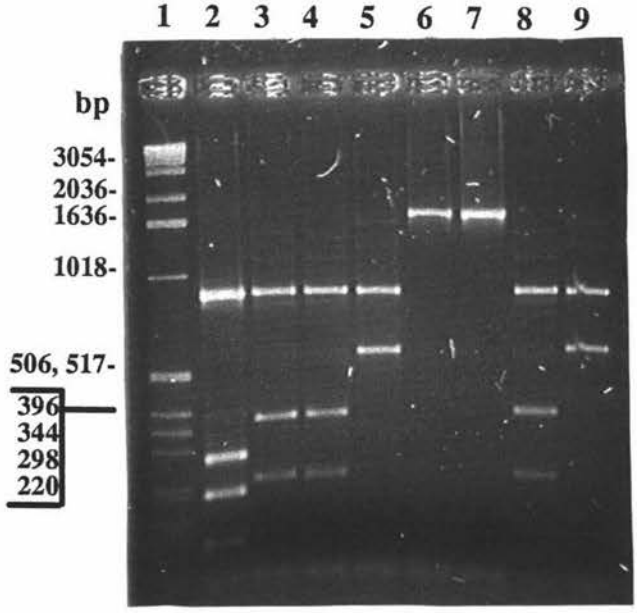


Figure 3.14

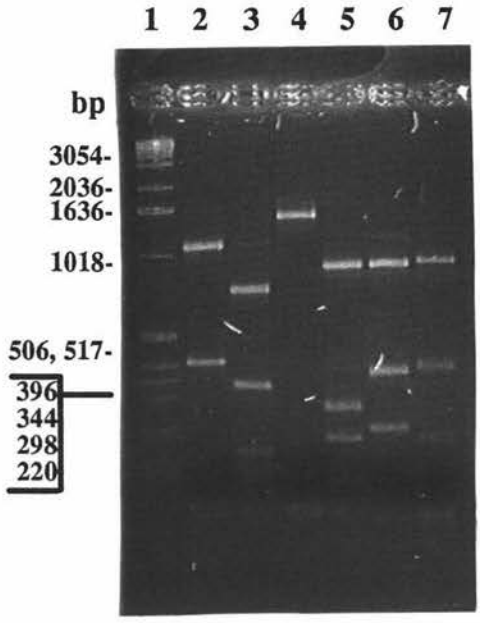


Figure 3.15 Restriction fragment length polymorphism patterns produced by *Pst*I and *Hin*dIII digestion of the *fla*B genes of five reference isolates.

Lane 1, BRL 1kb ladder; lane 2, *Pst*I digest of positive control (C9) P3; lane 3, *Pst*I digest of *C. jejuni* subsp. *jejuni*, P11; lane 4, *Pst*I digests of *C. coli*, P2; lane 5, *Hin*dIII digest of positive control (C9), H3; lane 6, *Hin*dIII digest of *C. jejuni* subsp. *jejuni*, H13; lane 7, *Hin*dIII digest of *C. coli*, H2.

C. jejuni subsp. *jejuni* subsp. *jejuni* had the P11/H13 type and *C. coli* had the P2/H2 type. The *Hin*dIII pattern H13, found in *C. jejuni* subsp. *jejuni* was not seen among the human isolates examined.

Figure 3.16 Stability of *Pst*I RFLP patterns.

Lane 1, BRL 1kb ladder; lane 2, Positive control (C9) P3; lane 3, C9 24 generations (day 0); lane 4, C10 24 generations (day 0); lane 5, C9, 120 generations (day 10); lane 6, C10 120 generations (day 10); lane 7, C9 240 generations (day 20); lane 8, C10 240 generations (day 20); lane 9, C9 360 generations (day 30); lane 10, C10 360 generations (day 30).

The *Pst*I patterns for isolates C9 and C10 have remained stable over approximately 360 generations.

Figure 3.17 Stability of *Hin*dIII RFLP patterns.

Lane 1, BRL 1kb ladder; lane 2, Positive control (C9) H3; lane 3, C9 24 generations (day 0); lane 4,C10 24 generations (day 0); lane 5, C9 120 generations (day 10); lane 6, C10 120 generations (day 10); lane 7, C9 240 generations (day 20); lane 8, C10 240 generations (day 20); lane 9, C9 360 generations (day 30); lane 10, C10 360 generations (day 30).

The *Hin*dIII patterns for isolates C9 and C10 have remained stable over approximately 360 generations.

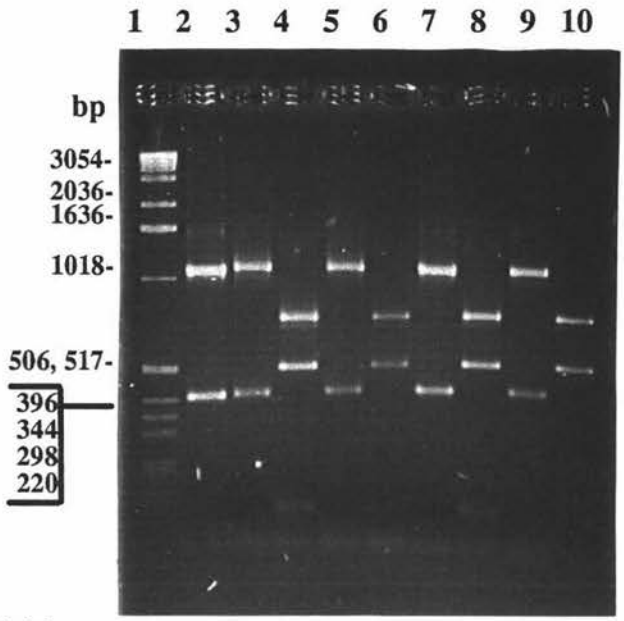


Figure 3.16

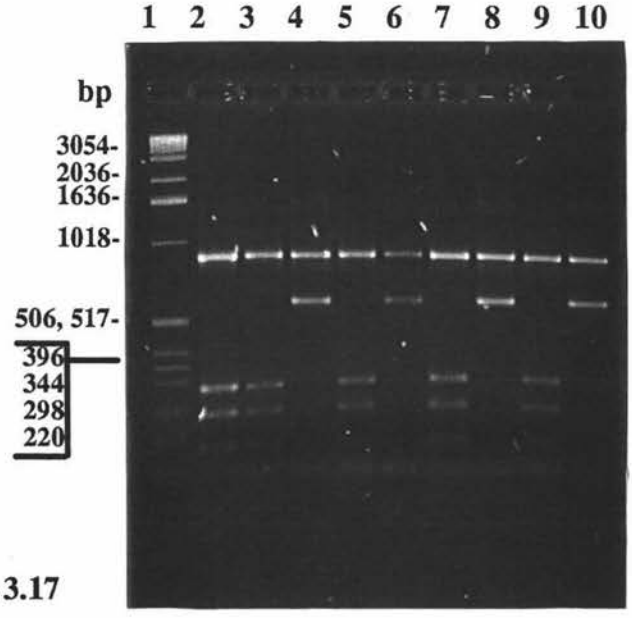
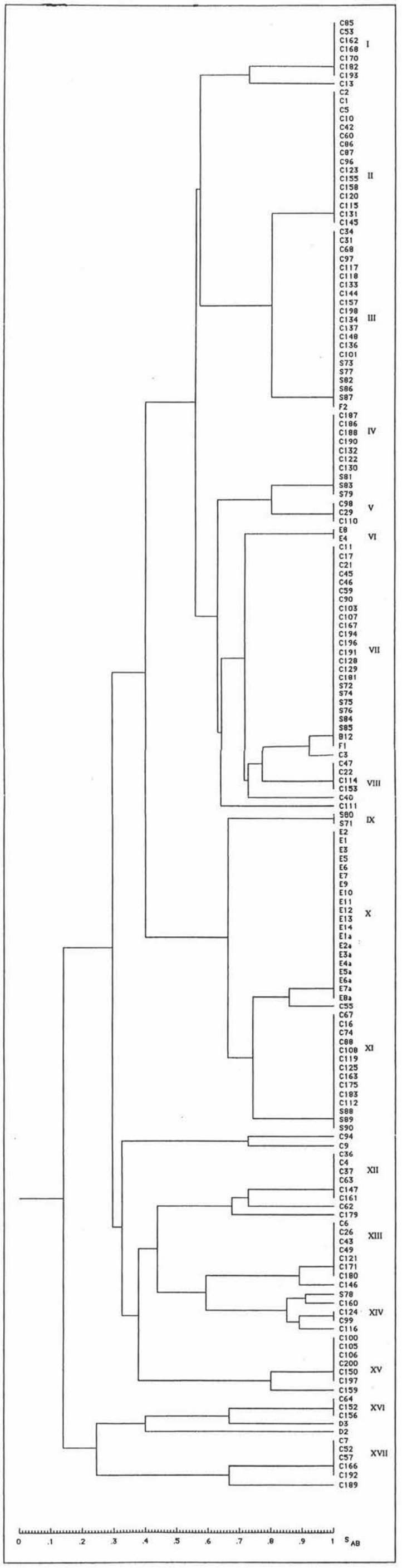


Figure 3.17

FIGURE 3.18 Dendogram of 168 *Campylobacter* isolates from humans and animals.



3.6.9 PCR Amplification of the *fla*A Gene

PCR primers pg50 and RAA19; designed by Alm, Guerry and Trust (1993a), were used to amplify a 1.45kb fragment of the *fla*A gene (Figure 3.19). The optimal PCR conditions were the same as those described in section 2.2.17.1 for the amplification of the *fla*B gene with one exception; the optimal annealing temperature for the *fla*A primers was 42°C.

3.6.10 *fla*A Gene RFLP Typing of Human Isolates

The *fla*A gene was amplified in 15 human isolates, representive of the 13 *Pst*I and 8 *Hin*dIII patterns found among the *fla*B genes. Thirteen distinct RFLP patterns were found when the amplicons were digested with *Pst*I (Figure 3.20). Two isolates, C131 and C8, produced RFLP patterns which were different from those found in the *fla*B gene. The fragment sizes of the *fla*A and *fla*B *Pst*I patterns of isolates C131 and C8 are compared in Table 3.8. Eight distinct RFLP patterns were produced when the amplicons were digested with *Hin*dIII (Figure 3.21). One isolate, C176 produced an RFLP pattern which was different to that found in the *fla*B gene. The fragment sizes of the *fla*A and *fla*B *Hin*dIII patterns of isolate C176 are compared in Table 3.8.

3.6.11 *fla*A Gene RFLP Typing of Outbreak Isolates

The *fla*A gene was amplified in 22 ESR isolates. Two distinct patterns were found when the amplicons were digested with *Pst*I (Figure 3.22). Two isolates (E4 and E8) had a pattern which was identical to P4 (*fla*B).The remaining isolates did not cut with *Pst*I. Two distinct *Hin*dIII patterns were found among the isolates (Figure 3.23). The patterns were identical to H9 and H10 found in the *fla*B genes.

TABLE 3.8 Comparison of RFLP patterns of the *fla*A and *fla*B genes[a]

Isolate	*fla*A (bp)	*fla*B (bp)
C131 (P1)	760 540 210	759 528 83
C8 (P3)	980 420 110	1220 409
C176 (H6)	565 308 151 120 102	523 304 250 159

[a] Some of the lower molecular weight bands did not photograph well but were present on the original gels.

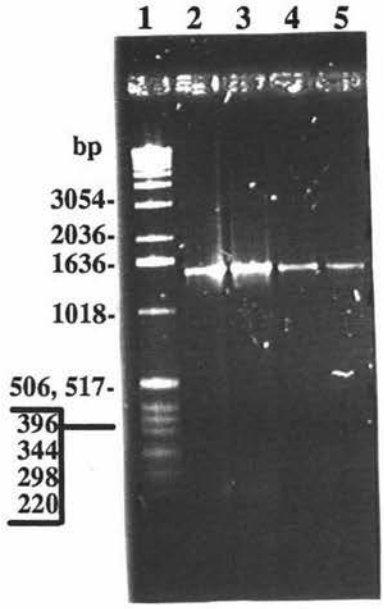


Figure 3.19 PCR amplification of the *fla*A gene with primers pg50 and RAA19.

Lane1, BRL 1kb ladder; lane 2, C9; lane 3, C10; lane 4, E4; lane 5, C9 *fla*B.

All samples show a single PCR product of a constant size ~ 1.45 kb. A *fla*B amplicon of isolate C9 (~1.46 kb) was included to compare the sizes of the two genes. The difference in the sizes of the PCR products was too small to be detected.

Figure 3.20 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*A genes of representative human *Campylobacter* isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), P3; lane 3, C131, P1; lane 4, C127, P2; lane 5, C8, P3; lane 6, C194, P4; lane 7, C40, P5; lane 8, C189, P6; lane 9, C179, P7; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, C160, P8; lane 3, C156, P9; lane 4, C159, P10; lane 5, C190, P11; lane 6, C193, P12; lane 7, C111, P13.

Thirteen distinct RFLP patterns were found when the amplicons were digested with *Pst*I. Two isolates, C31 and C8 produced different *Pst*I patterns to those found in the *fla*A gene.

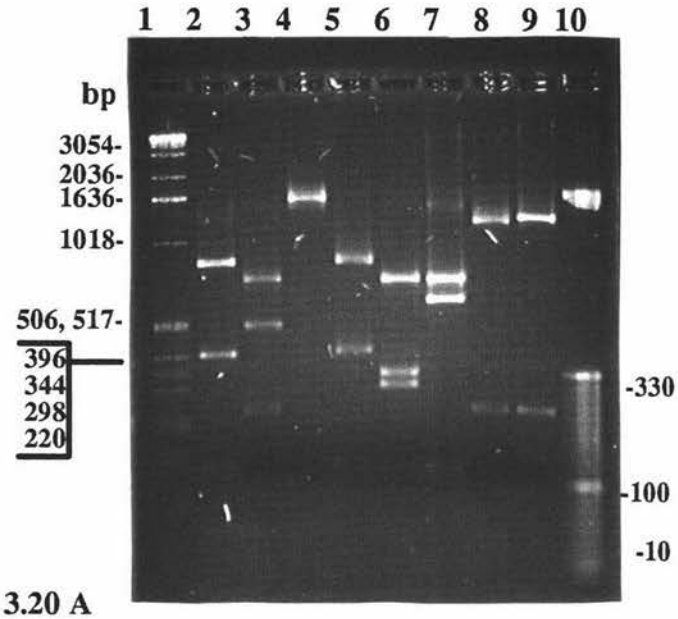


Figure 3.20 A

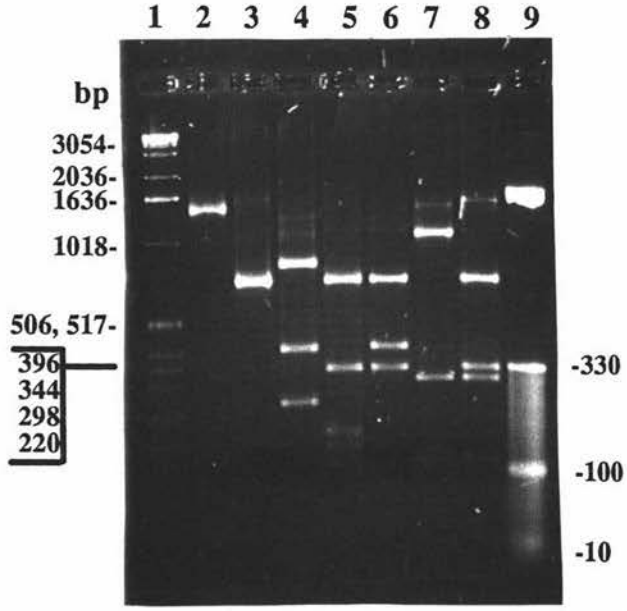


Figure 3.20 B

Figure 3.21 Restriction fragment length polmorphism patterns produced by *Hin*dIII digestion of the *fla*A genes of representative human *Campylobacter* isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), H3; lane 3, C131, H1; lane 4, C194, H2; lane 5, C8, H3; lane 6, C127, H4; lane 7, C159, H5; lane 8, C176, H6; lane9, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, C189, H7; lane 3, C192, H8.

Eight distinct RFLP patterns were produced when the amplicons were digested with *Hin*dIII. One isolate, C176 had a different pattern from that found with *fla*B.

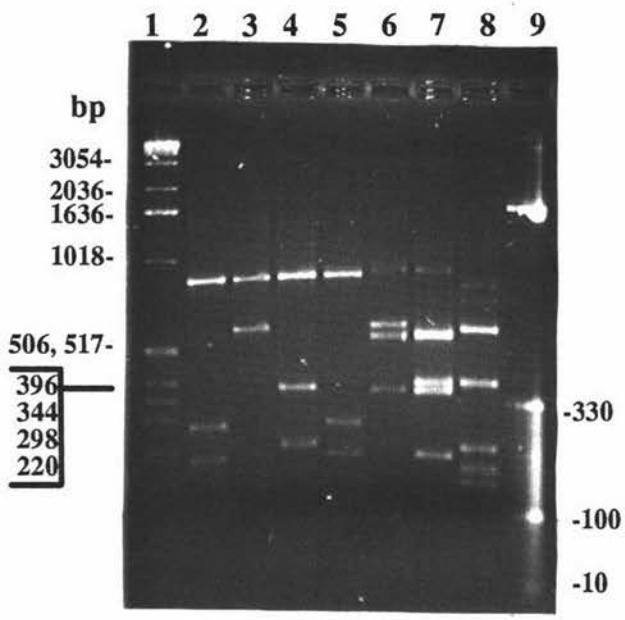


Figure 3.21 A

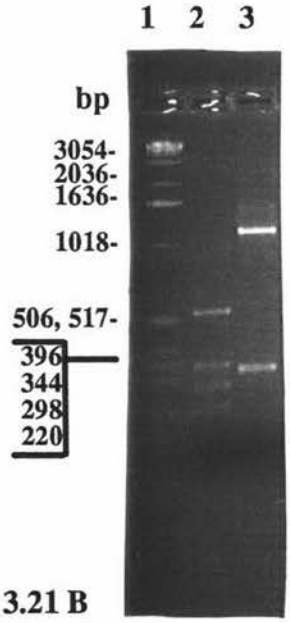


Figure 3.21 B

Figure 3.22 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*A genes of the ESR isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), P3; lane 3, E1, P6; lane 4, E2, P6; lane 5, E3, P6; lane 6, E4, P4; lane 7, E5, P6; lane 8, E6, P6; lane 9, E7, P6; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, E8, P4; lane 3, E9, P6; lane 4, E10, P6; lane 5, E11, P6; lane 6, E12, P6; lane 7, E13, P6; lane 8, E14, P6; lane 9, BRL 10bp ladder.
(C) Lane 1, BRL 1kb ladder; lane 2, E1a, P6; lane 3, E2a, P6; lane 4, E3a, P6; lane 5, E4a, P6; lane 6, E5a, P6; lane 7, E6a, P6; lane 8, E7a, P6; lane 9, E8a, P6; lane 10, BRL 10bp ladder.

Two distinct *Pst*I patterns were found among the ESR isolates. Two isolates, E4 and E8, had a pattern which was identical to P4. The remaining isolates did not cut with *Pst*I.

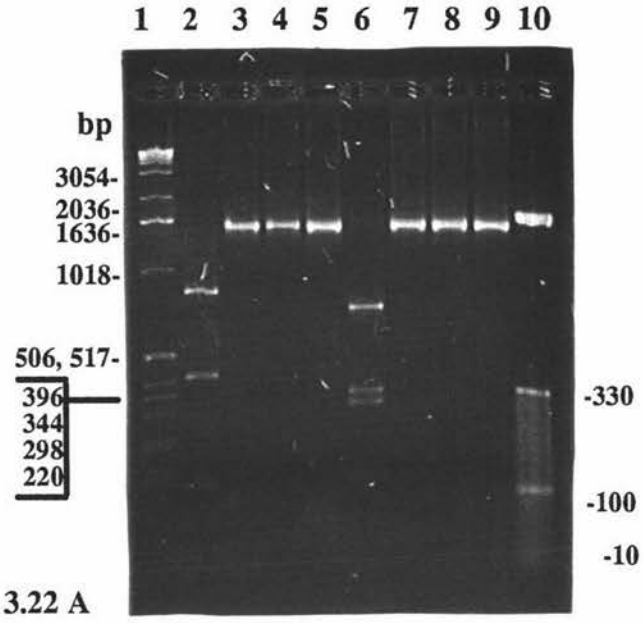


Figure 3.22 A

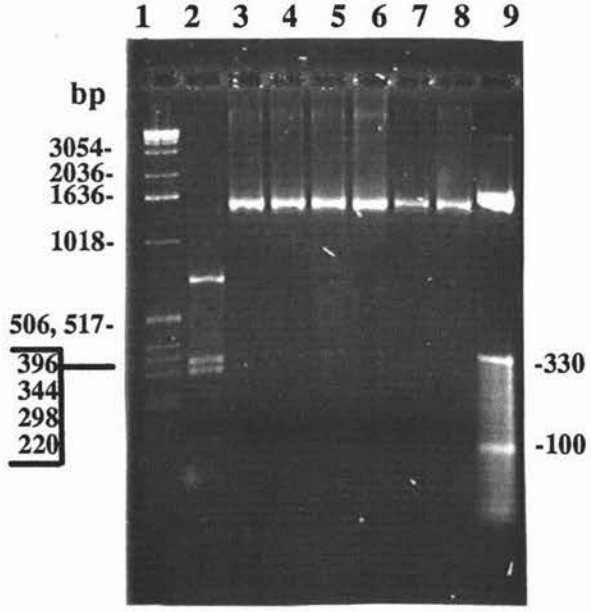


Figure 3.22 B

Figure 3.22 Restriction fragment length polmorphism patterns produced by *Pst*I digestion of the *fla*A genes of the ESR isolates.

(C) Lane 1, BRL 1kb ladder; lane 2, E1a, P6; lane 3, E2a, P6; lane 4, E3a, P6; lane 5, E4a, P6; lane 6, E5a, P6; lane 7, E6a, P6; lane 8, E7a, P6; lane 9, E8a, P6; lane 10, BRL 10bp ladder.

Figure 3.23 Restriction fragment length polmorphism patterns produced by *Hin*dIII digestion of the *fla*A genes of the ESR isolates.

(A) Lane 1, BRL 1kb ladder; lane 2, positive control (C9), H3; lane 3, E1, H9; lane 4, E2, H9; lane 5, E3, H9; lane 6, E4, H10; lane 7, E5, H9; lane 8, E6, H9; lane 9, E7, H9; lane10, BRL 10bp ladder.
(B) Lane 1, BRL 1kb ladder; lane 2, E8, H10; lane 3, E9, H9; lane 4, E10, H9; lane 5, E11, H9; lane 6, E12, H9; lane 7, E13, H9; lane 8, E14, H9; lane 9, BRL 10bp ladder.
(C) Lane 1, BRL 1kb ladder; lane 2, E1a, H9; lane 3, E2a, H9; lane 4, E3a, H9; lane 5, E4a, H9; lane 6, E5a, H9; lane 7, E6a, H9; lane 8, E7a, H9; lane 9, E8a, H9; lane 10, BRL 10bp ladder.

Two distinct *Hin*dIII patterns were found among the ESR isolates. The patterns were identical to those found with the *fla*B gene.

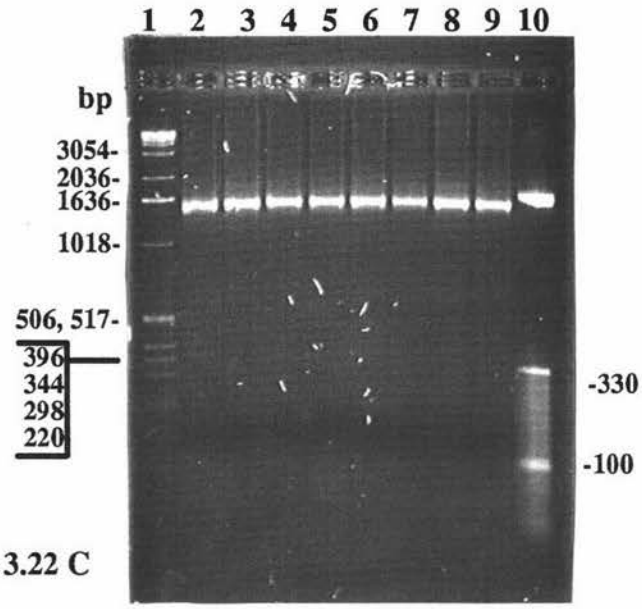


Figure 3.22 C

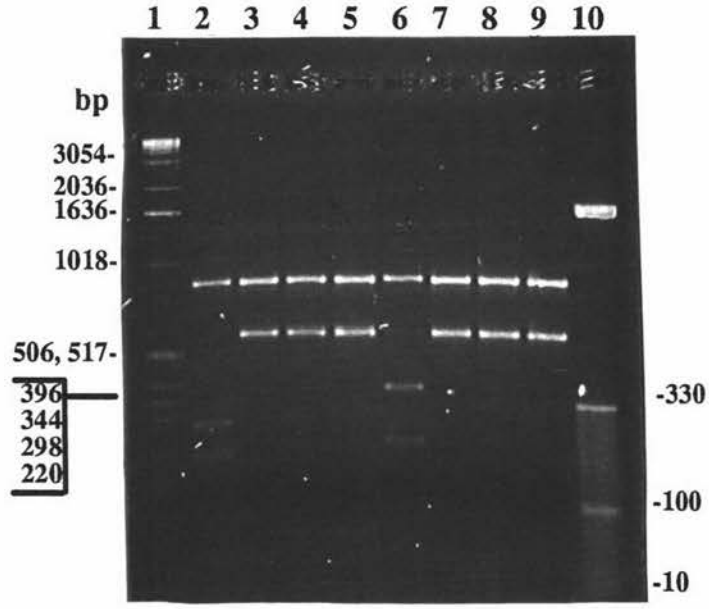


Figure 3.23 A

Figure 3.23 Restriction fragment length polmorphism patterns produced by *Hin*dIII digestion of the *fla*A genes of the ESR isolates.

(B) Lane 1, BRL 1kb ladder; lane 2, E8, H10; lane 3, E9, H9; lane 4, E10, H9; lane 5, E11, H9; lane 6, E12, H9; lane 7, E13, H9; lane 8, E14, H9; lane 9, BRL 10bp ladder.
(C) Lane 1, BRL 1kb ladder; lane 2, E1a, H9; lane 3, E2a, H9; lane 4, E3a, H9; lane 5, E4a, H9; lane 6, E5a, H9; lane 7, E6a, H9; lane 8, E7a, H9; lane 9, E8a, H9; lane 10, BRL 10bp ladder.

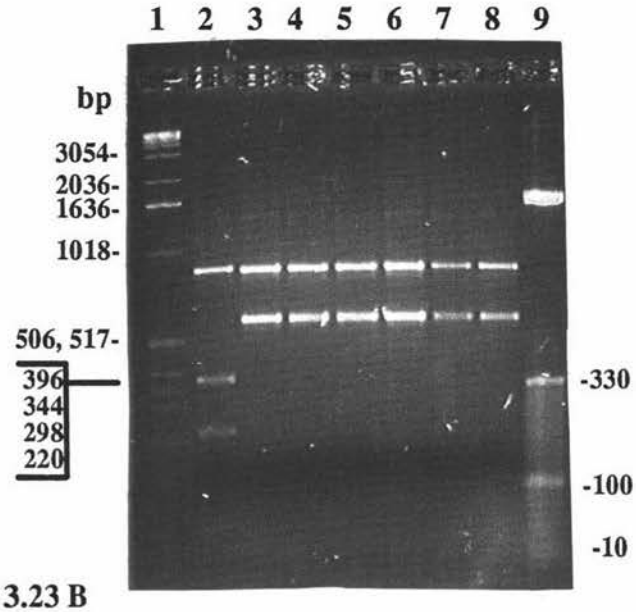


Figure 3.23 B

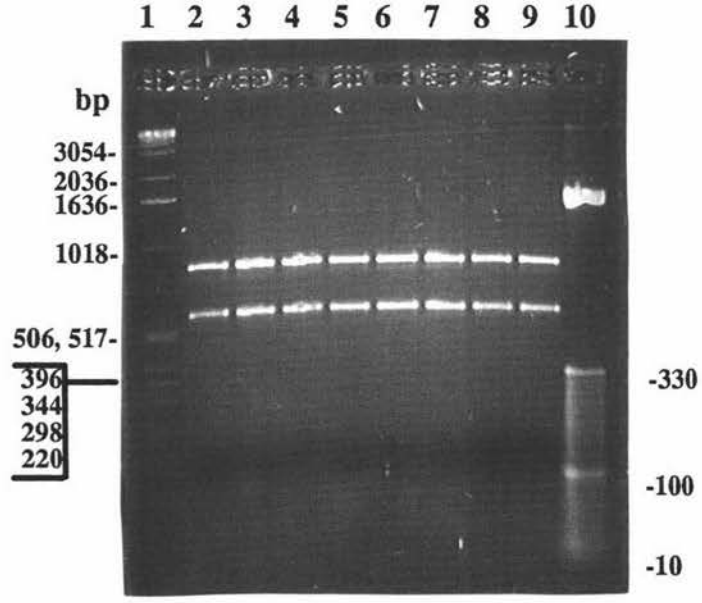


Figure 3.23 C

CHAPTER 4

DISCUSSION

Campylobacter species were identified with a series of biochemical tests. Among the human isolates from the Palmerston North region, the proportion of *C. coli* (11%) compared with *C. jejuni* (89%) was higher than is usually observed in developed countries. In Europe, Canada and the United States *C. coli* infections generally account for less than 5% of all *Campylobacter* infections (Taylor, 1992; Karmali *et al.*, 1983), although the proportion of *Campylobacter* infections due to *C. coli* can be much higher in developing countries (Taylor, 1992). The high number of *C. coli* isolates from humans in the Manawatu may reflect the sources of human infections in this region. Alternatively, it may be a sampling anomaly due to the relatively small number of isolates (140) examined.

A wider range of *Campylobacter* species were found among the animal isolates but, unlike the human isolates, were not directly linked to disease. Most were cultured from the faeces or carcasses of healthy animals or from animals infected with other gastrointestinal pathogens.

Biochemical identification is a logical starting point for the typing of strains but provides too little discrimination for use as an independant typing system (Fayos *et al.*, 1995).

Restriction endonuclease analysis was the first of three genotypic typing methods examined in this study. This method has been used in a number of studies to discriminate between *Campylobacter* strains (Collins and Ross, 1984; Kakoyiannis *et al.*, 1984; Owen *et al.*, 1989; Fox *et al.*, 1989; Patton *et al.*, 1991; Lind *et al.*, 1996), and requires a large amount of intact DNA.

Three methods were used to extract genomic DNA from *Campylobacter* cells. DNA extraction method #3 (alkaline lysis) was the most effective of the three methods. One explanation for the poor DNA yields using DNA extraction methods #1 and #2 is that the cells may not have lysed completely. Another reason for greater yields with DNA extraction method #3 may be that the extract was precipitated with ethanol rather than

isopropanol and was left at -20°C overnight to precipitate. All methods produced sheared DNA. This is most likely the result of the repeated pipetting and mixing necessary for the multiple phenol/chloroform/iso-amyl alcohol extractions. The DNA yields varied greatly between isolates. This may have been due to varying levels of DNase production by the isolates.

In the present study, the restriction endonucleases *Hae*III and *Cla*I were used to examine over 100 human *Campylobacter* isolates. These enzymes cut the genome frequently, hence the restriction patterns contained many fragments. As a result the patterns were hard to interpret. Due to the difficulties in extracting sufficient intact DNA from *Campylobacter* cells, and the complexity of the restriction patterns, this method was considered unsuitable for further use.

RAPD PCR typing was investigated as an alternative to restriction endonuclease analysis. This method uses PCR with random primers to obtain profiles of DNA fragments. The PCR reaction was standardised by varying the $MgCl_2$ and DNA template concentrations. However, having standardised the conditions, it was still difficult to obtain reproducible results. Several studies have found that RAPD fingerprinting provides a discriminatory and rapid means of comparing *Campylobacter* isolates (Mazurier *et al.*, 1992; Fayos *et al.*, 1993; Hernandez *et al.*, 1995; Madden *et al.*, 1996) although, Hernandez and collegues (1995) described a lack of interlaboratory reproducibility with this method. It was concluded that, for the purposes of this study (Section 3.5.2), RAPD PCR would not be a reliable method of discriminating between *Campylobacter* strains.

Subsequently, RFLP typing of the flagellin gene was examined as a means of distinguishing between *Campylobacter* isolates. Restriction fragment length polymorphisms within the *fla*B gene were examined by restriction endonuclease digestion with *Pst*I and *Hin*dIII. The fragment sizes for a particular RFLP pattern varied slightly from gel to gel. In keeping with previous studies, RFLP patterns were considered identical if the fragment sizes varied by less than 5% (Owen *et al.*, 1993a; Owen *et al.*, 1994; Nachamkin *et al.*,1996). The total size of the PCR product deduced from the summation of the constituent fragments also varied between RFLP patterns. This finding was also described by Owen and co-workers (1993 and 1994), and could be attributable to inaccuracies in sizing.

The digested amplicons were size fractionated through 3% agarose gels in order to visualise the smaller DNA fragments. However, at this percentage of agarose some of the higher molecular weight DNA fragments were larger than expected. For example, Figure 3.4 shows three uncut *fla*B amplicons visualised on a 1.6% agarose gel. The amplicon (~1.46kb) is clearly smaller than the 1636bp fragment of the 1kb ladder. By contrast, Lane 4 of Figure 3.5A shows a *fla*B amplicon that did not cut with *Pst*I, visualised on a 3% agarose gel. In this photograph, the uncut amplicon appears to be the same size as the 1636bp fragment of the 1kb ladder. The reason the 1636bp fragment itself appears relatively unaffected by the increase in agarose concentration may be due to the smaller amount of DNA in this band.

Despite the anomalies in comparing fragment sizes, the RFLP patterns were reproducible. A number of studies (Alm *et al.*, 1993a; Nachamkin *et al.*, 1993: Owen *et al.*, 1993a; Owen *et al.*, 1994; Mohran *et al.*, 1996; Nachamkin *et al.*, 1996; Santesteban *et al.*, 1996) have also shown that flagellin gene RFLP typing is a reproducible method of distinguishing between *Campylobacter* isolates.

A total of 26 *Pst*I/*Hin*dIII combined types were found among 140 human *Campylobacter* isolates. These results indicate that there is considerable heterogeneity among the *fla*B genes of the human isolates and are consistent with previous studies (Alm *et al.*, 1993b; Mohran *et al.*, 1996).

A number of human isolates (8.5%) produced DNases and could not be amplified directly from bacterial colonies. A diverse range of RFLP types were found among the DNase producing isolates; three isolates had unique combined RFLP types (Table 3.5). DNase production is a major distinguishing feature of the Lior biotyping scheme which divides *C. jejuni* into four biotypes, two of which produce DNases, and divides *C. coli* into two biotypes, one of which produces DNases (Lior, 1984). The Lior biotyping scheme relies on just three phenotypic tests, and is a relatively coarse method of discriminating between strains. The results of the present study support the findings of Owen and collegues (1993a and 1994) that there is little association between Lior biotype and flagellin gene RFLP type.

The current study provides evidence of conservation of RFLP types among *C. jejuni* and *C. coli* in that two RFLP types were unique to *C. coli* and eighteen RFLP types contained *C. jejuni* exclusively. These results contrast with those of Nachamkin and co-workers (1996) who found no conservation of *fla*A RFLP types among *C. jejuni* and *C. coli* isolates. This may reflect the relatively small number of isolates examined in the present study.

RFLP analysis of the *fla*A gene was performed on 15 human isolates representative of the 13 *Pst*I and 8 *Hin*dIII types found among the *fla*B genes. The majority gave identical patterns but three isolates showed different polymorphic patterns between the *fla*A and *fla*B genes. It appears that the *fla*A gene of isolate C131 has a *Pst*I restriction site at a different location to that of the *fla*B gene (Table 3.8). The *fla*A gene of isolate C8 has an additional *Pst*I site which cuts the 1220bp (*fla*B) fragment into two fragments of 980bp and 110bp. The *fla*A gene of isolate C176 contains an additional *Hin*dIII site. The *fla*B pattern for this isolate has a 250bp fragment which in the *fla*A pattern has been digested to give two fragments of 120bp and 102bp. Overall, these results are consistent with those of Alm and co-workers (1993) and Mohran and collegues (1996) who reported that for most strains the *fla*A gene gave identical polymorphic patterns to the *fla*B gene.

Five reference strains , *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. coli*, *C. fetus* subsp. *fetus* and *C. lari*, were included in this study. However, the *fla*B gene amplified in only two species- *C. jejuni* subsp. *jejuni* and *C. coli*. The *fla*B gene of thirteen animal isolates did not amplify, of which 9 were *C. fetus*, 2 were *C. hyointestinalis*, 1 was *C. jejuni* and 1 was *C. coli*. Studies on the distribution of the *fla*B gene have been confined to *C. jejuni* and *C. coli* and it is not known whether other *Campylobacter* species possess this gene. As the primers used in the present study were designed for the conserved regions of the *fla*B gene of *C. coli* VC167, it is likely they did not anneal to the flagellin genes of *Campylobacter* species, other than *C. jejuni* subsp. *jejuni* and *C. upsaliensis*.

The finding that one of the isolates which failed to produce a PCR product was *C. jejuni* and another was *C. coli* is more difficult to explain. These isolates may have been misidentified. The limitations and lack of reproduciblity of biochemical identification were discussed previously (Section 1.3.1).

Five of the eight RFLP types found among the animal isolates were also found in humans. The dendogram (Figure 3.18) provided a means of visualising the similarities between the isolates. The finding that the two *C. upsaliensis* isolates from dogs are only distantly related to *C. jejuni* and *C. coli* isolates (S_{AB} values of 0.40 to 0.66) indicates that the dendogram reflects real relationships. It can be seen that some of the animal isolates are more closely related to certain human isolates than some of the human isolates are to each other. These results indicate that sheep, cows and calves may be an important source of infection in humans.

Over 98% of *C. jejuni* and *C. coli* isolates were typeable by analysis of the *fla*B gene. These results contrast with those of serotyping studies in which up to 20% of human isolates were untypeable (Nicol and Wright, 1997; Steinhauserová *et al.*, 1997)

The potential of *fla*B RFLP typing as an epidemiological tool was demonstrated through the blind testing of 22 isolates (provided by C. Nicol, Institute of Environmental Science and Research), fourteen of which were from an outbreak of Campylobacteriosis. Two distinct *Pst*I/*Hind*III combined types were found among the isolates and all 14 outbreak isolates had identical restriction patterns. The serotypes of the outbreak isolates correlated with the *fla*B RFLP types. RFLP typing of the non-outbreak isolates was more discriminatory than serotyping. These findings are consistent with the observations of Owen and collegues (1994) and Nachamkin and co-workers (1996).

It has been suggested that, because of their similarity, *Campylobacter* flagellin genes undergo intragenic recombination. This has lead Harrington and co-workers (1997) to propose that flagellin gene typing may not be a stable method for the long-term monitoring of *Campylobacter* populations. This suggestion is not supported by the finding in this study that, *in vitro*, the *fla*B RFLP patterns were stable over ~360 generations in two separate *C. jejuni* isolates. Nineteen of the isolates included in this study represented repeated samples taken from 17 patients. The RFLP patterns for the majority of these isolates were identical to those of the original samples but repeated isolates from two patients had *Pst*I patterns which differed from the original samples (P5/H1 and P4/H1 in one patient; P5/H1 and P12/H1 in another patient). One explanation for this is that these individuals were simultaneously infected with more than one *Campylobacter* strain. This has been described for *Giardia duodenalis* infection of humans (Upcroft and Upcroft, 1994). An alternative explanation, supported by the fact

that the *Hin*dIII patterns did not change, is that point mutations or intragenic recombination have introduced additional *Pst*I sites into the *fla*B genes of the repeated samples. These findings support those of Harrington and co-workers (1997) that flagellin gene typing may not be a stable method of typing *Campylobacter* strains.

Flagellin gene RFLP typing has an advantage over other genotypic methods such as ribotyping, MEE and PFGE, in that it is relatively quick and simple to perform while providing a high level of discrimination. This method is able to type a significantly larger number of isolates than serotyping. However, flagellin gene typing may not be stable due to recombination within the flagellin genes. These difficulties highlight the need to use more than one typing method to discriminate between strains.

This research has confirmed the distribution and heterogeneity of the *fla*B gene in *C. jejuni* and *C. coli*. The results of this study indicate that sheep, cows and calves may be important sources of *Campylobacter* infection in humans. However, further research on a larger number of isolates from a wider range of animals is needed to determine the exact sources of *Campylobacter* infections in humans in New Zealand.

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