Campylobacter abortion in sheep:

a study of strain types and vaccine protection.

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy in Veterinary Pathology

at Massey University, Palmerston North, New Zealand.

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2003

Abstract

The bacteria *Campylobacter fetus* subsp. *fetus* and *C. jejuni* are important causes of abortion in sheep, and the majority of *Campylobacter* sheep abortion in New Zealand is associated with *C. fetus* subsp. *fetus*. Campylovexin® (Schering-Plough Animal Health Ltd., Upper Hutt) is a single-strain, killed vaccine available in New Zealand for the prevention of abortion in sheep due to *C. fetus* subsp. *fetus*. The present study was initiated in response to questions regarding the protection offered by this vaccine against other strains of *C. fetus* subsp. *fetus* causing sheep abortion in New Zealand.

Campylobacter isolates from aborted sheep foetuses were collected after culture in veterinary diagnostic laboratories from sheep flocks in the Hawke's Bay region in 1999. This was extended in 2000, when *Campylobacter* sheep abortion isolates cultured in the same way from farms throughout New Zealand were collected. In total, 374 *C. fetus* subsp. *fetus* isolates, 30 *C. jejuni* isolates, and one *C. coli* isolate were collected from 249 farms. These isolates were sub-typed using the genetic technique, pulsed-field gel electrophoresis (PFGE). A total of 26 distinct PFGE types were identified amongst the *C. fetus* subsp. *fetus* sheep abortion isolates, and one type, PFGE type B1, predominated. Of the farms from which *C. fetus* subsp. *fetus* was isolated, PFGE type B1 was found on 19 out of the 25 farms in the Hawke's Bay study (75%) and 132 out of the 200 farms in the national study (66%).

Nine distinct PFGE types were found amongst the 30 *C. jejuni* isolates from the Hawke's Bay and national studies. No single *C. jejuni* PFGE type was identified from sheep abortion most frequently. Investigation of a large sheep abortion outbreak initially attributed to *C. jejuni* revealed that the initial diagnosis was misleading due to contamination of the foetal sample, and that the outbreak was more likely to have been associated with *C. fetus* subsp. *fetus*.

Seven *C. fetus* subsp. *fetus* isolates representative of the genotypes found amongst New Zealand sheep abortion isolates in a 1987 study were lodged with the New Zealand Reference Culture Collection, Medical Section (de Lisle et al. 1987). These isolates were typed in the present study using PFGE, and were found to be similar or

indistinguishable from the PFGE types identified in the Hawke's Bay or national studies. The frequency of isolation of the types also appeared to be similar between 1987 and 2000, as the prevalent type from 1987 was identified as PFGE type B1.

A nationwide call for suspected cases of Campylovexin® breakdown resulted in the identification of one flock in which abortions due to *C. fetus* subsp. *fetus* were reported in ewes apparently vaccinated that season. Approximately 40 ewes aborted out of a flock of 1100 ewes, and it was thought that four ewes that had aborted were vaccinated that season. The high stocking density management of this flock meant that there was likely to have been a high challenge to vaccinated ewes from unvaccinated aborting ewes. Investigation of two other apparent Campylovexin® breakdown cases revealed that ewes had not been vaccinated that season and were also run at a high stocking density. In addition, the *C. fetus* subsp. *fetus* isolates in these two cases were the same PFGE type as the vaccine strain, PFGE type A1, which suggested that in these cases apparent vaccine breakdown was not due to lack of cross-protection against other strains. Despite the widespread occurrence of distinct strains of *C. fetus* subsp. *fetus*, abortion due to *C. fetus* subsp. *fetus* in ewes vaccinated with Campylovexin® appeared to be rare and was not associated with a particular PFGE type.

Serum from 15 ewes vaccinated with Campylovexin® was collected and used in Western blotting experiments to investigate antibody binding with the proteins of the various PFGE types of *C. fetus* subsp. *fetus*. Serum from vaccinated ewes contained antibodies that bound to the proteins of the different *C. fetus* subsp. *fetus* PFGE types. This *in vitro* study suggested that sheep vaccinated with Campylovexin® develop antibodies that recognise proteins of each of the *C. fetus* subsp. *fetus* strain types.

Vaccine challenge experiments were performed using pregnant guinea pigs as a model. Initially, it was shown using this model that Campylovexin® provided a statistically significant level of protection from homologous challenge with the vaccine strain. Then, using an isolate of PFGE type B1, it was demonstrated that Campylovexin® also provided a statistically significant level of protection against heterologous challenge.

Despite the range of *C. fetus* subsp. *fetus* PFGE types identified in this study, there was no evidence to suggest that the different strain types compromised Campylovexin®

efficacy in protection against *C. fetus* subsp. *fetus* sheep abortion. The protein recognition by serum from ewes vaccinated with Campylovexin®, the lack of a robust vaccine breakdown case, and the demonstration of cross-protection in the guinea pig model, provide evidence that Campylovexin® appears to offer cross-protection against strains of *C. fetus* subsp. *fetus* causing abortion in sheep in New Zealand.

Acknowledgements

I thank my team of supervisors:

Dr. Dave West	Associate Professor Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North
Dr. Stan Fenwick	Senior Lecturer in Veterinary Public Health School of Veterinary Clinical Science, Murdoch University, Perth, Australia
Dr. Roger Marchant	Veterinary Adviser Schering-Plough Animal Health Limited, Upper Hutt
Dr. Kevin O'Connell	Technical Manager R&D Global Vaccine Unit Schering-Plough Animal Health Limited, Upper Hutt

I thank Stan and Roger for dreaming up this project, constructing a plan and obtaining the funding from Technology New Zealand. I would like to thank them both for their support and especially Roger for his endless enthusiasm. Dave has been a fabulous strength in this project and I thank him for his support and for getting me to think. Trudy Owen (Regulatory Affairs Manager, SPAH) has been an invaluable source of advice and support and provided useful input at the project meetings. I would also like to recognise the time and support of Marty Phillips (Division Manager, SPAH) towards this project.

I thank the friendly crowd at SPAH for making me feel welcome, particularly the staff of R&D, QA, and QC. I would especially like to thank Philip Wallace (Wally) and Jacquie Clarke for providing a home-away-from-home for me in the Hutt.

I thank Megan Leyland (PFGE Typing Laboratory technician, IVABS) for sharing her considerable PFGE expertise, including help with Diversity Database and with the production of endless dendrograms. I would like to thank the microbiology staff of the veterinary diagnostic laboratories who supplied *Campylobacter* abortion isolates. I thank Kylie Walker (MicroLab technician, IVABS) for storing the *Campylobacter*

isolates from the Hawke's Bay in 1999 and the staff at Veterinary Services Hawke's Bay Ltd. for their continued support of this project.

Thank you to the veterinarians who advised us of suspected breakthrough cases: Sonya Jackson (North Canterbury Veterinary Services) and Carolyn Hamill (Edendale Vet Club), and the farmers for their cooperation in this investigation.

I would like to thank IVABS veterinarians Drs. Anne Ridler, Mark Owen, Dave West, Alison Quinn, Alastair Johnston, Mark Collett, and especially Dr Kathy Parton (IVABS Laboratory Animal Veterinarian), for advice and assistance with guinea pig procedures of a veterinary nature. I thank Debbie Chesterfield (Small Animal Production Unit, Massey University) and Rob Hazelwood (SPAH Animal Breeding Unit) for advice and assistance. I would like to thank Dr Nigel Perkins (Epidemiologist, IVABS) for statistical advice and Dr Alan Murray for helpful suggestions. I thank Dr Anne Midwinter for assistance with the Petroff-Hausser counter and Mike Hogan (Post-mortem room technician, IVABS) for assistance.

Finally, but not least, I would like to thank my family and friends for their support, particularly Anne Ridler, Julie Thompson, and Rowena Taylor for reviewing thesis drafts.

This work was supported by a Technology New Zealand Technology for Industry Fellowship in conjunction with Schering-Plough Animal Health Ltd.

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Literature Review

1.1 Introduction

The first isolation of *Campylobacter* species from sheep abortion was in Britain in 1909 when McFadyean and Stockman cultured a "vibrio" from an aborted sheep foetus (McFadyean and Stockman 1913). Since then, the *Campylobacter* species *C. fetus* subsp. *fetus* and *C. jejuni* have been found to be a significant cause of abortion in sheep in most of the sheep-producing countries. These include Australia (Robards et al. 1969; Clark and Monsbourgh 1974; Broadbent 1975; Dennis 1975), United States of America (Marsh et al. 1954), South Africa (Schutte et al. 1971), Britain (Watson et al. 1967), and New Zealand. Sheep abortions due to *Campylobacter* spp. were first identified in New Zealand in 1950 (McFarlane et al. 1952).

Although *C. fetus* subsp. *fetus, C. jejuni*, and *C. coli* have been implicated in sheep abortion (Marsh and Firehammer 1953; Berg et al. 1971), *C. fetus* subsp. *fetus* causes the majority of the *Campylobacter* abortions in New Zealand (Poland 2002).

1.2 The Genus *Campylobacter*

The genera *Campylobacter* and *Arcobacter* form the family Campylobacteriaceae. This family and the genera *Wollinella* and *Helicobacter* form rRNA Superfamily VI (Skirrow 1994).

Campylobacter spp. are relatively slow growing, fastidious, nonsporing, Gram negative, curved, helical, or gull-shaped bacteria (Skirrow 1994). They usually have one unsheathed polar flagellum, or one at each end, and motility is rapid and darting in a corkscrew manner (Penner 1988). *Campylobacter* spp. occupy a diverse range of niches: from intestines, genital tracts and the oral cavity, to roots of salt marsh plants (Penner 1988; On 1996). They cause an equally diverse range of diseases in a varied range of hosts: from abortion in sheep, to gastritis and septicaemia in humans (Penner 1988; On 1996).

1.2.1 *Campylobacter jejuni* and *C. coli*

Campylobacter jejuni and *C. coli* are significant causes of gastroenteritis in humans, usually by ingestion of contaminated milk, water or food, particularly chicken (Friedman et al. 2000). The New Zealand incidence of campylobacteriosis for the year to May 2001 was 222.4 cases per 100,000 population, four times the rate of salmonellosis at 52.4 cases (Anon 2001a). *Campylobacter jejuni* is divided into two subspecies: *jejuni* and *doylei*. The isolation of *C. jejuni* subsp. *doylei* has been limited to cases of human gastroenteritis (Vandamme 2000), and in this thesis *C. jejuni* refers to *C. jejuni* subsp. *jejuni*.

Campylobacter jejuni and *C. coli* are commonly found in intestinal contents or faeces of healthy sheep (Manser and Dalziel 1985; Stanley et al. 1998; Jones et al. 1999). However, there is some evidence to suggest that *C. jejuni* may be associated with diarrhoea in lambs or hoggets (Russell 1955; Stansfield et al. 1986).

1.2.2 *Campylobacter fetus*

Campylobacter fetus subsp. *fetus* is most closely related to the other *C. fetus* subspecies: *C. fetus* subsp. *venerealis* (Harvey and Greenwood 1983a). *Campylobacter fetus* subsp. *fetus* primarily causes abortion in sheep (see Section 1.4), but also occasionally causes sporadic abortion in cattle and sporadic infections in humans. *Campylobacter fetus* subsp. *venerealis* causes abortion and infertility in cattle.

1.2.2.1 *Campylobacter fetus* subsp. *fetus* infections in humans

Human disease due to *C. fetus* subsp. *fetus* is rare (Penner 1988), and mostly sporadic (Blaser 1998). Most *C. fetus* subsp. *fetus* infections in humans occur in patients with an underlying medical condition and immune deficiency, for example cancer, chronic liver disease, AIDS (Skirrow 1994; Meier et al. 1998), systemic lupus erythematosus, diabetes, agammaglobulinemia (Morrison et al. 1990; Ichiyama et al. 1998), and chronic alcoholism (Dronda et al. 1998). Although *C. fetus* subsp. *fetus* causes a variety of infections in humans, the most common is bacteraemia (Morrison et al. 1990; Meier et al. 1998). Also reported are gastrointestinal disease (Harvey and Greenwood 1983b; Rennie et al. 1994), meningitis in infants and adults (Morooka et al. 1996; Dronda et al. 1998), cellulitis (Ichiyama et al. 1998), abortion (Skirrow 1994; Steinkraus and Wright

1994), and cardiovascular diseases (Morrison et al. 1990; Montero et al. 1997). Most cases of disease due to *C. fetus* subsp. *fetus* cannot be traced to an identifiable source, although occasionally ingestion of contaminated food or water or contact with infected animals can be demonstrated (Meier et al. 1998; Ichiyama et al. 1998). A New Zealand farmer from an area where *C. fetus* subsp. *fetus* abortions were occurring in sheep developed bacteraemia due to *C. fetus* subsp. *fetus* after surgery to remove cancer (Gumbrell 1985a).

1.2.2.2 Abortion and infertility in cattle

Campylobacter fetus subsp. *venerealis* is reported to be a major cause of infertility and financial loss in the cattle industry (Hum 1996). However, abortion and infertility due to *C. fetus* subsp. *venerealis* has not been demonstrated to be a significant problem in cattle in New Zealand (Loveridge and Gardner 1993). *Campylobacter fetus* subsp. *venerealis* is adapted to the bovine genital tract and has a limited host range (Penner 1988). This organism is transmitted venereally to cows from infected bulls. Bulls contract the infection from infected females or from contaminated bedding. The infection in the prepuce of bulls is persistent, localised, and asymptomatic. The organism multiplies in the vagina and spreads through the cervix to the uterus, where a chronic inflammation develops, and abortion may result (Dekeyser 1984). The embryo normally dies very early in gestation and the cow reverts to oestrus (Border and Firehammer 1980). After sufficient immunity is developed, the cow is able to carry a foetus to term (Border and Firehammer 1980), but some may be sterile (Dekeyser 1984).

While most of the *Campylobacter* abortions in cattle are caused by *C. fetus* subsp. *venerealis*, abortion due to the closely related *C. fetus* subsp. *fetus* may occur (Skirrow 1994), and *C. jejuni* has also occasionally been implicated (Van Donkersgoed et al. 1990). Cattle infections with *C. fetus* subsp. *fetus* do not generally result in infertility (Hum 1996), and this organism may be found in the intestine of healthy cattle (Manser and Dalziel 1985).

1.3 Identification of *Campylobacter* species

Two aspects of research into *Campylobacter* abortions in sheep have contributed to confusion in the literature. One is nomenclature and the other is the ability to reliably distinguish the *Campylobacter* spp.

1.3.1 Nomenclature

Following the original isolation by McFadyean and Stockman in 1909, Smith and Taylor (1919) isolated a bacterium from infectious abortion in cattle. The bacterium was assumed to be the same as that causing infectious abortion in sheep and was named *Vibrio fetus* (Smith and Taylor 1919). In 1959, Florent distinguished two varieties of *Vibrio fetus* on the basis of two different diseases in cattle; *Vibrio fetus* var. intestinalis originated in the intestine and caused sporadic abortion, whereas *Vibrio fetus* var. venerealis was venereally transmitted and caused infertility (Karmali and Skirrow 1984).

In 1963, microaerophilic vibrios were separated from the facultative anaerobes and allocated a new genus, *Campylobacter*, from the Greek for "curved rod" (Sebald and Veron 1963). In 1973, Veron and Chatelaine proposed that *C. fetus* var. intestinalis become the type species for the genus, and be renamed *C. fetus* subsp. *fetus*. In addition, they proposed that *C. fetus* var. venerealis become *C. fetus* subsp. *venerealis*. However, another researcher simultaneously chose *C. fetus* var. venerealis as the type species and assigned this species the name *C. fetus* subsp. *fetus* (Smibert 1974). Accordingly, *C. fetus* var. intestinalis became *C. fetus* subsp. *intestinalis*. At the same time, *C. coli* and *C. jejuni* were not considered different enough to be separate species and were renamed *C. fetus* subsp. *jejuni*. This reclassification of these species appeared in Bergey's Manual of Determinative Bacteriology in 1974 (Smibert 1974), but was not widely adopted. Now Bergey's Manual of Systematic Bacteriology uses the classification scheme of Veron and Chatelain 1973 (Smibert 1984), as does the "Approved lists of bacterial names" (Skerman et al. 1980). However, the literature from the 1970s and 1980s needs to be examined carefully to determine which species and subspecies is being referred to. The nomenclature changes relating to *C. fetus* subsp. *fetus* are summarised in Table 1.1.

Table 1.1 Nomenclature of *Campylobacter fetus* (Adapted from Garcia et al. 1983 and Karmali and Skirrow 1984).

Official name Veron and Chatelain 1973	**Past name**	**Reference**
C. fetus subsp. *fetus*	*Vibrio fetus*	Smith and Taylor 1919
	Vibrio fetus var. intestinalis	Florent 1959
	C. fetus subsp. *intestinalis*	Smibert 1974
C. fetus subsp. *venerealis*	*Vibrio fetus*	Smith and Taylor 1919
	Vibrio fetus var. venerealis	Florent 1959
	C. fetus subsp. *fetus*	Smibert 1974
C. jejuni subsp. *jejuni*	*Vibrio jejuni*	Jones et al. 1931
	Related vibrios	King 1957
	C. fetus subsp. *jejuni*	Smibert 1974
C. coli	*Vibrio coli*	Doyle 1944
	Related vibrios	King 1957
	C. fetus subsp. *jejuni*	Smibert 1974

1.3.2 Identification of *Campylobacter* species associated with sheep abortion

1.3.2.1 Serotyping to distinguish *Campylobacter* species

Serotyping was often used to identify *Campylobacter* spp. and there were at least nine different serotyping or biotyping schemes in use between 1953 and 1971. Research during this period often referred to the isolate serotype, rather than species name as it is known today. *Campylobacter fetus* subsp. *fetus* has been serotyped at various times as 2, A2, B, II, III, and V. *Campylobacter fetus* subsp. *venerealis* has been serotyped as 1, sub 1, A-1, A-sub 1, A, I, III, and V. *Campylobacter jejuni* has been serotyped as C, I, and 13. It may be unclear exactly what species is referred to unless the reference is

Table 1.2 Serotypes, biotypes and group names assigned to *Campylobacter* spp. Reproduced from Garcia et al. 1983.

***Campylobacter* species**	**Serotype**				**Biotype**	**Group**		
	Marsh & Fireharnmer 1953	Mitscherlich & Liess 1958	Morgan 1959	Berg et al. 1971	Bryner et al. 1962	Mohanty et al. 1962	Plastridge et al. 1964	Akkermans et al. 1956
C. fetus subsp. *venerealis*	III	1	A	A-1	1	I	I	I
	III, V	1	A	A-sub 1	sub 1	III		
C. fetus subsp. *fetus*	V III			A-2	2	II	II	III
	II	2	B	B				
C. jejuni and *C. coli*	I	13		C				
C. sputorum subsp. *bubulus*		IV				II	IV	

given for the serotyping scheme used. For example, a study showing that the American magpie could carry and excrete *Vibrio fetus* serotype I for at least 213 days (Meinershagen et al. 1965) was referring to *C. jejuni* using the serotyping scheme of Marsh and Firehammer (1953). The names of serotypes and the species they refer to are summarised in Table 1.2, reproduced from Garcia et al. (1983).

1.3.2.2 Phenotypic tests to distinguish *Campylobacter* species

Sheep abortion due to *Campylobacter* spp. is primarily diagnosed by the isolation of *Campylobacter* organisms from foetal stomach contents. *Campylobacter* organisms may also be visualised by direct microscopy of foetal stomach contents after staining with dilute carbol fuchsin or Gram stain (Quinn et al. 1994). *Campylobacter fetus* subspecies have small (1mm), round, smooth, translucent colonies on blood agar (Quinn et al. 1994). *Campylobacter jejuni* colonies are normally flat and larger than *C. fetus* colonies, and may be watery and spreading on moist plates (Quinn et al. 1994).

Typically identification of *Campylobacter* sheep abortion isolates is by:

- colony morphology after microaerobic incubation at 37°C
- microscopic morphology: Gram negative curved, S-shaped or helical rods
- a positive oxidase test (Quinn et al. 1994). The oxidase test detects the presence of the enzyme oxidase, which catalyses the oxidation of cytochrome c by molecular oxygen (MacFaddin 2000a).

Species and subspecies identification of *Campylobacter* is generally performed by phenotypic testing:

- catalase activity
- susceptibility or resistance to nalidixic acid or cephalothin
- hydrogen sulphide production
- growth at 25°C or 42°C (Quinn et al. 1994)

The phenotypic tests that distinguish selected *Campylobacter* spp. are summarised in Table 1.3. *Campylobacter fetus* subsp. *venerealis*, *C. lari* and *C. hyointestinalis* have been included here in addition to the *Campylobacter* spp. implicated in sheep abortion: *C. fetus* subsp. *fetus*, *C. coli* and *C. jejuni*. *Campylobacter fetus* subsp. *venerealis* is closely related to *C. fetus* subsp. *fetus* (Harvey and Greenwood 1983a). *Campylobacter*

jejuni, *C. coli*, *C. lari* and *C. hyointestinalis* have been isolated from sheep faeces (Stanley et al. 1998). *Campylobacter* spp. are biochemically relatively inactive; they do not ferment sugars, but have a respiratory metabolism and use amino acids and tricarboxylic acid cycle intermediates. Species differentiation can be difficult due to tolerance to growth conditions of strains within species and also due to problems with reproducibility of some tests (Skirrow 1994).

Table 1.3 Phenotypic tests of selected *Campylobacter* species (collated from Quinn et al. 1994 and Quinn et al. 2002). The table summarises catalase activity; the production of hydrogen sulphide (H_2S) with triple sugar iron agar (TSI) and lead acetate (PbAc); growth in 1% glycine; susceptibility (S) or resistance (R) to the antibiotics nalidixic acid (N) and cephalothin (C); growth at 25°C and 42°C; and hippurate hydrolysis (Hip) of selected *Campylobacter* species.

Species	Catalase	H_2S TSI	H_2S PbAc	Glycine 1%	N	C	25°C	42°C	Hip
C. fetus subsp. *fetus*	+	-	+	+	R	S	+	-	-
C. fetus subsp. *venerealis*	+	-	-	-	R	S	+	-	-
C. jejuni	+	-	+	+	S	R	-	+	+
C. coli	+	-	+	+	S	R	-	+	-
C. lari	+	-	+	+	R	R	-	+	-
C. hyointestinalis	+	+	+	+	R	S	+	+	-

Presence of catalase

Catalase is an enzyme that converts hydrogen peroxide into water (MacFaddin 2000b). The catalase test is a simple test which separates the genus *Campylobacter* into catalase positive and negative species. *Campylobacter fetus* subsp. *fetus*, *C. jejuni*, *C. coli*, *C. fetus* subsp. *venerealis*, *C. hyointestinalis* and *C. lari* are all catalase positive. Catalase

negative *Campylobacter* spp. tend to be more oxygen sensitive than catalase positive *Campylobacter* spp., and are able to reduce nitrites and produce hydrogen sulphide in triple sugar iron media, in general contrast to the catalase positive *Campylobacter* spp. (Karmali and Skirrow 1984).

Antibiotic sensitivity

The antibiotics nalidixic acid and cephalothin are useful in differentiating *C. fetus* subsp. *fetus* from *C. jejuni* and *C. coli*. *Campylobacter fetus* subsp. *fetus* is susceptible to cephalothin and resistant to nalidixic acid, in contrast to *C. jejuni* and *C. coli*, which have the opposite susceptibility. However, each test should not be looked at in isolation (On 1996), as atypical *C. fetus* subsp. *fetus* isolates have been found with resistance to cephalothin (Edmonds et al. 1985) and non-standardisation of the method used may influence the outcome of this test (On and Holmes 1991b).

Growth at different temperatures

Growth at 25°C is the other useful test to distinguish *C. fetus* subsp. *fetus* from *C. jejuni* and *C. coli*. *Campylobacter jejuni* and *C. coli* are able to grow at 42°C, but not at 25°C. Conversely, *C. fetus* is able to grow at 25°C, but not at 42°C. However, growth at 25°C has been shown to be more discriminating of species than at 42°C, as many typical *C. fetus* subsp. *fetus* isolates are able to grow at 42°C (Skirrow and Benjamin 1980; Edmonds et al. 1985; On and Harrington 2001).

Hippurate hydrolysis

Campylobacter jejuni subsp. *jejuni* and subsp. *doylei* are the only *Campylobacter* spp. capable of hydrolysing sodium hippurate (Harvey 1980; On 1996). This test is used to differentiate *C. jejuni* from *C. coli*, however, some hippurate negative *C. jejuni* have been isolated (Totten et al. 1987).

Hydrogen sulphide production

Measurement of hydrogen sulphide production is one test to distinguish the *C. fetus* subspecies, as *C. fetus* subsp. *fetus* produces a small amount of hydrogen sulphide, detectable with a lead acetate strip, and *C. fetus* subsp. *venerealis* does not produce any (Quinn et al. 1994).

There are two tests for detecting the production of hydrogen sulphide, as different enzymes catalyse the production of hydrogen sulphide with the use of different substrates, and different media may be used to detect this production. Hydrogen sulphide may be produced from thiosulfate or cysteine, and maybe both. Thiosulphate reductase produces hydrogen sulphide from thiosulphate, and cysteine desulfhydrase produces hydrogen sulphide from cysteine (Barrett and Clark 1987). The first test is production of hydrogen sulphide after inoculation into Triple Sugar Iron agar (TSI), which contains sodium thiosulphate and ferric ammonium citrate. A positive result is blackening of the medium by the formation of iron sulphide. The second test uses a lead acetate strip, which is extremely sensitive in hydrogen sulphide detection. The media is semi-solid Brucella or Heart Infusion Broth with 0.02% cysteine. Blackening of the lead acetate strip indicates a positive reaction (Quinn et al. 1994). For clarity, the medium and detection system used need to be specified when reporting results.

Growth in glycine

Growth on media containing 1% glycine is the other test used to distinguish *C. fetus* subspecies, as *C. fetus* subsp. *venerealis* is unable to grow in the presence of 1% glycine (Quinn et al. 2002). This test has also been rather controversial because tolerance to glycine can be transferred between bacteria (Chang and Ogg 1971). However, others have found long-term isolates have retained glycine intolerance (Roop et al. 1984). Also, there have been reports of glycine-tolerant *C. fetus* subsp. *venerealis*, which were termed biovar intermedius (Cameron 1982; Salama et al. 1992). However, false positive results may be obtained if the concentration of bacteria is too large in the inoculum and depending on the media used (On and Holmes 1991a and 1991b). When subjected to this phenotypic test using standardised methods, isolates previously identified as biovar intermedius were not glycine tolerant and were identified as *C. fetus* subsp. *venerealis* (On and Harrington 2001).

1.3.2.3 Genetic tests to distinguish *Campylobacter* species

The problems associated with phenotypic testing to distinguish *Campylobacter* spp., such as the necessity to first culture the bacteria and reliance on a small number of tests to distinguish between species, have led to the investigation of genetic tests to differentiate *Campylobacter* species.

Nucleic acid probes

The relative stability of chromosomal DNA lends itself to the use of nucleic acid probes for taxonomic and diagnostic determination of bacteria (On 1996). The probe is a sequence of nucleic acids which bind specifically to DNA from the target bacteria. This binding may be detected in a number of ways.

In 1983, a study examined the DNA relatedness of catalase positive *Campylobacter* isolates for taxonomy purposes (Harvey and Greenwood 1983a). Several isolates identified phenotypically were subjected to DNA hybridisation with a set of confirmed species and subspecies. This investigation confirmed that *C. fetus* subsp. *fetus* and subsp. *venerealis* are closely related, and that the *C. fetus* subspecies are less closely related to *C. coli* and *C. jejuni* (Harvey and Greenwood 1983a). Probes to 16S ribosomal RNA gene sequences do not distinguish between *C. fetus* subsp. *fetus* and subsp. *venerealis*, as they are too closely related and the sequences differ by just one base (Wesley et al. 1991).

Genus-specific probes to *Campylobacter*, *Arcobacter* and *Helicobacter* have been designed and probes to 16S rRNA are commercially available for *Campylobacter* spp. such as *C. jejuni* and *C. coli*. Some probes may be used directly on clinical samples, which decreases the time delay due to culture of the bacteria and may potentially be a more sensitive detection method than culture. However, a negative result is not very helpful as to the identification of the isolate and this technique has been suggested as confirmatory rather than diagnostic (On 1996).

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) involves specific amplification of a region of DNA from the target bacteria using specific, short sequences of nucleotides, called primers. The amplified DNA is then visualised after electrophoresis. The technique has been described for the identification of various *Campylobacter* spp. (On 1996).

PCR using primers specific to the 16S rRNA sequence has been shown to be a useful tool to distinguish *C. fetus* from other *Campylobacter* spp., and from other genera, such as *Escherichia coli*, *Salmonella*, *Arcobacter skirrowii*, and *Listeria monocytogenes*. Because of the homology between the 16S rRNA of *C. fetus* subspecies, this does not

discriminate between these subspecies (Oyarzabal et al. 1997). However, a PCR assay has also been developed that distinguishes between the *C. fetus* subspecies. The assay involves a PCR reaction with two sets of specific primers. The reaction confirms the species as *C. fetus*, and an extra band is produced if the subspecies is *venerealis* (Hum et al. 1997).

Recently, the technique of PCR-bioprobes has been applied to identification of *Campylobacter* spp. (Logan et al. 2001). This involves a PCR assay to detect the presence of *Campylobacter* spp., using primers specific for the conserved region of the 16S rRNA gene. The PCR is followed by annealing the amplified DNA to a bioprobe and detecting the temperature at which this duplex dissociates. This melting temperature is characteristic for different species due to varying degrees of mismatches between the sequences of the probe and amplified DNA. This technique has been applied to identification of *Campylobacter* spp. from DNA extracted from clinical samples. However, this technique fails to differentiate the *C. fetus* subspecies and *C. jejuni* from *C. coli* (Logan et al. 2001).

Genomic sizing using pulsed-field gel electrophoresis (PFGE)

Some attempts have been made to differentiate *Campylobacter* spp. and *C. fetus* subspecies by determining the size of the genome (Chang and Taylor 1990; Salama et al. 1992; Fujita and Amako 1994). This method uses the technique of pulsed-field gel electrophoresis, which is detailed later. Briefly, genomic DNA is digested with restriction enzymes and the DNA fragments are separated on a gel. The sizes of the DNA fragments are calculated and summed to give the size of the entire genome for the organism. In 1990, the genome size of *C. jejuni* was found to be 1.721Mb, *C. coli* 1.714Mb, *C. lari* 1.451Mb, and *C. fetus* subsp. *fetus* 1.267Mb (Chang and Taylor 1990). A later study found that *C. fetus* subsp. *fetus* was 1.12-1.14Mb and *C. fetus* subsp. *venerealis* was 1.3Mb (Salama et al. 1992). However, errors may be made with this method, such as fragments of similar sizes migrating to the same position on the gel and appearing as a single band, and the poor resolution of small, closely positioned bands. Indeed, a later study that acknowledged and tried to solve these problems found the *C. fetus* subsp. *fetus* genome size to be 2.016Mb (Fujita and Amako 1994). Therefore, the genomic sizing results obtained with this technique need to be interpreted with caution (On 1996). Genomic sizing is not particularly helpful in definitively assigning species

identity to isolates given the difficulties associated with this method compared with other methods.

Numerical analysis of the PFGE profiles of *C. fetus* isolates was found to be an effective method of distinguishing the subspecies (On and Harrington 2001). Cluster analysis of the PFGE profiles was generally shown to correlate with the results of PCR and phenotypic testing; isolates identified as *C. fetus* subsp. *venerealis* by phenotypic and PCR testing had PFGE profiles that clustered by similarity with the type strain for *C. fetus* subsp. *venerealis*, and vice versa. This was true for 29 out of the 31 isolates. However, two isolates identified as *C. fetus* subsp. *fetus* by cluster analysis of PFGE profiles were identified as *C. fetus* subsp. *venerealis* by either PCR or phenotypic testing (On and Harrington 2001). As PFGE is time-consuming and requires expensive, specialised equipment (Lahti 1996), phenotypic or PCR methods to identify subspecies of *C. fetus* may be more suitable for the routine diagnostic laboratory.

1.4 Epidemiology of *Campylobacter* abortion in sheep

Campylobacter spp. are an important cause of sheep abortions in New Zealand, and the majority of cases are due to *C. fetus* subsp. *fetus* (Table 1.4). Another important cause of abortion in sheep is the protozoan, *Toxoplasma gondii*, and recently *Salmonella* Brandenburg has emerged as a significant cause of sheep abortion in the South Island (Clark et al. 2000).

A survey in the Ashburton area of New Zealand (Canterbury region) of 36 farms affected by *C. fetus* subsp. *fetus* abortions in 1980 found that most farms had fewer than 5% of ewes affected (20 farms). On 12 farms 5-10% of the ewes aborted, one farm had between 10-15% abortions and on three farms 15% of the ewes aborted (Anon 1980).

Much of what is known about the pathogenesis and epidemiology of *Campylobacter* abortion in sheep is based on the details contained in the Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to inquire into Epizootic Abortions: Part III, and Appendix to Part III, Abortion in Sheep, by

McFadyean and Stockman in 1913. This work was consolidated in the 1950s and 1960s, and as a consequence the literature on the pathogenesis and epidemiology of *Campylobacter* abortion in sheep dates from this period. It is not always clear which species of *Campylobacter* is referred to in the literature during this time, for reasons already discussed. However, more recent investigation into the role of *C. jejuni* abortion in sheep suggests the pathogenesis is similar to that described for *C. fetus* subsp. *fetus* (see section 1.4.2).

Table 1.4 Number of sheep abortion submissions and number of diagnoses of *C. fetus* subsp. *fetus*, other *Campylobacter* species and *Toxoplasma gondii*, for the years 1995-2001 (collated from the annual Ministry of Agriculture and Forestry Biosecurity Authority Animal Health Surveillance Reports).

Year	No. abortion submissions	*C. fetus* subsp. *fetus*	Other *Campylobacter* species	*Toxoplasma gondii*	Reference
1995	431	87 (43%)	15 (7%)	100 (50%)	Anon 1996
1996	473	117 (51%)	18 (8%)	94 (41%)	Anon 1997
1997	609	102 (43%)	22 (9%)	114 (48%)	Anon 1998
1998	725	86 (37%)	58 (25%)	91 (39%)	Anon 1999
1999	1,218	111 (31%)	85 (24%)	158 (45%)	Anon 2000
2000	1,246	173 (49%)	67 (19%)	110 (31%)	Anon 2001b
2001	799	107 (48%)	37 (17%)	79 (35%)	Poland 2002

1.4.1 Pathogenesis of *Campylobacter* abortions in sheep

Upon infection, a bacteraemia develops (Miller et al. 1959) which is followed by placentitis in pregnant ewes and death of the foetus (Jensen et al. 1961). Late in infection the uterus and placenta may be oedematous. The foetus and placenta may decompose before abortion (Jensen et al. 1961), which normally occurs in the last six weeks of gestation (Hartley and Kater 1964). If lambs are carried to term they may be

born dead or die soon after (Hartley and Kater 1964). The foetus usually has subcutaneous oedema and fluid in the body cavities (Hartley and Kater 1964; Gill and Clark 2000). In approximately one quarter of the cases, the foetal liver has circular white lesions, which are foci of infection containing large numbers of *Campylobacter* cells (Figure 1.1; Hartley and Kater 1964; Gill and Clark 2000). *Campylobacter* organisms can be seen by microscopy of foetal stomach contents stained with dilute Carbol fuchsin (McFarlane et al. 1952; Quinn et al. 1994). The foetal heart, blood, liver and lung (McFarlane et al. 1952) and the placenta and vaginal discharges may also be heavily infected with *Campylobacter* organisms (Jensen et al. 1961). Occasionally ewes may develop secondary metritis, which can be fatal (Jensen et al. 1961).

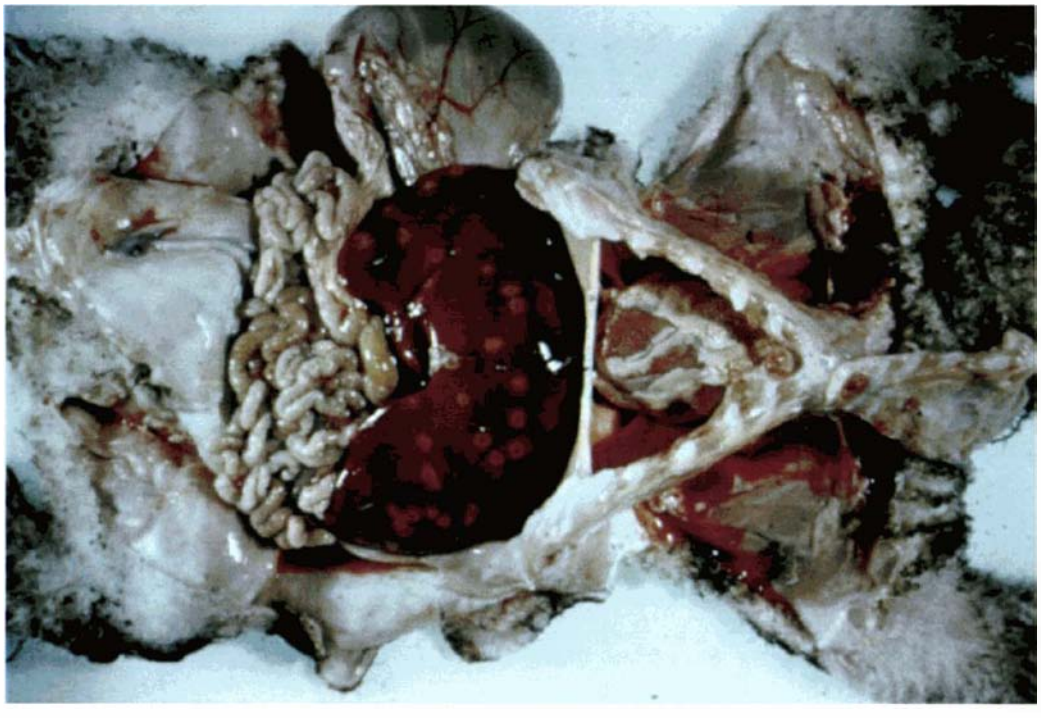


Figure 1.1 Circular, white lesions on the foetal liver found in approximately one quarter of *Campylobacter fetus* subsp. *fetus* abortion cases.

Ewes that abort due to *Campylobacter* infection remain fertile and are subsequently immune to *Campylobacter* abortions (Marsh et al. 1954; Jensen et al. 1957; Meinershagen et al. 1969). This was demonstrated by an oral challenge of groups of ewes in the fourth month of gestation with *C. fetus* subsp. *fetus* (Meinershagen et al. 1969). The 41 ewes from a flock that had been affected by *C. fetus* subsp. *fetus* abortions the previous season had either aborted or lambed normally and none aborted when artificially challenged. However, 13 out of 20 ewes (65%) that had no known previous exposure to *C. fetus* subsp. *fetus* aborted when challenged and *C. fetus* subsp. *fetus* was isolated from the foetuses (Meinershagen et al. 1969). Immunisation of ewes was demonstrated in an experiment where three groups of pregnant ewes were challenged with infected foetal tissue. The 44 ewes that had had *Campylobacter* abortions the previous season had four dead lambs (9%) and the 44 ewes that had been fed infected foetal material as hoggets had three dead lambs (7%). The 21 ewes that had not been previously exposed to *Campylobacter* had 16 dead lambs (76%). *Campylobacter* was recovered from "many" of the 16 dead lambs and from two lambs from each of the other groups (Jensen et al. 1957).

1.4.2 The role of *C. jejuni* in sheep abortion

The literature that convincingly implicates *C. jejuni* in sheep abortion is relatively recent, as before 1980 the identification of *Campylobacter* spp. from sheep abortion was often inadequate. However, two studies in the 1970s, in which *Campylobacter* isolates were serotyped, show that *C. jejuni* was cultured from sheep abortion cases. Berg et al. (1971) classified three *Campylobacter* isolates cultured from sheep foetuses and one from a sheep placenta as serotype C (Berg et al. 1971), which was later identified as *C. jejuni* (Garcia et al. 1983). In another study, 89 *Campylobacter* isolates collected from 69 outbreaks of "ovine vibriosis" throughout Australia between 1956 and 1971 were serotyped according to the scheme of Marsh and Firehammer (1953) (Clark and Monsbourgh 1974). The isolates from most of the outbreaks were serotype V (*C. fetus* subsp. *fetus*), but those from 10 outbreaks were serotype I (Clark and Monsbourgh 1974), later identified as *C. jejuni* (Garcia et al. 1983).

More recently, sheep abortion samples from 27 farms were submitted to the Caine Veterinary Teaching and Research Centre at the University of Idaho, USA for abortion

investigation (Delong et al. 1996). *Campylobacter* spp. were cultured from foetal samples from 14 farms. Fifteen isolates were analysed from 13 of the farms from which *Campylobacter* spp. were cultured. Fourteen of these isolates were *C. jejuni* and one isolate was *C. fetus* subsp. *fetus*. Other causes of abortion were detected on other farms in the study, such as *Chlamydia* species, *Toxoplasma gondii*, *Actinomyces pyogenes* and *Pasteurella haemolytica*, and on five farms there was no diagnosis (Delong et al. 1996).

A report in 1993 of the cases documented over 10 years at the South Dakota Animal Disease Research and Diagnostic Laboratory in USA showed that *Campylobacter* spp. were implicated in 184 out of 702 cases of infectious sheep abortion (Kirkbride 1993). Of these *C. fetus* subsp. *fetus* was diagnosed in 125 cases and *C. jejuni* in 57 cases. The report noted that the relative prevalence of each of these species was changing and in 1988-1989 *C. jejuni* was implicated in 26 cases while *C. fetus* subsp. *fetus* was diagnosed in only nine (Kirkbride 1993).

Two case studies in the 1980s also implicate *C. jejuni* in sheep abortion. In 1986, there was a report of 25 ewes out of a flock of 200 ewes that aborted in late pregnancy in Turkey (Diker and Istanbulluoglu 1986). Pure cultures of *C. jejuni* were obtained from foetal liver, lungs and stomach contents from all three aborted foetuses that were submitted for post mortem and microbiological examination. No other known cause of abortion could be found in the flock (Diker and Istanbulluoglu 1986).

In 1987, there was a report of abortions in two flocks of ewes from which *C. jejuni* was isolated in USA (Hedstrom et al. 1987). Fifteen ewes out of a flock of 200 ewes aborted and one placenta and one foetus were submitted for examination. *Campylobacter jejuni* was isolated from the placenta, but not the foetus. From the second flock, approximately 80 ewes out of 100 ewes aborted. Three foetuses and one placenta were submitted for examination and *C. jejuni* was isolated from the placenta and the liver, lung and stomach contents of all three foetuses. In this flock 80% of the ewes aborted, which is unusually high and it was noted that "other causes of abortion cannot be entirely excluded" (Hedstrom et al. 1987).

The *C. jejuni* isolate from the placenta in flock 1 above was used to intravenously inoculate seven pregnant ewes, using a 5mL dose of a suspension containing 10^8-10^9

colony forming units (cfu) per mL (Hedstrom et al. 1987). All of the ewes developed an elevated temperature and aborted within 12 days of inoculation. The pathology of the abortions of the artificially inoculated ewes was found to be similar to that from the two original flocks. The gross and histologic findings were similar to those obtained from the study of Jensen et al. (1961) in which the pathology of *C. fetus* subsp. *fetus* abortion in sheep was examined following artificial inoculation of ewes with *C. fetus* subsp. *fetus* (Hedstrom et al. 1987).

Thus, it appears that abortion in sheep due to *C. jejuni* occurs and that the pathology is similar to abortion due to *C. fetus* subsp. *fetus*.

1.4.3 Transmission of *Campylobacter* abortions to sheep

Campylobacteriosis can be transmitted by oral inoculation of pregnant ewes with infected foetal tissue. Experimentally, oral or intraperitoneal inoculation during each month of gestation resulted in abortions during the third, fourth, and fifth months (Jensen et al. 1957). Transmission of *C. fetus* subsp. *fetus* from orally infected lambs to uninoculated pen mates has also been demonstrated. Both orally inoculated and uninoculated lambs developed bacteraemia and shed *C. fetus* subsp. *fetus* in faeces (Bryans and Shephard 1961). After the initial *Campylobacter* abortion occurs, other pregnant ewes are exposed to large numbers of organisms from the aborted material, which may result in further abortions. Prevention of spread of the infection is important for control of the outbreak, such as prompt removal of aborting ewes and foetuses and moving stock from contaminated pasture (Jensen et al. 1957). Similarly, an epidemiological study of *Campylobacter* abortions in New Zealand found that high stock density rotational grazing management systems are associated with a higher *Campylobacter* abortion rate than low stock density set-stocking management practices (Quinlivan and Jopp 1982).

1.4.3.1 Investigations into venereal transmission of infection

The role of the ram in transmission of *Campylobacter* abortions in sheep was investigated as it was known that campylobacteriosis in cattle was venereally transmitted by bulls to cows. No abortions could be induced in ewes by mating with rams fed infected lamb tissues, nor with inoculation of *Campylobacter* organisms into

the cervix at the time of mating (Jensen et al. 1957). *Campylobacter fetus* subsp. *fetus* could not be isolated from the preputial mucus or semen of rams that had been mated with flocks subsequently affected by *Campylobacter* abortions. However, a *Campylobacter* isolate was cultured that was identified as *Vibrio bubulus* (Firehammer and Lovelace 1961), which is now called *C. sputorum* biovar bubulus (Penner 1988). This organism has no known pathological role (Penner 1988).

Campylobacter fetus subsp. *fetus* may be excreted from the vagina of the ewe for up to four weeks after abortion. After this time the commensal organism *C. sputorum* biovar bubulus may be isolated (Firehammer and Lovelace 1961). The *Campylobacter* isolates obtained from vaginal mucus for 18 months after an abortion outbreak were misidentified as the outbreak strain (Eide and Helle 1958), and were probably also *C. sputorum* biovar bubulus.

The results of this work suggest that *C. fetus* subsp. *fetus* is not venereally transmitted.

1.4.4 Sheep as a reservoir of *C. fetus* subsp. *fetus* and *C. jejuni*

1.4.4.1 Investigations into intestinal carriage

It is generally assumed that some sheep become intestinal carriers of *C. fetus* subsp. *fetus* after infection (Gilmour 1983; Penner 1988; Delong et al. 1996; Grogono-Thomas et al. 2000). However, this has not been unequivocally proven. It was claimed that *C. fetus* subsp. *fetus* was isolated from faeces and intestinal contents of 36 out of 214 (17%) healthy sheep (Smibert 1965a and 1965b). However, the isolates from this study grew at 42°C, but were unable to grow at 25°C (Smibert 1965b), which indicates that they were *C. jejuni*. A later article by the same author stated that *C. fetus* subsp. *fetus* was not in fact isolated from sheep intestines or faeces, and that the isolates obtained were *C. jejuni* (Smibert 1978).

Campylobacter jejuni is a common isolate from faecal or rectal samples from sheep, along with the other thermophilic *Campylobacter* species, *C. coli* and *C. lari*. The most commonly isolated species is *C. jejuni* at 82-90% of the isolates, then *C. coli* at 8-13%, followed by *C. lari* ~2% and *C. hyointestinalis* (Manser and Dalziel 1985; Stanley et al. 1998; Jones et al. 1999). Isolation rates ranging from 22% of sheep (Manser and Dalziel

1985) to 100% of sheep (Jones et al. 1999) have been found. The difference in isolation rates may be partially explained by a study which found that shedding of *Campylobacter* spp. varied from 0% of sheep in January to 100% in April/May (Northern Hemisphere) and was maximal at lambing, weaning, and movement to new pasture (Jones et al. 1999). *Campylobacter sputorum* biovar fecalis (formally *C. fecalis* Roop et al. 1985) was reported to have been cultured from sheep faeces in 1965 (Firehammer 1965), but this species was not detected in the later studies (Manser and Dalziel 1985; Stanley et al. 1998; Jones et al. 1999). *Campylobacter sputorum* biovar fecalis is not thought to be pathogenic (Penner 1988).

Ewes have been experimentally infected with *C. fetus* subsp. *fetus* and faecal shedding measured. One study claimed that *C. fetus* subsp. *fetus* could be isolated from sheep faeces for up to 25 days after artificial inoculation. However, the isolates all grew at 42°C and not at 25°C (Firehammer 1965), and it is likely that these isolates were *C. jejuni*. The longest demonstrated shedding of *C. fetus* subsp. *fetus* in faeces was after experimental challenge of 11 ewes with 10^8 organisms, which resulted in 91% abortions and excretion of *C. fetus* subsp. *fetus* in all animals for up to 42 days postchallenge (Grogono-Thomas et al. 2000). It is unknown whether the organism was cleared from the intestine or whether excretion of *C. fetus* subsp. *fetus* after 42 days was so low that a more sensitive technique than bacterial isolation would be necessary to detect it.

While it is possible that sheep may become long-term intestinal carriers of *C. fetus* subsp. *fetus*, to date there is no evidence of intestinal carriage past 42 days after infection.

1.4.4.2 Investigations into gall bladder carriage

The gall bladder was proposed to be a reservoir for *C. fetus* subsp. *fetus* in sheep, after *Campylobacter* spp. were found in the gall bladders of experimentally inoculated guinea pigs in 1953 (Ristic and Morse 1953). However, the early experiments investigating this were inconclusive due to inadequate identification of the isolates. One ewe slaughtered seventeen days after abortion had a *Campylobacter* spp. cultured from the liver, bile, and hepatic lymph node. Direct injections of *Campylobacter* organisms into the gall bladders of ten ewes resulted in the isolation of *Campylobacter* in one ewe (the first one slaughtered at 39 days post injection) (Firehammer et al. 1962). At an abattoir in Iowa,

ten sheep out of a total of 186 from nine flocks had a *Campylobacter* spp. cultured from the gall bladder that were identified serologically as *C. fetus* subsp. *fetus*. However, three out of the ten isolates grew at 43°C and one isolate did not grow at 22°C (Bryner et al. 1972), which indicates that at least one isolate was possibly not *C. fetus* subsp. *fetus*.

In a survey of bile samples from 1015 sheep at a Melbourne slaughter house, three sheep were reported to be positive for *C. fetus* subsp. *fetus* and 30 for *C. jejuni* (Clark and Monsbourgh 1979). There was no information about the tests performed to identify the *Campylobacter* isolates to the species level, the exposure of the sheep to *C. fetus* subsp. *fetus*, or the abortion history of the sheep that were culture positive. However, the survey was carried out in "early 1976" and sporadic campylobacteriosis was reported for the area the sheep came from (Clark and Monsbourgh 1979). Therefore, it is possible that the sheep from which the *Campylobacter* spp. were isolated had aborted the previous lambing season.

Campylobacter jejuni has been shown to multiply and survive in human bile samples kept at 37°C for 2-3 months (Blaser et al. 1980).

It appears that *C. fetus* subsp. *fetus* may be isolated from the gall bladder of sheep. However, as the duration of carriage of *C. fetus subsp. fetus* in the gall bladder has not been elucidated, it is unknown whether infection can be transmitted to other sheep by the excretion of *C. fetus* subsp. *fetus* from the gall bladder in future seasons.

1.4.4.3 Transmission studies using previously exposed sheep

Transmission of *Campylobacter* infection from previously exposed sheep to naive ewes has been attempted in two experiments without success (Marsh et al. 1954). In the first experiment, fifty maiden ewes from a flock free from *Campylobacter* abortions for nine years were bred, wintered and lambed with 42 ewes that had *Campylobacter* abortions the previous season. No *Campylobacter* abortions resulted. In the other experiment, 24 ewes which had lambed normally the previous season in a flock affected by 26% *Campylobacter* abortions, were bred with eight ewe hoggets, one ewe lamb and one 3-year old ewe from a "presumably" *Campylobacter*-free flock. Again, no abortions due to *Campylobacter* spp. resulted (Marsh et al. 1954). However, the results of this study

do not eliminate the possibility of the carrier ewe, as the number of animals involved in these limited experiments was small.

1.4.5 Birds as a reservoir of *C. fetus* subsp. *fetus* and *C. jejuni*

The American magpie (*Pica pica*) was proposed to be a vector of *Campylobacter* infection because of its scavenging feeding habits and close association with livestock (Waldhalm et al. 1964). It was shown that after experimental inoculation of the magpie with a *Campylobacter* sheep abortion isolate, the magpies could carry and excrete the organism for at least 213 days. Faecal cultures were positive for *Campylobacter* spp., and produced *Campylobacter* abortions in ewes after oral inoculation (Meinershagen et al. 1965). Phenotypic tests on the *Campylobacter* isolates were not reported in this study, but the isolates were serotype I using the serotyping scheme of Marsh and Firehammer (1953), later identified as *C. jejuni* (Garcia et al. 1983). However, as *C. jejuni* is frequently isolated from the intestines of wild birds (Smibert 1969), the persistence of *C. jejuni* in the magpies is not unexpected. Similarly, *Campylobacter* isolates were cultured from sparrows, starlings, pigeons, blackbirds, chickens and turkeys and these isolates were identified as *C. fetus* subsp. *fetus* (Smibert 1969), but were later found to be *C. jejuni* (Smibert 1978). A similar study in Great Britain isolated *C. jejuni* from the carrion crow, *Corvus corone corone* (Watson et al. 1967). An Australia study recovered *Campylobacter* spp. from 65% of 106 ravens (*Corvus coronoides*). However, the isolates obtained were not identified satisfactorily. Pregnant ewes orally challenged with raven faeces produced live but weak lambs at term, and *Campylobacter* spp. were recovered from their tissues (Dennis 1967).

The role of wild birds as a reservoir of *C. fetus* subsp. *fetus* infection has not been demonstrated. However, it is possible that birds may act as mechanical vectors. In New Zealand the black backed gull (*Larus dominicanus*) and the Australasian harrier hawk (*Circus approximans*) scavenge aborted material in the same way as the American magpie, and hawks have been seen to physically convey aborted material from one farm to the next (Smart 2000). In addition, black backed gulls commonly ingest placenta and foetal tissue (Clark et al. 1999). In a study of the role of black backed gulls in the spread of *Salmonella* Brandenburg sheep abortion in New Zealand, this organism was found in the intestines of 42-52% of the birds sampled (Clark et al. 1999). Up to 25 million

Salmonella Brandenburg organisms per gram of intestinal contents were found in black backed gulls sampled from farms affected by *Salmonella* Brandenburg sheep abortions (Clark et al. 1999). As these birds may fly up to 50 km from their nests, they have the potential to spread the disease by defecating on neighbouring farms (Clark et al. 1999). It is possible that black backed gulls may also act as a vector for *C. fetus* subsp. *fetus* in this way in New Zealand.

1.4.6 Other reservoirs of *C. fetus* subsp. *fetus* and *C. jejuni*

Campylobacter jejuni is frequently isolated from the intestines or faeces of healthy animals, including birds (Watson et al. 1967; Smibert 1969), cattle (Manser and Dalziel 1985; Atabay and Corry 1998) and sheep (Smibert 1965b; Manser and Dalziel 1985; Stanley et al. 1998; Jones et al. 1999). In addition, *C. coli* has been cultured from faecal samples from healthy sheep, cattle and pigs (Manser and Dalziel 1985).

The only species that has been demonstrated to harbour *C. fetus* subsp. *fetus* in the faeces of healthy animals is cattle (Manser and Dalziel 1985; Atabay and Corry 1998). In a study of 111 faecal samples from cattle in England, 22 (20%) were positive for *Campylobacter* species. *Campylobacter jejuni* was the most commonly isolated species (73%), followed by *C. coli* (13%) and *C. fetus* subsp. *fetus* (7%) (Manser and Dalziel 1985). In a study of faecal samples from 136 cattle in three herds in England, the number of cattle in each herd positive for *Campylobacter* spp. varied from 38-79%. *Campylobacter hyointestinalis* was isolated from approximately one-third of the cattle, *C. sputorum* biovar *paraureolyticus* from 21%, *C. fetus* subsp. *fetus* from 11% and *C. jejuni* from 7% (Atabay and Corry 1998).

Water is a potential reservoir for *C. jejuni*. *Campylobacter jejuni* and *C. coli* have been isolated from rivers draining land used for agriculture in New Zealand (Hudson et al. 1999; Savill et al. 2001).

1.4.7 Environmental survival of *C. fetus* subsp. *fetus* and *C. jejuni*

The environmental survival of *C. fetus* subsp. *fetus* has not been determined. A brief report in 1948 claimed that *Campylobacter* spp. could be reisolated from hay, soil and manure after 20 days if kept at 6°C (Lindenstruth and Ward 1948). However, it is not

clear which *Campylobacter* spp. were used in the inoculum and the identity of the isolates cultured was not detailed sufficiently. In addition, the hay, soil and manure were not sterilised before inoculation, and *Campylobacter* spp. are known to be ubiquitous (Penner 1988).

Campylobacter jejuni has been shown to survive in water for up to four weeks when at a temperature of 4°C, but to die within four days at 25°C (Blaser et al. 1980).

1.5 Strain typing of *Campylobacter* sheep abortion isolates

Three criteria are important when sub-typing isolates belonging to the same species. Firstly, all isolates must be typeable by the chosen method. Second, there must be differentiation between strains, and finally, the results must be reproducible (Lahti 1996; Olive and Bean 1999). Various methods are used for typing *Campylobacter* species, including serotyping, phage typing, flagellin typing (*fla* typing), pulsed-field gel electrophoresis (PFGE), ribotyping, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and multiplex PCR-RFLP analysis. These methods were evaluated in the reviews of Newell et al. (2000) and Wassenaar and Newell (2000). The three typing methods that have been applied to *Campylobacter* sheep abortion isolates are serotyping, restriction endonuclease analysis (REA), and pulsed-field gel electrophoresis (PFGE). These three typing methods are reviewed here. Pulsed-field gel electrophoresis was the typing method used in this doctoral study.

1.5.1 Serotyping

Serotyping is based on the recognition of antibodies in antisera to the antigens on the surface of the bacterial cells (Arbeit 1995). Serotyping is a phenotypically-based typing technique that has been applied to differentiate strains of *C. jejuni*, *C. coli* (Jacobs-Reitsma et al. 1995; Newell et al. 2000) and *C. fetus* subsp. *fetus* (Bird et al. 1984; Varga 1991). However, serotyping of *C. fetus* subsp. *fetus* has poor discriminatory power, as *C. fetus* subsp. *fetus* can only be serotyped into two groups, Type A or B, according to an outer membrane lipopolysaccharide (Bird et al. 1984; Varga 1991). When serotyping was used in New Zealand veterinary diagnostic laboratories from the early 1980s to distinguish strains of *C. fetus* subsp. *fetus* from sheep abortion, two

serotypes were established. One was 5915, the serotype of the Campylovexin® vaccine strain. The other serotype was 7438, or non-vaccine strain (Jopp 1982; Pauling 1988).

The Penner or Lior serotyping schemes are most commonly applied when serotyping *C. jejuni* and *C. coli* isolates (Newell et al. 2000). The discriminatory power of this technique when applied to *C. jejuni* or *C. coli* is significantly greater than serotyping of *C. fetus* subsp. *fetus*, as 48 serotypes of *C. jejuni* and 17 serotypes of *C. coli* have been identified with the Penner scheme (Newell et al. 2000). However, many isolates were unable to be typed with this method (Jacobs-Reitsma et al. 1995; Gibson et al. 1997). In addition, the molecular typing technique pulsed-field gel electrophoresis has been found to be even more discriminatory than serotyping of *C. jejuni* isolates (Gibson et al. 1995). There have been no reports of strain differentiation of *C. jejuni* or *C. coli* sheep abortion isolates using serotyping.

1.5.2 Restriction endonuclease analysis (REA)

Molecular typing techniques have proved to be useful in overcoming some of the problems of the phenotypically-based typing methods and are generally more discriminatory (Wassenaar and Newell 2000). Restriction endonuclease analysis (REA) is a molecular technique which results in a DNA profile of an isolate. The DNA of the isolate is digested using a restriction enzyme (endonuclease). The restriction enzymes used in REA cut the DNA at frequent sites, which results in many DNA fragments from ~0.5 to 50kb in length, and these fragments are separated by size using agarose gel electrophoresis (Arbeit 1995). The DNA banding pattern (or profile) can be visualised under UV light after staining the gel with ethidium bromide. The DNA profiles of the isolates are compared and isolates with distinct profiles are considered to be a different strain or type. However, there are problems with the REA typing method, including plasmid DNA contamination of profiles and the difficulty in comparing the complex profiles of poorly separated DNA bands between different isolates. For this reason, REA typing has been largely superseded by the development of other molecular typing techniques (Arbeit 1995). Nevertheless, REA has been used in most of the previous typing studies of *Campylobacter* sheep abortion isolates.

1.5.2.1 Restriction endonuclease analysis of *Campylobacter* sheep abortion isolates

Three REA typing studies have examined *C. fetus* subsp. *fetus* sheep abortion isolates from New Zealand and were carried out at the Wallaceville Animal Research Centre in Upper Hutt.

In a report from 1984, the restriction profiles of 43 *C. fetus* subsp. *fetus* sheep abortion isolates recovered from ten New Zealand farms over three years were compared (Collins and Ross 1984). Four different types of profiles were found amongst the isolates. A single restriction type was isolated from a single farm over three successive years (Collins and Ross 1984).

A larger study of 51 *C. fetus* subsp. *fetus* sheep abortion isolates from 51 New Zealand farms during 1983 found seven REA types (Collins and de Lisle 1985). These were designated REA types a-g. Most of the isolates (32 out of 51, 63%) were the same type (REA type b) and this type was found throughout New Zealand. The strain used in the production of the vaccine to prevent abortion in sheep due to *C. fetus* subsp. *fetus* (Campylovexin®, Schering-Plough Animal Health Ltd., Upper Hutt) was found to be REA type a. Type a was the second most common REA type in this study. However, it was noted that the collection of isolates in this study was non-random. Selection of the isolates was biased towards those that did not react in the slide agglutination test with antiserum produced against the Campylovexin® vaccine strain (Collins and de Lisle 1985).

In a further report, 70 *C. fetus* subsp. *fetus* sheep abortion isolates from 67 New Zealand farms were typed using REA (de Lisle et al. 1987). Again, seven different REA types were found and REA type b was by far the most common (57%) followed by REA type a (22%). Representative isolates of each of the restriction types (a-g) were lodged with the New Zealand Reference Culture Collection (de Lisle et al. 1987). Unlike the report by the same authors involving 51 isolates (Collins and de Lisle 1985) which is described in the previous paragraph, this report does not state from which lambing season these 70 isolates were collected (de Lisle et al. 1987). However, it is mentioned that the isolates were "maintained by subculturing at 4-6 month intervals" (de Lisle et al. 1987). It must be assumed that the 51 isolates in the previous study which were non-

randomly selected from the 1983 abortion season were not amongst the 70 isolates in the later study.

There has also been a report of REA typing of *C. jejuni* sheep abortion isolates (Delong et al. 1996). Five distinct REA types were found amongst 14 *C. jejuni* sheep abortion isolates from 12 farms in the USA. There were two isolates analysed from each of two farms and in each case, both isolates from the same farm were indistinguishable (Delong et al. 1996).

1.5.3 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a molecular typing technique that has been used to type various pathogens in epidemiological studies (Tenover et al. 1995). Pulsed-field gel electrophoresis is based on the same principle as REA, in that genomic DNA is digested using a restriction enzyme and the DNA fragments are separated to form a profile specific for that isolate. In contrast to REA, PFGE uses restriction enzymes that cut the DNA rarely, which results in a small number of large DNA fragments (5-20 fragments of 10-800kb). Fewer DNA fragments means that PFGE profiles are more simple and easier to interpret than REA profiles (Arbeit 1995; Newell et al. 2000).

However, large DNA molecules are susceptible to shearing damage and are not separated by normal agarose gel electrophoresis (Smith and Cantor 1987). To protect the DNA from shearing, the bacteria are embedded whole in agarose, then are lysed *in situ* to expose the DNA to restriction digestion. The agarose "plug" of digested DNA from the isolate is then inserted into the wells of an agarose gel for electrophoresis (Smith and Cantor 1987). To separate the large DNA molecules generated by rare cutting enzymes, electrophoresis is performed under an electric current that alternates (pulses) for specific time periods at an angle (normally 60°) to the overall direction of travel of the DNA (Chu et al. 1986; Smith and Cantor 1987; Birren et al. 1988). This method enables the clear separation of the very large fragments of DNA obtained with genomic DNA digestion.

1.5.3.1 Sub-typing using pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis is reproducible (On and Harrington 2001) and highly discriminatory (Owen et al. 1989; Gibson et al. 1995; On and Vandamme 1997; On 1998; Wassenaar et al. 1998; Newell et al. 2000). In theory, all bacteria should be typable using PFGE, providing the bacteria are able to be cultured (Arbeit 1995; Lahti 1996). However, some strains of *C. jejuni* require inactivation of DNAse activity to prevent degradation of DNA during preparation for PFGE (Gibson et al. 1994).

Only the subtyping method amplified fragment length polymorphism (AFLP) has been reported to have more discriminatory power than PFGE when typing *C. jejuni* strains (Lindstedt et al. 2000; de Boer et al. 2000). This method involves the selective amplification of restriction fragments of chromosomal DNA and has recently been adapted for genotyping *C. jejuni and C. coli* (Duim et al. 1999; Kokotovic and On 1999) and to distinguish the subspecies of *C. fetus* (Wagenaar et al. 2001). However, the studies that concluded that AFLP was more discriminatory than PFGE used only the restriction enzyme *Sma*I for analysis of PFGE (Lindstedt et al. 2000; de Boer et al. 2000). It has been shown that the PFGE profiles of some *C. jejuni* isolates that are indistinguishable when *Sma*I is used can be further differentiated with the use of *Kpn*I (Gibson et al. 1995; On et al. 1998). Therefore, it is recommended that more than one restriction enzyme be used in PFGE analysis (Gibson et al. 1997; On et al. 1998). Furthermore, comparison of AFLP with PFGE using more than one restriction enzyme showed the discriminatory power to be equal between the two methods (Kokotovic and On 1999). However, AFLP clearly has the advantage of achieving the level of discrimination in one step equivalent to that achieved in multiple steps with PFGE.

1.5.3.2 Interpretation of PFGE profiles

There are no standard criteria for determining the relatedness of isolates based on analysis of PFGE profiles (Lahti 1996).

Tenover et al. (1995) proposed a set of guidelines for the interpretation of PFGE profiles for epidemiological studies. These guidelines were intended for the analysis of a small set of isolates (≤30) that were epidemiologically related in a disease outbreak, collected over a short period of time (1-3 months) and for which at least 10 DNA bands in the PFGE profile were obtained. Table 1.5 (reproduced from Tenover et al. 1995)

summarises the criteria for categorising isolates from an outbreak. Using these criteria, isolates were considered to be indistinguishable to the outbreak strain when there were no differences detected in the PFGE profiles. Closely related isolates were those with PFGE profiles that differ by two or three bands from the outbreak strain. Two or three band differences were considered to be generated by either a point mutation in the DNA resulting in the creation or loss of a restriction site, or deletion or insertion of DNA that does not contain a restriction site. In this way strains with two or three band differences were considered to be closely related because they differ by a single genetic event. Possibly related isolates differ by two independent genetic events (between four and six band differences in the PFGE profile), and unrelated isolates differ by three or more independent genetic events (seven or more band differences). A band difference is defined as the absence of a band present in the profile of the outbreak strain or the presence of a band that is absent in the outbreak strain profile (Tenover et al. 1995).

Table 1.5 Interpretation of PFGE profiles (reproduced from Tenover et al. 1995).

Category	No. of genetic differences compared with outbreak strain	Typical no. of fragment differences compared with outbreak profile	Epidemiologic interpretation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate is probably part of the outbreak
Possibly related	2	4-6	Isolate is possibly part of the outbreak
Different	≥3	≥7	Isolate is not part of the outbreak

Many studies involving the analysis of PFGE profiles of isolates do not fit within the criteria of Tenover et al. (1995) of ≤30 epidemiologically related isolates collected over 1-3 months. Computer analysis of PFGE profiles is often used in these circumstances, however there are no standard criteria for this (Owen et al. 1989; Gibson et al. 1997; On

1998; de Boer et al. 2000; On and Harrington 2001). Often similarity between PFGE profiles is calculated with the Dice coefficient, where the numbers of bands in common between profiles is divided by the total number of bands (On 1998; de Boer et al. 2000; On and Harrington 2001). Cluster analysis may then be performed and the clusters of similarity of the profiles may be illustrated with a dendrogram (Owen et al. 1989; Gibson et al. 1997; On 1998; de Boer et al. 2000; On and Harrington 2001).

1.5.3.3 Pulsed-field gel electrophoresis and genetic instability

Concern has been raised regarding genetic instability of *Campylobacter* spp. and the effect on PFGE profiles in epidemiological studies (Wassenaar et al. 1998; Wassenaar et al. 2000). The PFGE profiles of *C. coli* isolates were found to have changed significantly after *in vitro* sub-culturing (On 1998). However, these isolates were sub-cultured extensively over a long period; 50 times over six months (On 1998). Similarly, two out of 12 *C. jejuni* strains passaged *in vivo* through chick intestines were shown to have different PFGE profiles (Hanninen et al. 1999). In contrast, stability of PFGE profiles has been reported for *C. jejuni* isolates subjected to limited *in vitro* sub-culturing steps (10 times) and after storage at –80°C (Wassenaar et al. 1998), which more closely resemble procedures routinely used in strain typing *Campylobacter* isolates.

1.5.3.4 Pulsed-field gel electrophoresis of *Campylobacter* sheep abortion isolates

Strain typing using PFGE has been performed in epidemiological studies on *C. jejuni* isolates from water, humans, poultry, and cattle (On et al. 1998; Wassenaar et al. 1998; Hudson et al. 1999). This method has also been used in a number of epidemiological studies of infections in humans caused by *C. fetus* subsp. *fetus*, such as an investigation of a diarrhoea outbreak (Rennie et al. 1994) and of a meningitis outbreak in a neonatal intensive care unit (Morooka et al. 1996).

To date, there has only been one report of analysis of *Campylobacter* sheep abortion isolates using PFGE. Pulsed-field gel electrophoresis was used in the characterisation of *C. fetus* subsp. *fetus* isolates cultured from an abortion outbreak in apparently vaccinated ewes in New Zealand (Fenwick et al. 2000). The PFGE profiles of four *C. fetus* subsp. *fetus* isolates cultured from aborted foetuses were compared to that of the

Campylovexin® vaccine strain. It was found that the outbreak was due to a single strain of *C. fetus* subsp. *fetus* that was dissimilar to the vaccine strain (Fenwick et al. 2000).

1.6 Prevention of *Campylobacter* abortions in sheep

Early experiments demonstrated that ewes develop immunity to *Campylobacter* abortion after abortion or exposure to the organism. Immunisation of ewes was demonstrated in an experiment in which three groups of pregnant ewes were artificially challenged by being fed infected foetal tissue (Jensen et al. 1957). The 44 ewes that had had *Campylobacter* abortions the previous season had four dead lambs (9%) and the 44 ewes that had been fed infected foetal material as hoggets had three dead lambs (7%). The 21 ewes that had not been previously exposed to the *Campylobacter* spp. had 16 dead lambs (76%). *Campylobacter* organisms were recovered from "many" of the 16 dead lambs and from two lambs from each of the other groups (Jensen et al. 1957).

A report in 1961 details an experiment in which ewes given a subcutaneous injection before mating of formalin-killed or live *Campylobacter* organisms were protected against abortion when challenged in the fifth month of gestation (Miller and Jensen 1961). Challenge of the ewes was by oral administration of a *Campylobacter* culture or by exposure to aborting ewes. None of the 44 ewes that had been vaccinated with the live vaccine aborted. Of the ewes vaccinated with the formalin-killed vaccine, seven out of the 25 (28%) orally-challenged ewes aborted and one out of the 22 (5%) ewes housed with aborting ewes aborted. Of the ewes that were not vaccinated, 21 out of the 25 (84%) orally-challenged ewes aborted and eight out of the 21 (38%) ewes housed with aborting ewes aborted (Miller and Jensen 1961).

1.6.1 Lack of vaccinal cross-protection between *C. jejuni* and *C. fetus* subsp. *fetus*

A report in 1964 showed that ewes vaccinated against *C. jejuni* abortion were not protected against artificial challenge with *C. fetus* subsp. *fetus*, and vice versa (Miller et al. 1964). Of 22 ewes vaccinated with an experimental vaccine prepared from a *C. jejuni* culture, nine aborted (41%) when challenged with a rumen inoculation of *C. fetus*

subsp. *fetus*. This was similar to the number of ewes that aborted after *C. fetus* subsp. *fetus* challenge when no vaccine was used (eight out of 19 ewes, 42%). The *C. fetus* subsp. *fetus* vaccine was found to be protective against challenge with *C. fetus* subsp. *fetus*, as none of the 22 ewes aborted when vaccinated and challenged with *C. fetus* subsp. *fetus*. But when *C. jejuni* was used as the challenge organism, 19 out of 22 (86%) ewes vaccinated with *C. fetus* subsp. *fetus* aborted. Twelve out of the 22 control unvaccinated ewes aborted after challenge with *C. jejuni* (55%) (Miller et al. 1964). In a separate experiment, an experimental vaccine containing both *C. jejuni* and *C. fetus* subsp. *fetus* was found to be effective against sheep abortion stimulated by artificial challenge by rumen inoculation with each species separately and together. One out of 82 ewes aborted in this experiment, compared with 15 out of 23 unvaccinated ewes challenged with *C. jejuni* and four out of 21 unvaccinated ewes challenged with *C. fetus* subsp. *fetus* (Miller et al. 1964).

A study in Edinburgh (Scotland) described the serological response of sheep after vaccination with an experimental trivalent vaccine containing one strain of *C. jejuni* and two strains of *C. fetus* subsp. *fetus*. Serum agglutination titres to all three strains were found to be equivalent to titres obtained when the strains were used separately (Thompson and Gilmour 1978).

A 1995 report from Turkey also showed that there was poor vaccinal cross-protection between *C. fetus* subsp. *fetus* and *C. jejuni*, but good cross-protection between *C. jejuni* and *C. coli*, using pregnant guinea pigs as an animal model for disease (Diker and Turutoglu 1995). The same trends of cross-protection were observed when the *in vitro* bactericidal activity of rabbit serum was tested against the strains of *C. fetus* subsp. *fetus*, *C. jejuni* and *C. coli* (Diker and Turutoglu 1995).

1.6.2 Vaccine development and testing using guinea pigs as a model

There have been a number of reports detailing the testing of various vaccines for the prevention of *Campylobacter* abortion in sheep, using pregnant guinea pigs as an animal model for the disease. In 1978, the efficacy of a commercial vaccine for the prevention of *Campylobacter* sheep abortion was compared to that of an experimental vaccine using the pregnant guinea pig model (Bryner et al. 1978). The commercial vaccine was

prepared with both *C. jejuni* and *C. fetus* subsp. *fetus*, and was presumably in use in the USA where this study was performed. The experimental vaccine was prepared with the challenge strain, which was *C. jejuni* obtained from an aborted foetus. After intraperitoneal challenge of the guinea pigs, 31 out of the 37 (84%) guinea pigs vaccinated with the commercial vaccine aborted. Four out of the 20 (20%) guinea pigs vaccinated with the experimental vaccine aborted. Antibody titres were shown to be lower for the guinea pigs given the commercial vaccine than the experimental vaccine (Bryner et al. 1978). This same commercial vaccine was tested using the pregnant guinea pig model with *C. fetus* subsp. *fetus* as the challenge strain (Bryner et al. 1979). Eleven out of 28 (39%) vaccinated guinea pigs aborted. A second commercial vaccine for *Campylobacter* sheep abortion, which was also prepared with both *C. fetus* subsp. *fetus* and *C. jejuni*, was tested using this model. After *C. fetus* subsp. *fetus* challenge, 15 out of 30 (50%) vaccinated guinea pigs aborted. All 80 of the unvaccinated control guinea pigs aborted. An experimental vaccine was prepared with the challenge strain of *C. fetus* subsp. *fetus* and one out of 42 (2%) vaccinated guinea pigs aborted (Bryner et al. 1979).

1.6.3 Vaccination of sheep after the abortion outbreak has started

A study in 1975 found that some protection from *C. fetus* subsp. *fetus* abortion may be obtained by vaccinating sheep close to lambing or immediately after the first abortion has been noticed (Gilmour et al. 1975). Six weeks prior to the start of lambing, vaccination of ewes and simultaneous oral challenge with homologous *C. fetus* subsp. *fetus* resulted in one of twenty sheep aborting (5%). Of the control group of twenty sheep challenged the same way, but not vaccinated, nine abortions occurred (45%). In a separate experiment, vaccination after the first abortion in the group resulted in fewer than expected abortions for the rest of the group. One or two out of 13 ewes aborted (8-15%) when the ewes were vaccinated after the first abortion, compared to seven out of 21 ewes aborting (33%) when the ewes were not vaccinated (Gilmour et al. 1975). However, these experiments were conducted under artificial conditions as the same *C. fetus* subsp. *fetus* strain for production of the vaccine and challenge inocula was used, and ewes were vaccinated immediately upon discovery of abortion.

1.6.4 Prevention of *C. fetus* subsp. *fetus* abortion in sheep in New Zealand - Campylovexin® vaccine

Campylovexin® is the only vaccine available in New Zealand to prevent abortion in sheep due to *C. fetus* subsp. *fetus*. Produced by Schering-Plough Animal Health Ltd. (Upper Hutt, New Zealand), Campylovexin® is a single strain, killed, alum adjuvanted, *C. fetus* subsp. *fetus* vaccine and has been available since the early 1980s. The vaccine strain (strain 5915) was isolated from a field case of sheep abortion in mid-Canterbury, New Zealand in 1978 (Gumbrell 1985b). It is recommended that unvaccinated ewes be vaccinated before mating, followed by a booster dose 4-8 weeks later. In addition, previously vaccinated ewes should receive an annual booster dose before mating (Schering-Plough Animal Health Ltd. Technical Bulletin).

A field trial of Campylovexin® took place in 1980 involving over 10,000 maiden two-tooth ewes on 16 farms in the Hawke's Bay region (Quinlivan and Jopp 1982). There was a control group of unvaccinated ewes on each property. The vaccinated and unvaccinated ewes were run separately after the fourth month of gestation. The ewes were not artificially inoculated, but left for natural challenge to take place. At docking, there were significant differences in lamb production between the vaccinated and unvaccinated ewes, with 7% more lambs at docking from the vaccinated ewes (Quinlivan and Jopp 1982).

Vaccination of ewes after abortions have started is not recommended as standard practice (Wallace 1982), however, there is some evidence to suggest that Campylovexin® use during abortion storms may help to reduce lamb losses. In 1983 on three farms in Canterbury, half of the ewes on each farm were vaccinated soon after diagnoses of *Campylobacter* abortions (between 38 and 44 days before the start of lambing) (Gumbrell et al. 1996). A booster injection was given ten days later. The reduction in abortions in the vaccinated ewes compared with the unvaccinated ewes was 8% and 15% on two of the farms, but there was no reduction in abortions on the third farm (Gumbrell et al. 1996).

There is some evidence to suggest that Campylovexin® may reduce early neonatal lamb losses even in the absence of observed abortions (Anderson 2001). A trial on three New

Zealand properties was performed where half of the flock was vaccinated and at docking the number of lambs belonging to each group were counted. The vaccinated and unvaccinated ewes were run together throughout the trial. There were over 1,400 ewes in the trial. On each property there was a 6-10% decrease in lamb losses amongst the vaccinated ewes compared to the unvaccinated ewes, but no abortions due to *Campylobacter* spp. were observed (Anderson 2001).

Campylovexin® is generally thought to be effective in protecting against *C. fetus* subsp. *fetus* abortions (Quinlivan and Jopp 1982; Pauling 1988; Gumbrell et al. 1996) and there have been few reports of Campylovexin® failure (Pauling 1988; Marchant 1999). However, in 1996 an apparent vaccine breakdown occurred in the Manawatu region, when fifteen ewes aborted out of a flock of 300 vaccinated two-tooth ewes (Fenwick et al. 2000). *Campylobacter fetus* subsp. *fetus* was isolated from six foetuses, and the isolates were shown to have a different PFGE profile to the vaccine strain. However, it was suggested that the ewes may have been exposed to high *C. fetus* subsp. *fetus* challenge when they were densely stocked each night in a small, "sacrifice" paddock (Fenwick et al. 2000).

1.7 Surface layer proteins (SLPs)

The cells of both subspecies of *C. fetus* have an outermost capsule made up of a layer of proteins (Pei et al. 1988; Tu et al. 2001). The layer of proteins is called the surface layer (S-layer) and the proteins themselves are called surface layer proteins (SLPs). Many other bacteria possess S-layers and various functions of S-layers have been demonstrated, such as acting as protective coats, molecular sieves, and molecule and ion traps, for cell adhesion, cell surface recognition, and for maintaining cell shape (Sleytr and Messner 1988).

The S-layer of *C. fetus* subspecies has been the subject of much research. The bacterial strains used in most of this research are simply referred to as *C. fetus*, although Blaser et al. (1985) characterised to the subspecies level some of the strains that have been commonly used and most of these were *C. fetus* subsp. *fetus*. Strains can spontaneously

lose the S-layer during laboratory sub-culturing and these spontaneous mutants have often been used in studies of the properties of the S-layer (Blaser et al. 1987; Pei and Blaser 1990; Grogono-Thomas et al. 2000).

1.7.1 Properties of surface layer proteins

Surface layer proteins are a family of proteins with molecular weights most commonly reported of 98-100kDa, 125-127kDa and 149kDa (Winter et al. 1978; Blaser et al. 1987; Pei et al. 1988). However, SLPs have also been reported with molecular weights of 85kDa, 110kDa, 131-135kDa and 140kDa (Dubreuil et al. 1988 and 1990; Wang et al. 1993). Surface layer proteins are secreted from the cell and self-assemble into a capsule surrounding the cell (Fujimoto et al. 1991). They are the predominant protein expressed by *C. fetus* at approximately 10^5 proteins per cell (Yang et al. 1992). Surface layer proteins are also the immunodominant protein antigen of *C. fetus* subsp. *fetus*, as Western blotting of whole cell preparations using antiserum prepared against whole cells showed that the predominant reaction was to SLPs (Dubreuil et al. 1988). An SLP of a particular size is normally expressed by a cell and the other SLPs are present as minor proteins (Fujimoto et al. 1991). It was found that a single *C. fetus* strain can simultaneously express four different SLPs of different sizes and different antigenic types (Dubreuil et al. 1990). Electron micrography detected hexagonal and tetragonal arrangements of S-layers and found that the pattern was dependent on the dominant SLP expressed (Fujimoto et al. 1991). Occasionally two different arrangements of SLPs were found on the surface of a single cell (Fujimoto et al. 1991). It has been shown that isolates can spontaneously change the predominant SLP expressed during *in vivo* or *in vitro* passage and that this occurs at a high frequency (Fujimoto et al. 1991; Wang et al. 1993; Garcia et al. 1995). It has been suggested that SLP switching enables *C. fetus* subsp. *fetus* to evade the host immune response, resulting in long-term colonisation (Wang et al. 1993; Grogono-Thomas et al. 1996; Dworkin and Blaser 1997).

1.7.2 Surface layer proteins and lipopolysaccharides

As mentioned previously (Section 1.5.1), *C. fetus* subsp. *fetus* can be serotyped into Types A or B according to an outer membrane lipopolysaccharide (Varga 1991). Surface layer proteins are bound to lipopolysaccharides in a type-specific way, that is SLPs from Type A strains only bind to lipopolysaccharides of Type A strains, and not

lipopolysaccharides of Type B strains (Yang et al. 1992). Subsequent to removal with distilled water, SLPs can reattach to cells in a type-specific way (Yang et al. 1992). The N-terminal regions of different sized SLPs from Type A strains are antigenically conserved. Similarly, the N-terminal regions of different sized SLPs from Type B strains are antigenically conserved (Wang et al. 1993). This N-terminal region is responsible for the type-specific binding to lipopolysaccharides (Dworkin et al. 1995a and 1995b) and Southern hybridisation studies have found that these N-terminal regions are practically identical at the sequence level (Dworkin et al. 1995b). Furthermore, the C-terminal regions of SLPs of the same size from Type A and B strains are antigenically conserved (Wang et al. 1993). Western Blotting and ELISA experiments have shown that antiserum raised to a purified SLP binds to SLPs of different sizes and from both serotype A and B strains (Pei et al. 1988; Fujimoto et al. 1991; Wang et al. 1993).

1.7.3 Surface layer protein gene expression

Surface layer protein gene expression has been investigated as it is an interesting mechanism for generating variability of proteins. There are eight conserved SLP gene cassettes in a small area of the genome (8%) and each codes for a separate SLP (Dworkin et al. 1995a). The SLP genes from *C. fetus* Type A strains are designated *sapA*, *sapA1*, *sapA2* etc. and the SLP genes from Type B strains: *sapB*, *sapB1*, *sapB2* etc. (Blaser and Gotschlich 1990; Dworkin et al. 1995a). There is a single strong (sigma-70 like) promoter for expression of all the SLP genes (Tummuru and Blaser 1992). In some spontaneous mutants lacking the S-layer this promoter region has been deleted (Tummuru and Blaser 1992; Fujita and Amako 1994; Dworkin et al. 1995a). The promoter is situated on a large (6.2kb) invertible element between two of the SLP gene cassettes, which are oppositely oriented (Dworkin and Blaser 1996). A site-specific exchange of SLP gene cassettes can also occur, which then positions them immediately after the promoter (Blaser et al. 1994; Dworkin and Blaser 1996). In this way, synthesis of the SLP from the one promoter can potentially be varied between any of the gene cassettes. The mechanism of inversion of the SLP gene cassettes is still to be elucidated, and is confounded by the finding that the inversion of the SLP gene cassettes is RecA dependent (Dworkin et al. 1997). RecA catalyses homologous base pairing and single-stranded DNA exchange and has not been found in DNA inversion mechanisms

before (Dworkin et al. 1997). It appears that RecA-independent inversion also occurs, although at a lower frequency, as a *recA*-mutant was found to have switched SLP expression (Ray et al. 2000).

1.7.4 The S-Layer as a virulence factor

The S-Layer is an important virulence factor for *C. fetus* subspecies in evading the non-specific immune response of a host, by conferring resistance to:

- the bactericidal membrane attack activity of serum
- phagocytosis by polymorphonuclear leukocytes.

1.7.4.1 Resistance to the bactericidal membrane attack activity of serum

It has been shown that lysis of bacterial cells by the complement cascade of normal human serum is ineffective against *C. fetus* cells with an S-layer (Blaser et al. 1987; Blaser et al. 1988). In an assay, spontaneous mutant *C. fetus* cells that lacked the S-layer were sensitive to killing by normal human serum, as greater than 90% of the cells were destroyed when incubated with 10% normal human serum at 37°C for 1 hour (Blaser et al. 1988). In contrast, virtually no wild-type *C. fetus* cells (with the S-layer) were killed in the assay (Blaser et al. 1988). Blaser et al. (1988) found that this resistance was due to the interruption of the complement cascade. Complement factor, C3b, is unable to bind to encapsulated *C. fetus* and if bound is subject to cleavage by serum proteins to the haemolytically inactive iC3b and C3dg. Even immune serum containing specific antibodies is unable to lyse *C. fetus* cells with an S-layer, indicating that antibody-directed complement binding does not occur with encapsulated strains (Blaser et al. 1988).

1.7.4.2 Resistance to phagocytosis by polymorphonuclear leukocytes

It has been shown that spontaneous mutant *C. fetus* cells that lack the S-layer are opsonised by normal human serum and are readily phagocytosed (Blaser et al. 1988). In contrast, *C. fetus* cells that are encapsulated by the S-layer resist phagocytosis by polymorphonuclear leukocytes unless they are opsonised by specific antibodies (Blaser et al. 1988). The specific antibodies involved in opsonisation may be to proteins other than the S-layer, for example flagella proteins (Blaser et al. 1988).

The studies using spontaneous mutants lacking the S-layer were confirmed with wild-type strains that had been treated with pronase (Blaser and Pei 1993). It was shown that treatment of cells with the enzyme pronase progressively removed the SLPs without affecting *in vitro* viability. Therefore pronase treated cells were genetically identical to the untreated *C. fetus* cells, but lacked the S-layer. *Campylobacter fetus* cells treated with pronase were susceptible to the bactericidal activity of serum and to phagocytosis by polymorphonuclear leukocytes, equivalent to spontaneous mutants lacking the S-layer (Blaser and Pei 1993).

1.7.5 Surface layer proteins and disease

The presence of the S-layer on *C. fetus* subsp. *fetus* was found to be essential for producing abortions in sheep (Grogono-Thomas et al. 2000). A spontaneous mutant lacking the S-layer was unable to cause abortion in any of seven sheep subcutaneously challenged with 10^8 cfu on day 105 of gestation and *C. fetus* subsp. *fetus* could not be recovered from the placentae. When the parent strain (with S-layer) was used as a challenge in the same way, ten out of eleven ewes aborted and *C. fetus* subsp. *fetus* was recovered from the foetal liver, intestine and placentae (Grogono-Thomas et al. 2000). A further experiment using an intraplacental challenge with the *C. fetus* subsp. *fetus* mutant lacking SLP resulted in placental colonisation and abortion similar to the effects of the wild-type strain (Grogono-Thomas et al. 2000). Grogono-Thomas et al. (2000) proposed that the S-layer is important for systemic spread of *C. fetus* subsp. *fetus* infection in pregnant sheep, rather than the foetopathogenic effects. The systemic spread of *C. fetus* subsp. *fetus* in a compromised host (such as a pregnant ewe) may be facilitated by the serum and phagocytosis resistance properties of the S-layer (Blaser et al. 1987).

Studies using mouse models also demonstrated the importance of the *C. fetus* S-layer in causing disease. In a mouse model a transient high-grade bacteraemia developed 30 minutes after oral challenge with 10^8 cfu *C. fetus* and lasted for at least 48 hours (Pei and Blaser 1990). The presence of the S-layer was shown to be essential for bacteraemia to develop, as challenge using spontaneous mutant *C. fetus* strains lacking the S-layer did not produce bacteraemia at all (Pei and Blaser 1990). A passive protection study was performed with rabbit antiserum to the S-layer and mice who received the rabbit

immune serum did not develop bacteraemia upon challenge (Pei and Blaser 1990). Mice were found to be more susceptible to intraperitoneal challenge than oral challenge. In a mouse model, 17 out of 20 (85%) mice died after intraperitoneal challenge with the wild-type *C. fetus* strain (with S-layer) (Pei and Blaser 1990). In this model, challenge with a strain lacking the S-layer was not lethal, as none of the 20 mice died. In a further experiment, challenge with a strain lacking the S-layer was administered with a preparation of free SLPs and three out of 10 mice died. However, none of the seven mice died that were given the preparation of free SLPs alone (Pei and Blaser 1990). This demonstrated that the presence of the S-layer was essential for producing disease in the mouse model, that free SLPs administered with spontaneous mutants lacking the S-layer may produce disease, but that free SLPs alone were not pathogenic.

The results of the mouse studies using spontaneous mutants lacking the S-layer were confirmed using wild-type strains that had been treated with pronase (Blaser and Pei 1993). Intraperitoneal challenge of ten mice with wild-type *C. fetus* cells treated with pronase resulted in the death of one mouse (10%), whereas challenge of ten mice with the untreated wild-type strain resulted in the death of seven mice (70%). The dose for this experiment was 8×10^7 cfu, but when the challenge was increased to 8×10^9 cfu the difference in mortality for the groups of mice was less significant; seven mice died when given pronase-treated cells and eight mice died with untreated (Blaser and Pei 1993).

1.7.6 Surface layer proteins and immunity

It has been demonstrated that prior exposure to *C. fetus* subsp. *fetus* (either by natural challenge or vaccination) is protective against disease in sheep (Section 1.6). It has been demonstrated in rabbits that vaccination with whole cell *C. fetus* subsp. *fetus* elicits the production of antibodies primarily against SLPs (Dubreuil et al. 1988), and in the mouse model that antibodies to SLPs are associated with protection from disease (Pei and Blaser 1990; Blaser and Pei 1993).

Two studies in sheep also suggest that vaccination with whole cell *C. fetus* subsp. *fetus* elicits the production of antibodies primarily against SLPs and that antibodies to SLPs

are associated with protection from disease (Myers et al. 1970; Grogono-Thomas et al. 2003).

In 1970, a study investigating antigens for inclusion in *C. fetus* subsp. *fetus* sheep vaccines found one protein which was present in each of the fractions of a *C. fetus* subsp. *fetus* culture, and which elicited protective antibodies in sheep (Myers et al. 1970). Four vaccines were prepared, and each vaccine was prepared from one of the four following fractions of a *C. fetus* subsp. *fetus* culture:

- Whole cell vaccine: the harvested bacteria from *C. fetus* subsp. *fetus* culture.
- Post-growth broth vaccine: the bacteria were removed from the culture medium by centrifugation and filtration, then the culture medium was lyophilised to concentrate it 100-fold.
- Sediment vaccine: the bacteria after washing, sonication and centrifugation steps.
- Supernatant fluid vaccine: the filtrate of the supernatant fluid after the washing, sonication and filtration steps.

Four groups of 10-11 pregnant ewes were vaccinated twice; each group of ewes was vaccinated with one of the four vaccines. The ewes were challenged with an intraruminal injection of 1.5×10^{10} cfu of the same strain of *C. fetus* subsp. *fetus* used in the preparation of the vaccines. No abortions occurred in any of the vaccinated ewes, but four out of 11 unvaccinated ewes aborted (36%) and *C. fetus* subsp. *fetus* was recovered from the foetuses and/or placentae. The vaccinated ewes were blood sampled at two week intervals. The post-culture broth vaccine was shown by electrophoresis to contain a single protein. Gel double-diffusion experiments showed that antibodies to the post-culture broth antigen were present in the serum of the ewes from each group. This antigen was the only protein present in each of the vaccine preparations (Myers et al. 1970). This post-growth broth antigen was later characterised (Myers 1971) and found to be SLP (McCoy et al. 1975; Winter et al. 1978; Dubreuil et al. 1988). This early study showed that ewes vaccinated with a whole cell *C. fetus* preparation produced antibodies to SLPs and that the ewes with antibodies to SLPs were protected from abortion upon artificial challenge with *C. fetus*.

In a report in 2003, it was shown that after oral or subcutaneous challenge with 10^8 cfu *C. fetus* subsp. *fetus* at day 126 of gestation, eleven out of eleven ewes produced antibodies (IgA, IgG1 and IgG2) that were specific to SLPs (Grogono-Thomas et al.

2003). These antibodies were detected in serum, bile, urine, milk and colostrum. In addition, anti-SLP IgA antibodies were detected in faecal samples of the five sheep that were challenged orally. Ten ewes challenged with a *C. fetus* subsp. *fetus recA* mutant strain (that was unable to alter the size of the expressed SLP) produced antibodies within one week of challenge, whereas the ewes challenged with the wild-type strain produced antibodies by two weeks after challenge (Grogono-Thomas et al. 2003). This study showed that systemic and mucosal anti-SLP antibodies were produced in sheep following exposure to *C. fetus* subsp. *fetus* and that there was a delay of up to one week in the production of these antibodies if the *C. fetus* subsp. *fetus* strain was able to alter the expressed SLP. This apparent delay is supportive of the hypothesis of evasion of host immune responses by SLP switching (Wang et al. 1993; Grogono-Thomas et al. 1996; Dworkin and Blaser 1997).

In a further experiment, it was shown that sheep vaccinated with *C. fetus* subsp. *fetus* produced antibodies specific to SLPs and were protected from abortion after artificial challenge (Grogono-Thomas et al. 2003). However, the differences in abortion rates between vaccinated and unvaccinated sheep in this report were not statistically significant due to the low level of abortions in the unvaccinated sheep. When six ewes were vaccinated with two doses of wild-type *C. fetus* subsp. *fetus* and challenged by subcutaneous injection with 10^8 cfu of the same strain at 105 days gestation, none of the ewes aborted. Similarly, 12 ewes were vaccinated with two doses of *C. fetus* subsp. *fetus recA* mutant strains, which were able to express an SLP but were unable to switch to expression of a different sized SLP. When these ewes were challenged at 105 days gestation with the same *recA* mutant or a *recA* mutant only capable of expressing a different SLP, none of the ewes aborted. It was shown that after vaccination with all these *C. fetus* subsp. *fetus* strains serum antibodies specific to SLPs were produced in these ewes, and that these antibody levels were sustained for the subsequent 31 weeks that they were monitored (Grogono-Thomas et al. 2003). However, lower levels of abortion were observed in the positive controls for this experiment than anticipated from previous results. Ten ewes were vaccinated with sterile broth or a *C. fetus* subsp. *fetus* strain that was unable to express any SLPs. When these ewes were also challenged at 105 days gestation with wild-type *C. fetus* subsp. *fetus*, there were only two abortions (20%) (Grogono-Thomas et al. 2003). This was a lower abortion rate in "naive" animals than was found earlier using the same challenge model when ten out of eleven ewes

aborted (91%) (Grogono-Thomas et al. 2000). The reason for the low level of abortion was not known but was proposed to be due to undetected prior exposure of the ewes (Grogono-Thomas et al. 2003). Nevertheless, two out of 10 unvaccinated animals aborted and none out of 18 vaccinated animals aborted which "approaches statistical significance" and this protection was associated with anti-SLP antibodies.

In an experiment described above, six ewes were vaccinated with a *recA* mutant strain which only expressed an SLP of a particular size, and were subsequently challenged with a *recA* mutant which only expressed an SLP of a different size, and none of the ewes aborted (Grogono-Thomas et al. 2003). This suggests that immune protection gained from anti-SLP antibodies is independent of SLP switching. In a further experiment, the serum IgG anti-SLP antibodies from sheep challenged with wild-type *C. fetus* subsp. *fetus* or the *recA* mutants were shown to bind strongly with amino acids 81-100 of the conserved N-terminal region of Type A SLP (Grogono-Thomas et al. 2003). These results are supported by those of Western Blotting and ELISA experiments which showed that antiserum raised to a purified SLP binds to SLPs of different sizes (Pei et al. 1988; Fujimoto et al. 1991; Wang et al. 1993), indicating that binding occurs to a conserved region. These findings are inconsistent with the theory of SLP switching enabling *C. fetus* subsp. *fetus* to evade the host immune response, resulting in long-term colonisation (Wang et al. 1993; Grogono-Thomas et al. 1996; Dworkin and Blaser 1997). Switching of SLPs may not enable *C. fetus* subsp. *fetus* to evade the host immune response long-term, but it has been shown to delay the production of anti-SLP antibodies by up to one week, which could potentially allow rapid establishment of infection (Grogono-Thomas et al. 2003).

Together these studies suggest that vaccination of sheep with whole cell *C. fetus* subsp. *fetus* elicits the production of antibodies primarily against the conserved SLP epitope involving amino acids 81-100 and that antibodies to SLPs are associated with protection from disease.

1.8 Animal challenge models for *C. fetus* subsp. *fetus* vaccine efficacy

Several animal models for the study of *Campylobacter* spp. have been detailed so far in this literature review, such as sheep, guinea pigs, rabbits and mice.

1.8.1 Rabbits

Rabbits are frequently used for the production of antiserum for *Campylobacter* research (Marsh and Firehammer 1953; Williams et al. 1976; Bryner et al. 1978; Bird et al. 1984; Yrios and Balish 1986b; de Lisle et al. 1987; Pei and Blaser 1990; Diker and Turutoglu 1995; Grogono-Thomas et al. 2000), but have not been established as a model for *C. fetus* subsp. *fetus* disease.

1.8.2 Mice

A mouse model was used to study strain virulence and the role of SLPs, as discussed in Section 1.7.5 (Pei and Blaser 1990; Blaser and Pei 1993). Mice have also been used as an intestinal disease model for *C. fetus* subsp. *fetus* (Yrios and Balish 1986a). Germ-free athymic and euthymic mice were orally inoculated with a *C. fetus* subsp. *fetus* isolate, which was found to colonise the colon in large numbers for 224 days but was not consistently recovered from other internal organs. The mice were apparently free from disease and ileal, caecal and colonic histological sections were normal (Yrios and Balish 1986a). There are no reports of mice being used as an animal model to investigate *C. fetus* subsp. *fetus* vaccine efficacy.

1.8.3 Sheep

The obvious animal in which to examine *C. fetus* subsp. *fetus* abortion is sheep, due to the occurrence of the disease in this species.

In early studies using sheep for the investigation of *Campylobacter* abortion, the sheep were often challenged orally or by rumen inoculation (Jensen et al. 1957; Miller et al. 1964; Myers et al. 1970; Bryner et al. 1972; Gilmour et al. 1975). Abortion rates varied from 36-76% of the ewes challenged in these studies. It was found that ewes challenged

intravenously often died from the inoculation (Osborne and Smibert 1963; Bryner et al. 1972). However, ewe death was not reported in a later study in which seven pregnant ewes were intravenously inoculated with 5mL of a suspension containing 10^8-10^9 cfu/mL of *C. jejuni* (Hedstrom et al. 1987).

Recently a model for *C. fetus* subsp. *fetus* challenge of sheep was reported which established that subcutaneous challenge with 10^8 cfu yielded more abortions than oral inoculation of the same dose (50-63% compared with 20-30%) (Grogono-Thomas and Woodland 1996). However, the reproducibility of these results is questionable as a later study showed subcutaneous challenge of 11 ewes with 10^8 cfu at day 105 of gestation resulted in 10 abortions (91%), whereas subcutaneous challenge of five ewes with 10^8 cfu of the same strain at day 126 of gestation resulted in one abortion (20%) (Grogono-Thomas et al. 2000). Similarly, a further study by the same authors in which ten ewes were subcutaneously challenged at 105 days gestation with 10^8 cfu, resulted in only two abortions (20%) (Grogono-Thomas et al. 2003). In this last study, previous exposure of the ewes to *C. fetus* subsp. *fetus* was suggested as a reason for the low abortion rate. However, this was undetected in the monitored pre-inoculation antibody levels of the sheep (Grogono-Thomas et al. 2003). For a number of reasons sheep may not be an ideal model for investigation of *Campylobacter* abortion and vaccine protection:

- the various potential routes of exposure of sheep have not been unequivocally elucidated, which means there are difficulties in ensuring the trial sheep are naïve
- previous exposure to *C. fetus* subsp. *fetus* may be difficult to detect
- a robust challenge model has not been developed
- sheep are generally seasonal breeders with oestrus activity occurring in autumn
- sheep have a relatively long gestation period.

1.8.4 Guinea pigs

The first detailed study using pregnant guinea pigs as a model for *Campylobacter* abortions was in 1953 (Ristic and Morse 1953). Since then, abortion in pregnant guinea pigs has been the principal animal model used for testing the efficacy of *Campylobacter* vaccines (Bryner et al. 1978, 1979, and 1988; Diker and Turutoglu 1995) or the virulence of strains (SultanDosa et al. 1983; Taylor and Bryner 1984; Coid et al. 1987).

Typically guinea pigs are 3-5 months old and weigh 600-770g when mated. Pregnancy can be detected after 18 days by manual palpation of the abdomen and guinea pigs have an average of three foetuses. Challenge is performed in the third or fourth week of pregnancy. Latterly, an intraperitoneal injection of 100×Minimum Abortive $Dose_{50}$ ($100\times MAD_{50}$) has been the standard challenge used for assessing the efficacy of a vaccine, where the MAD_{50} is the minimum number of viable cells needed to produce abortion in 50% of unvaccinated guinea pigs (Bryner et al. 1988; Diker and Turutoglu 1995). Guinea pig challenge studies using various *C. fetus* subsp. *fetus* strains have determined MAD_{50} values of between 100-1000 cfu (145 cfu by Bryner et al. 1979; 1000 cfu by Bryner et al. 1978; 100-1000 cfu by Bryner et al. 1988; and 1000 cfu by Diker and Turutoglu 1995). Abortion is preceded by vaginal bleeding and takes place 1-12 days post-challenge. In order to ensure detection of abortion, guinea pigs should be weighed daily and on average lose >50g upon abortion (Bryner et al. 1978 and 1979; SultanDosa et al. 1983; Diker and Turutoglu 1995).

As guinea pigs have a short gestation time (63-69 days), are susceptible to *C. fetus* subsp. *fetus* and *C. jejuni* abortions, can be effectively immunised against abortion, and a vaccine can be evaluated in guinea pigs in three months, they are a useful model for testing *C. fetus* subsp. *fetus* vaccine efficacy (Bryner et al. 1978 and 1979).

1.9 Objectives of the present study

This project was initiated in response to questions regarding the protection offered by the single-strain *C. fetus* subsp. *fetus* sheep abortion vaccine, Campylovexin® (Schering-Plough Animal Health Ltd.) against other *C. fetus* subsp. *fetus* sheep abortion strains in New Zealand.

Since the development of Campylovexin® in the 1980s, it has been known that there were at least two "strains" of *C. fetus* subsp. *fetus* causing abortion in sheep in New Zealand. Around this time, Animal Health Diagnostic Laboratories routinely serotyped *C. fetus* subsp. *fetus* sheep abortion isolates as "vaccine strain" or "non-vaccine strain". A report in 1988 detailed the apparent emergence of the non-vaccine strain (Pauling 1988). Strain typing of *C. fetus* subsp. *fetus* sheep abortion isolates using restriction endonuclease analysis identified that the vaccine strain was different from the most commonly found type, REA type b (Collins and de Lisle 1985). In addition, some REA type b isolates were serologically different to the vaccine strain (Collins and de Lisle 1985). This was followed in 1987 by a larger study in which more isolates were typed and the same result was found (de Lisle et al. 1987).

Despite some doubt regarding the protection against these different strains offered by Campylovexin®, there have been few reported cases of *C. fetus* subsp. *fetus* abortions in vaccinated ewes (Pauling 1988; Marchant 1999). However, in 1996 an apparent vaccine breakdown occurred, when fifteen ewes aborted out of a flock of 300 vaccinated two-tooth ewes (Fenwick et al. 2000). *Campylobacter fetus* subsp. *fetus* was isolated from six foetuses, and the isolates were shown to have a different PFGE profile to the vaccine strain. However, it was suggested that the ewes may have been exposed to high *C. fetus* subsp. *fetus* challenge when they were densely stocked each night in a small, "sacrifice" paddock (Fenwick et al. 2000).

The overall aim of this project was to evaluate the efficacy of Campylovexin®, particularly relating to protection against other *C. fetus* subsp. *fetus* sheep abortion strains, with the following objectives:

- Establish the strain variation of *C. fetus* subsp. *fetus* in New Zealand, by typing isolates from sheep abortions using PFGE.
- Study the serological reactivity of serum from sheep vaccinated with Campylovexin® against different *C. fetus* subsp. *fetus* strain types.
- Conduct a national survey to detect and investigate cases of suspected Campylovexin® breakdown.
- Test Campylovexin® efficacy against selected strains using an animal challenge model.

Chapter 2

General Materials and Methods

2.1 Manufacturers' addresses

BBL	Becton Dickinson & Co., Sparks, MD, USA
BDH Laboratory Supplies	Poole, England
Becton Dickinson Vacutainer Systems	Franklin Lakes, NJ, USA
bioMerieux	Marcy-l'Etoile, France
BioRad	Hercules, CA, USA
Difco	Detroit, MI, USA
Fort Dodge Animal Health	Overland Park, KS, USA
Fort Richards	Auckland, New Zealand
Gibco Invitrogen Corporation	Grand Island, New York, USA
Kem-En-Tec	Copenhagen, Denmark
Medvet Science Pty Ltd.	Thebarton, South Australia
Merck	Darmstadt, Germany
New England Biolabs (NEB)	Beverley, MA, USA
Oxoid Ltd	Basingstoke, Hampshire, England
Roche Applied Science	Mannheim, Germany
Schering-Plough Animal Health Ltd.	Upper Hutt, New Zealand
Sigma Chemical Co.	St. Louis, MO, USA
Schleicher & Schuell	Dassel, Germany
Tasman Medical and Scientific Ltd	Auckland, New Zealand

2.2 Media, stains and solutions

2.2.1 Media

Blood Agar (BA)

Dissolve 44 g Columbia Blood Agar Base (Difco) in 950 mL distilled water, autoclave. When cooled to 45°C, add 50 mL defibrinated sheep blood (Gibco) and mix (5%). Pour 20 mL into petri dishes and leave to set.

Blood Agar with Campylobacter Selective Supplement

Prepare Blood Agar medium as above. Before pouring into petri dishes add one vial of Oxoid Campylobacter Selective Supplement (Skirrow) per 500 mL media. Campylobacter Selective Supplement contains Vancomycin, Polymyxin B, and Trimethoprim Lactate.

Heart Infusion Broth (HIB)

Dissolve 2.5 g Heart Infusion Broth (Difco) in 100 mL distilled water. Dispense 3 mL aliquots into bijou bottles, autoclave.

Heart Infusion Broth / Cysteine media

Dissolve 5 g Heart Infusion Broth (Difco), 0.04 g Cysteine (Sigma), and 0.3 g Bacto agar (Becton, Dickinson and Co.) in 200 mL distilled water. Dispense 5 mL aliquots into universal bottles, autoclave.

Triple sugar iron slope (TSI slope)

Dissolve 65 g triple sugar iron agar (Merck) in 1 L distilled water. Dispense 15 mL aliquots into tubes, autoclave, and leave to solidify at an angle.

2.2.2 Grams stains

Crystal violet

Dissolve 0.5% crystal violet (Sigma) in distilled water.

Lugols iodine

Dissolve 20 g potassium iodide (BDH) in 250 mL distilled water. Add 10 g iodine (BDH) and dissolve. Make up to final volume of 1 L.

Safranin

Dissolve 0.5% safranin (BDH) in distilled water.

2.2.3 Pulsed-field gel electrophoresis solutions

PettIV buffer

1 M NaCl, 10 mM Tris.Cl, pH 8.0, 10 mM EDTA, pH 8.0

ESP-Lysis buffer

0.5% lauroyl sarcosine, 250 mM EDTA, pH 9.0, 0.5 mg/ml Proteinase K

TE buffer

10 mM Tris, 1 mM EDTA, pH 8.0

TBE buffer

45 mM Tris, 45 mM Boric acid, 1 mM EDTA

2.2.4 SDS-polyacrylamide gel electrophoresis solutions

Coomassie Blue R Stain

0.2% (w/v) Coomassie Blue R (Sigma) in methanol. Use 1:1 with 20% acetic acid.

Coomassie Destain

50% (v/v) methanol, 10% (v/v) acetic acid

10× Running Buffer

250 mM Tris, 2 M Glycine, 1% SDS

Sample Buffer

62.5 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 10% (w/v) SDS, 0.01% bromophenol blue. Add 5% ß-mercaptoethanol just before use.

2.2.5 Western Blotting solutions

anti-sheep antibody

alkaline phosphatase conjugated Donkey anti-sheep IgG, Sigma.

AP Solution (Alkaline Phosphatase Solution)

100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$

BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate)

Dissolve 50 mg/mL BCIP (Sigma) in water.

NBT (Nitro Blue Tetrazolium)

Dissolve 10 mg/mL NBT (Sigma) in water.

PonceauS

0.01% PonceauS in 5% acetic acid.

TBS (Tris-buffered saline)

40 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween20, 0.001% Thimerosol.

TBSM (Tris-buffered saline with milk powder)

40 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween20, 0.001% Thimerosol, 1% skim milk powder.

Transfer Buffer

25 mM Tris, 192 mM Glycine, 20% Methanol.

2.3 *Campylobacter* spp. growth and identification

2.3.1 Growth and identification of *Campylobacter* spp.

Campylobacter spp. were incubated for all growth steps in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) generated by CampyGen sachets (Oxoid Ltd) in a sealed jar (Tasman Medical and Scientific Ltd). Incubation was at 35°C, unless stated.

Foetal stomach contents were plated directly onto selective media (either Skirrows from Fort Dodge Animal Health or Blood Agar with Campylobacter Selective Supplement, Oxoid Ltd). Growth after 48 hours was identified as *Campylobacter* spp. by colony morphology (Figure 2.1), by Gram stain (Figure 2.2), and a positive oxidase test.

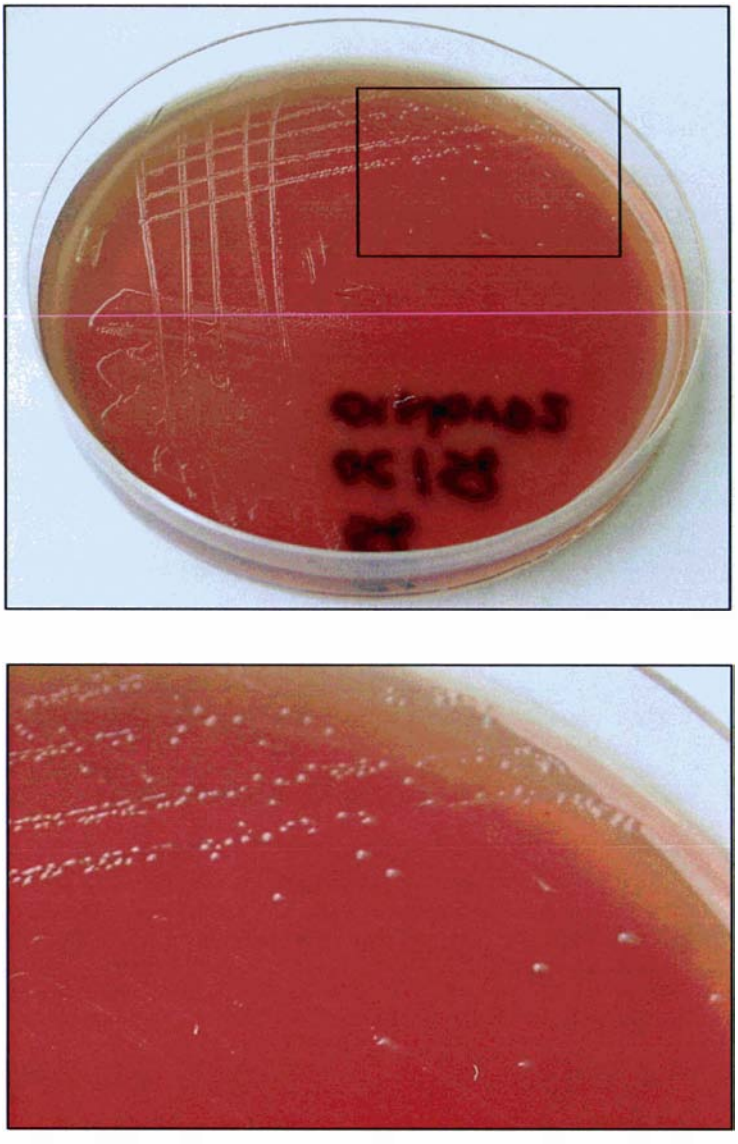


Figure 2.1 *Campylobacter fetus* subsp. *fetus* colonies on a blood agar plate.

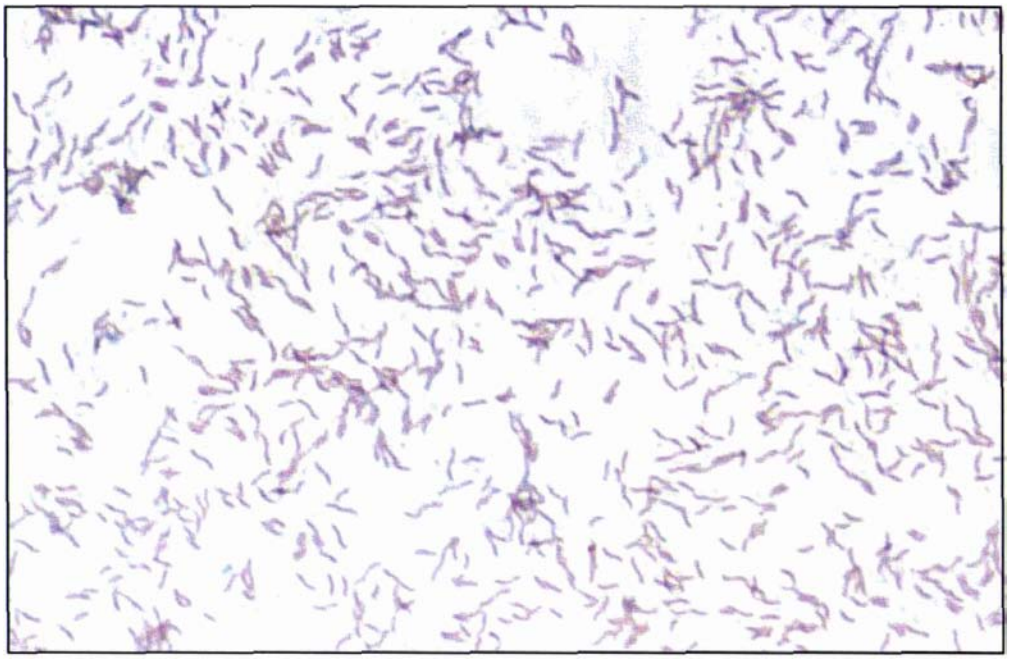


Figure 2.2 Gram stain of *C. fetus* subsp. *fetus*.

2.3.1.1 Gram stain

Heat fix bacterial growth on a microscope slide. Flood slide with Crystal Violet for 30 seconds, rinse with water, then flood slide with Lugols Iodine for 30 seconds, and rinse with water. Destain with acetone (Merck) briefly and rinse with water. Counterstain with Safranin for one minute, rinse with water. Blot slide and view under oil at 1000× magnification.

2.3.1.2 Oxidase test

Touch the bacterial growth with a piece of Oxidase Detection Strip (Medvet Science Pty Ltd). A change in colour of the strip from white to purple within 5 seconds indicates a positive oxidase test.

2.3.2 Speciation of *Campylobacter* isolates

Isolates were identified as *C. fetus* subsp. *fetus* by (Quinn et al. 1994):

- catalase activity
- production of hydrogen sulphide in 0.02% w/v semi-solid cysteine medium (lead-acetate strip detection)
- lack of hydrogen sulphide production in triple sugar iron medium
- susceptibility to cephalothin
- resistance to nalidixic acid
- growth at 25°C

Isolates were identified as *C. jejuni* by (Quinn et al. 1994):

- catalase activity
- production of hydrogen sulphide in 0.02% w/v semi-solid cysteine medium (lead-acetate strip detection)
- lack of hydrogen sulphide production in triple sugar iron medium
- susceptibility to nalidixic acid
- resistance to cephalothin
- lack of growth at 25°C
- a positive hippurate hydrolysis test

Isolates were identified as *C. coli* by the same results as *C. jejuni*, except that *C. coli* have a negative hippurate hydrolysis test.

2.3.2.1 Catalase test

Drop a generous loop of bacterial growth onto a microscope slide (avoid contamination with Blood Agar). Add one drop of 3% hydrogen peroxide solution. The production of gas (bubbles) indicates catalase activity.

2.3.2.2 Hydrogen sulphide production

Lead acetate strip

Inoculate Heart Infusion Broth/Cysteine media with a small loop of growth from a BA plate. Suspend a lead acetate strip (BDH Laboratory Supplies) above the medium, securing it in place with the cap. The cap should be slightly loose to allow the inside of

the universal to become microaerobic. Incubate for 2-5 days. A positive reaction is characterised by blackening of the lead acetate strip.

Triple sugar iron slope

Harvest some bacterial growth with a straight wire and plunge to the bottom of a triple sugar iron (TSI) butt. On the way to the top of the tube, wipe the wire over the surface of the slope. Incubate for 2-5 days. Blackening of the butt or the fluid at the base of the slope indicates strong production of hydrogen sulphide. Change in colour of the butt or slope indicates acidity or alkalinity of growth. No change should be observed with *C. fetus* subsp. *fetus*, *C. coli*, or *C. jejuni*.

2.3.2.3 Nalidixic acid and cephalothin susceptibility

Pre-wet a swab and harvest some bacterial growth from a BA plate into HIB and emulsify to produce an even suspension. Wipe the swab over the surface of a fresh BA plate in three directions while twirling the swab. Place the 30 μg antibiotic discs (Oxoid Ltd) onto the plate with flamed tweezers. Incubate for two days. A zone of growth inhibition around an antibiotic disc indicates susceptibility.

2.3.2.4 Growth at 25°C

Pick a single colony from a BA plate and streak onto a fresh BA plate. Incubate at 25°C for three days.

2.3.2.5 Hippurate hydrolysis test

Pre-wet a swab and harvest some bacterial growth from a BA plate into 400 μL distilled water in a test tube, and emulsify to produce an even suspension. Add one BBL Taxo Differentiation Disc Hippurate and incubate at 37°C for two hours. Add four drops of ninhydrin reagent (bioMerieux) to the tube, mix gently, and incubate at 37°C for 15 minutes. A change in colour of the suspension to purple indicates a positive hippurate hydrolysis reaction.

2.4 Pulsed-field gel electrophoresis (PFGE)

Agarose plug preparation and PFGE were performed by modification of the method of Chang and Taylor (1990) using the switch times optimised by Ahmed (1999).

2.4.1 Genomic DNA preparation and digestion

Bacterial growth after 48 hours on blood agar was harvested into 3 mL HIB. The volume of the suspension equivalent to 150 µL of culture at density OD_{610nm} = 1.4 was centrifuged for 5 minutes at 13 krpm (ie. 1.4 × 150 µL =210, $210/OD_{610}$ = volume (µL) of cells). The cell pellet was washed with 150 µL ice-cold PettIV buffer, before harvesting the cells by centrifugation and resuspending them in 50 µL ice-cold PettIV buffer. To this, 100 µL cooled, molten 1% low-melt PFGE-grade agarose (BioRad) in PettIV buffer was added, quickly mixed and dispensed into the plug mould. Plugs were left to solidify for 1 hour on ice.

Lysis of the bacteria took place overnight in ESP-Lysis buffer at 50°C. Plugs were washed on ice three times for 30 minutes in TE buffer with 1 mM phenylmethylsulphonyl fluoride (PMSF, Roche Applied Science), then three times for one hour in TE buffer alone.

2.4.1.1 *Sma*I digestion

One third of each plug was equilibrated with 1.2× NEB Buffer 4 for 45 minutes on ice. The plug slice was then equilibrated for 45 minutes on ice with 1× NEB Buffer 4 and 30 units of NEB restriction enzyme *Sma*I, before incubation overnight at 25°C.

Remaining two-thirds of plug may be stored at 4°C in 1 mL TE until required.

2.4.2 Pulsed-field gel electrophoresis (PFGE)

A 1% PFGE-grade agarose (BioRad) in 0.5× TBE buffer gel was pre-electrophoresed for 1.5 hours at 6 Volts/cm, switch time 10 s, angle 120°, at 14°C in 2.5 L 0.5× TBE buffer in a BioRad Contour-clamped Homogeneous Electric Field (CHEF) Mapper II system. Digested plug slices were inserted into the wells and sealed with cooled molten agarose, before electrophoresis for 26 hours at 6 Volts/cm, switch times 1-28 s, angle

120°, at 14°C (Figure 2.3). The gel was stained for 10 minutes in 0.01% v/v ethidium bromide (BDH), rinsed briefly in distilled water, and the PFGE profiles were visualised and the digital image was stored using the BioRad GelDoc 2000 system. The molecular size standards used were Lambda Ladder PFG Marker and Low Range PFG Marker (NEB).

Figure 2.3 Pulsed-field gel electrophoresis tank.

2.4.3 Analysis of PFGE profiles

The PFGE profiles were analysed with BioRad Diversity Database software. A maximum band position tolerance of 2% was used to compensate for between-gel variance. A similarity matrix between the PFGE profiles was calculated using the Dice coefficient. The dendrogram showing clusters of similarity of PFGE profiles was produced using the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis. Using this analysis, isolates with indistinguishable PFGE profiles were grouped together as the same type, for example all A1 isolates were indistinguishable by PFGE. Clusters of PFGE types that were ≥84% similar were considered to belong to the same PFGE group, and the PFGE types within a group were named sequentially in order of identification, for example A1, A2, A3, A4, A5, A6, then A7. The limit of 84%

corresponded to the PFGE profiles within each group consisting of at least 11 invariable bands. Pulsed-field gel electrophoresis types that were <84% similar by cluster analysis were considered to belong to a different PFGE group, for example A, B, C, D, E, and F.

2.5 SDS-polyacrylamide gel electrophoresis

2.5.1 Protein sample preparation

Growth after 48 hours on Blood Agar was harvested into 3 mL Heart Infusion Broth. The density of bacteria in the suspension was estimated by measurement of OD_{600nm}. Bacteria were pelleted by centrifugation at 13,000 rpm for 5 minutes, and the pellet was resuspended in a volume equivalent to $200 \times OD_{600nm}$ Sample Buffer and stored at –70°C.

2.5.2 Protein electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was adapted from the method of Laemmli (1970). 2 µL of the protein sample in a total volume of 8 µL was loaded per well of a 15-well BioRad Tris-HCl Ready Gel® precast gel (7.5% resolving gel, 4% stacking gel). Diluted samples were heated in a boiling water bath for 5 minutes prior to loading. Electrophoresis was performed at 200 V for approximately 45 minutes in Running Buffer, using a BioRad Mini-Protean II electrophoresis system (Figure 2.4).

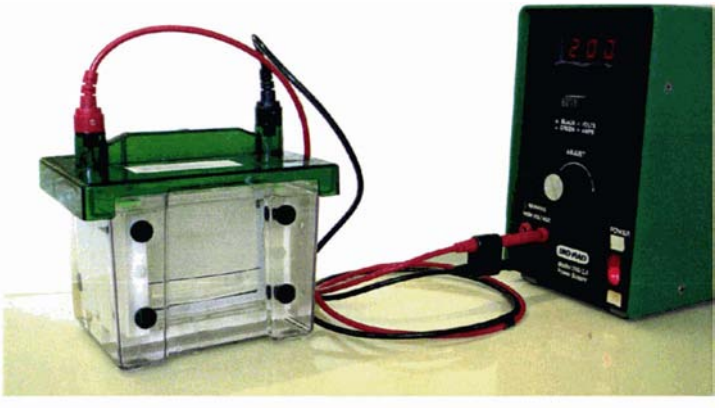
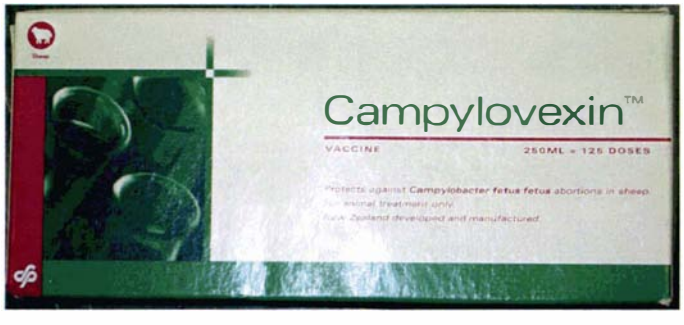


Figure 2.4 Mini-Protean II electrophoresis system.

Protein standards were BioRad SDS-PAGE Broad Range Molecular Weight Standards. Gels were either stained or used for Western Blotting (Section 2.7). Gels were stained with a 1:1 solution of Coomassie Blue R Stain and 20% acetic acid for 1 hour at room temperature with mixing by rocking. Gels were destained in Coomassie Destain for 1 hour at room temperature with rocking. The stained gels were equilibrated in water and dried between sheets of cellophane (Kem-En-Tec).

2.6 Collection of serum from vaccinated ewes

Serum was collected from 15 mixed-age Romney ewes before and after vaccination with Campylovexin® (Schering-Plough Animal Health Ltd.). Ewes were blood sampled immediately before the first Campylovexin® injection (pre-immune sera). The ewes were vaccinated according to the manufacturers' instructions (Campylovexin® batch number 533A, exp. 08/2002, 2 mL, subcutaneous injection in the anterior neck). Thirty days later the ewes were given a second Campylovexin® injection (2 mL, subcutaneous injection in the anterior neck, from the same vaccine pack stored at 4°C).



Three weeks later the ewes were blood sampled (immune sera). Blood was collected in a 10 mL blood tube (Vacutainer® No Additive, Becton Dickinson Vacutainer Systems), and left to clot at room temperature for 1-2 hours. Clots were left to contract for a minimum of 1 hour at 4°C. Blood was centrifuged at 3,200 rpm for 10 minutes and the serum removed. An equal volume of pre-immune sera from the 15 sheep was pooled and this was used in Western blotting at a dilution of 1:250. An equal volume of

immune sera from the 15 sheep was pooled and this was used at a dilution of 1:250. Sera were stored at –70°C.

Animal Ethics

Approval for this work was gained from the Massey University Animal Ethics Committee. Protocol number: 00/176.

2.7 Western Blotting

Western blotting was performed by adaptation of the method of Towbin et al. (1979). After SDS-PAGE, separated proteins were transferred to 0.45 µm nitrocellulose membrane (Schleicher and Schuell) in a BioRad Mini Trans-Blot® Cell at 100 V for 1 hour in Transfer Buffer (Figure 2.5).

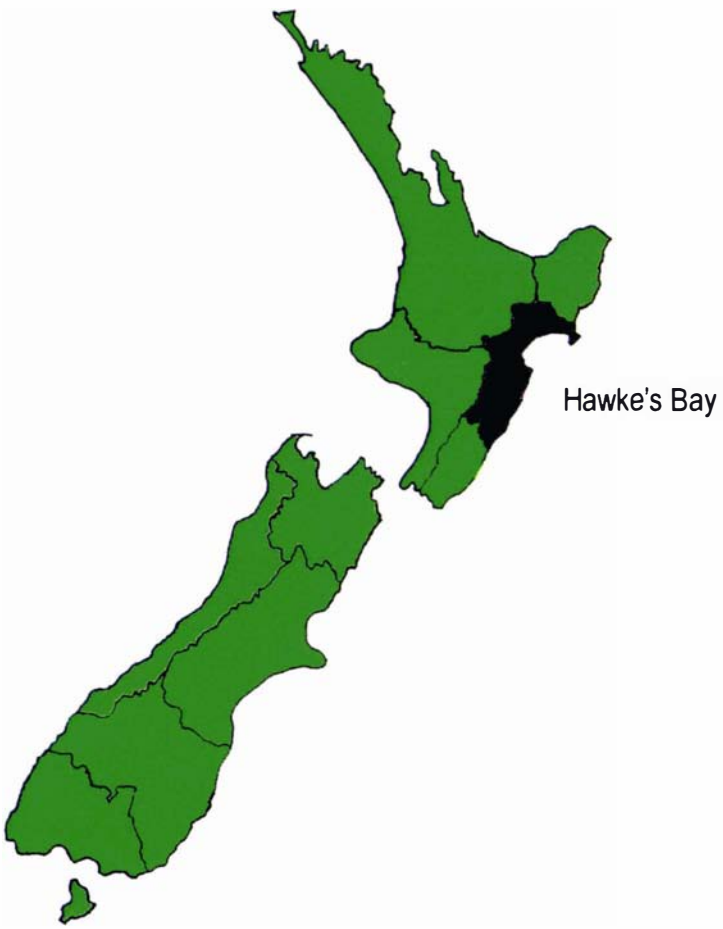
Figure 2.5 Western Blotting apparatus.

The membrane was stained with PonceauS and bands of the protein standards were marked with pencil. PonceauS was washed off with water and the membrane was blocked overnight in Tris-buffered saline with milk powder (TBSM). After a brief rinse in fresh TBSM, the membrane was incubated with a dilution of 1:250 sheep serum in TBSM (rocking at room temperature for 2 hours). The membrane was washed 5 times for 5 minutes with TBSM and incubated with a 1:30,000 dilution of anti-sheep antibody

in TBSM (rocking at room temperature for 2 hours). The membrane was washed 5 times for 5 minutes with Tris-buffered saline (TBS). Alkaline phosphatase colour reaction was developed by briefly incubating the membrane in 33 μL BCIP and 330 μL NBT in 10 mL AP Solution. Colour development was stopped by rinsing with water.

Chapter 3

Typing of *Campylobacter fetus* subsp. *fetus* from sheep abortions using pulsed-field gel electrophoresis: a pilot study of Hawke's Bay isolates



Results of this work are published in:

Mannering SA, Marchant RM, Middelberg A, Perkins NR, West DM and Fenwick SG (2003) Pulsed-field gel electrophoresis typing of *Campylobacter fetus* subsp. *fetus* from sheep abortions in the Hawke's Bay region of New Zealand. *New Zealand Veterinary Journal* 51 (1) 33-37.

3.1 Introduction

Three criteria are important in sub-typing bacterial isolates belonging to the same species (Olive and Bean 1999). Firstly, all isolates must be typeable by the chosen method. Second, there must be differentiation between strains, and finally, the results must be reproducible. Traditionally, serotyping has been used as a method of typing *C. fetus* subsp. *fetus* isolates. However, using this phenotypic technique, only two serotypes have been identified for this organism (Bird et al. 1984; Varga 1991).

Molecular typing techniques have proved to be useful in overcoming some of the problems of the phenotypically-based methods and are generally more discriminatory (Wassenaar and Newell 2000). Pulsed-field gel electrophoresis (PFGE) is a molecular typing technique that has been used to type various pathogens in epidemiological studies (Tenover et al. 1995), including *C. fetus* subsp. *fetus* (Fujita et al. 1995). Pulsed-field gel electrophoresis is based on the same principle as an earlier method, restriction endonuclease analysis (REA); but in the case of PFGE genomic DNA is digested using rare-cutting restriction enzymes and the small number of large DNA fragments are separated to form a profile specific for that isolate. This overcomes the difficulty inherent with REA of comparing the complex profiles of poorly separated DNA bands between different isolates (Arbeit 1995).

It has been known since the 1980s that there were at least seven strain types of *C. fetus* subsp. *fetus* causing abortion in sheep in New Zealand. Initially, Collins and Ross (1984) found four different types when they used REA to type 43 isolates. In subsequent studies of 51 and 70 isolates, seven REA types were found (Collins and de Lisle 1985; de Lisle et al. 1987).

The importance of the multiple strains in the epidemiology of the disease was unknown, as the single-strain *C. fetus* subsp. *fetus* vaccine, Campylovexin® (Schering-Plough Animal Health Ltd.), appeared to provide cross-protection (Pauling 1988). However, in 1996 *C. fetus* subsp. *fetus* abortions occurred in a flock apparently vaccinated with Campylovexin® (Fenwick et al. 2000). The isolates cultured from the affected flock were typed using PFGE and were found to be a different type to the vaccine strain. For

these reasons, the strain variation of *C. fetus* subsp. *fetus* implicated in sheep abortion in New Zealand was investigated using the technique PFGE. This knowledge was then used in further studies of the *in vitro* and *in vivo* efficacy of the Campylovexin® vaccine against these strains (Chapters 6-8).

Initially, a pilot study was undertaken involving *C. fetus* subsp. *fetus* isolates cultured from sheep abortions in the Hawke's Bay region of New Zealand in 1999. This chapter describes the typing results of these isolates using PFGE.

3.2 Materials and Methods

3.2.1 Isolation of *Campylobacter* spp. from sheep abortions

Aborted foetuses (1-5 per flock) were submitted for *Campylobacter* isolation from 50 commercial sheep flocks in the Hawke's Bay region in which *Campylobacter* was clinically suspected to be the cause of the abortion. Information was also sought on whether the affected sheep had been vaccinated with the *C. fetus* subsp. *fetus* vaccine, Campylovexin®.

Campylobacter isolates were cultured from the foetal stomach contents by either Gribbles Veterinary Pathology Animal Health Laboratory, Palmerston North (then known as AgriQuality Farm Network Animal Health Laboratory) or Massey University Diagnostic Microbiology Laboratory, Palmerston North, according to the method detailed in Chapter 2. Briefly, foetal stomach contents were plated directly onto selective media and incubated for 48 hours at 37°C in a microaerobic atmosphere. *Campylobacter* spp. identification was confirmed by Gram stain or dark field microscopy and isolates were frozen in 15% glycerol for further analysis.

3.2.2 Speciation of *Campylobacter* isolates

Isolates were subsequently identified to species level by standard microbiological methods detailed in Chapter 2. Isolates were identified as *C. fetus* subsp. *fetus* by production of hydrogen sulphide in 0.02% w/v semi-solid cysteine medium (lead-acetate strip detection), lack of hydrogen sulphide production in triple sugar iron medium, catalase activity, susceptibility to cephalothin, resistance to nalidixic acid, and growth at 25°C. Isolates were identified as *C. jejuni* by production of hydrogen sulphide in 0.02% w/v semi-solid cysteine medium (lead-acetate strip detection), lack of hydrogen sulphide production in triple sugar iron medium, catalase activity, susceptibility to nalidixic acid, resistance to cephalothin, lack of growth at 25°C, and a positive hippurate hydrolysis test (Quinn et al. 1994).

3.2.3 Pulsed-field gel electrophoresis of *C. fetus* subsp. *fetus* isolates

Preparation and digestion of genomic DNA and PFGE of *C. fetus* subsp. *fetus* isolates was performed according to the method detailed in Chapter 2. In addition to digestion with the restriction enzyme *Sma*I, digestion with the restriction enzyme *Sal*I was performed as follows. One third of each plug was equilibrated for 45 minutes on ice with 1.2× Roche Applied Science Buffer H. The plug slices were then equilibrated with 1× Roche Buffer H and 30 units of Roche restriction enzyme *Sal*I. Digestion reactions were incubated overnight at 37°C.

3.2.4 Analysis of PFGE profiles

The PFGE profiles of the isolates were analysed using BioRad Diversity Database software according to the method detailed in Chapter 2. The PFGE types were named according to the method detailed in Chapter 2.

3.2.5 *Campylobacter fetus* subsp. *fetus* vaccine strain

The *C. fetus* subsp. *fetus* strain used to produce the Campylovexin® vaccine (strain 5915) was typed using PFGE in the same way as the abortion isolates from this study.

3.3 Results

3.3.1 *Campylobacter* isolations

Campylobacter isolates were cultured from abortion samples from 28 out of the 50 Hawke's Bay farms. Three or more foetuses were submitted from 17 out of these 28 farms (60%). In total, 85 *Campylobacter* isolates were cultured, each isolate being cultured from a different aborted foetus. Of these, 81 were *C. fetus* subsp. *fetus* from 25 farms and four isolates were *C. jejuni* from the other three farms.

The results relating to the four *C. jejuni* isolates from three affected properties are presented in Chapter 5: Pulsed-field gel electrophoresis of *Campylobacter jejuni* sheep abortion isolates.

3.3.2 Pulsed-field gel electrophoresis analysis of *C. fetus* subsp. *fetus* abortion isolates

Ten distinct PFGE profiles were identified amongst the 81 *C. fetus* subsp. *fetus* isolates (Figure 3.1). The PFGE profiles consisted of 13-16 DNA bands, which ranged in size between 31.2 and 417.7 kb. Seven of these bands were invariable between all the PFGE profiles and the profiles were all at least 60% similar when a similarity matrix was calculated using the Dice coefficient (Appendix 1). A dendrogram showing similarity of the PFGE profiles of the isolates was produced using cluster analysis (Figure 3.2). The PFGE profiles ≥84% similar by cluster analysis were considered to belong to the same PFGE group, as these profiles differed by the position of only one or two bands. Conversely, the PFGE profiles less than 84% similar by cluster analysis were considered to belong to different PFGE groups. The profiles formed six PFGE groups, which were named A, B, C, D, E, and F.

Distinct PFGE profiles within the same PFGE group were further organised into types. Four of the PFGE groups (B, D, E, and F) each contained two PFGE types, which were named B1 and B2, D1 and D2, E1 and E2, and F1 and F2, respectively (Figure 3.1). There were at least 12 invariable bands between the two PFGE types within each group. The similarity between the PFGE profiles of B1 and B2, D1 and D2, and E1 and E2 was each 93%, and that of F1 and F2 was 89% (Figure 3.2). Isolates of the same PFGE type

had indistinguishable PFGE profiles, for example the PFGE profiles of all the isolates of PFGE type B1 were indistinguishable.

Pulsed-field gel electrophoresis using the restriction enzyme *Sal*I confirmed the strain typing of the isolates as above, except that PFGE types B1 and B2 were not differentiated by *Sal*I. No additional differentiation of types was generated by the use of *Sal*I.

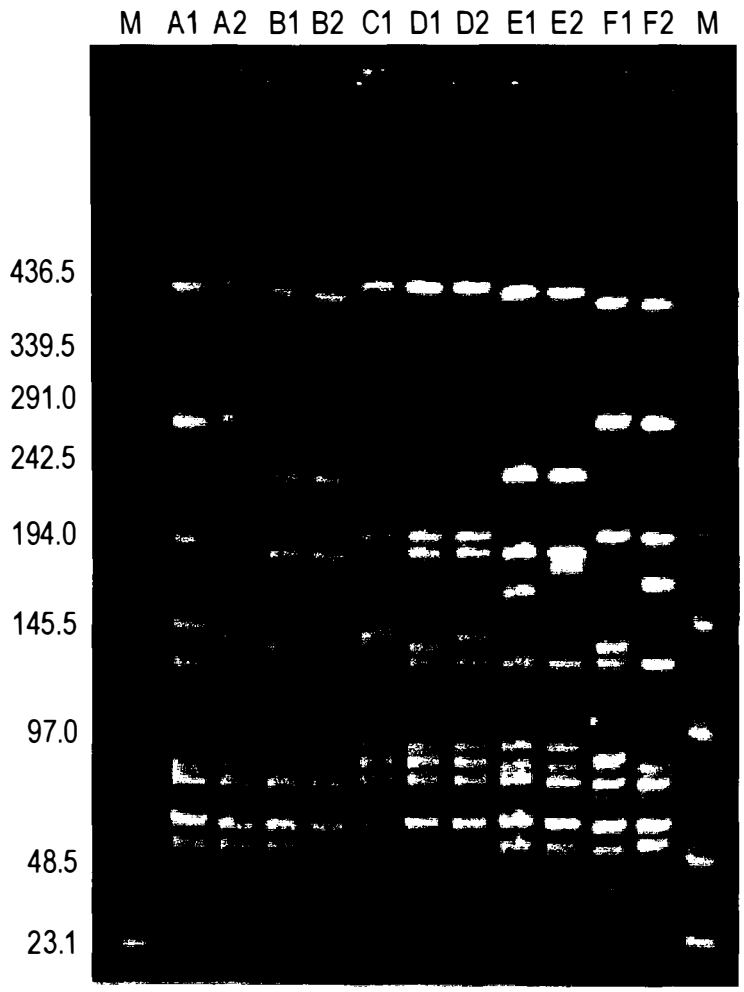


Figure 3.1 Pulsed-field gel electrophoresis (PFGE) using the restriction enzyme *Sma*I of the ten PFGE types isolated from sheep abortions in Hawke's Bay in 1999, and the Campylovexin vaccine strain (A1). Lanes of *C. fetus* subsp. *fetus* are labelled with the assigned name of the PFGE type. M = molecular size marker (lambda ladder), DNA standard bands are labelled with sizes (kb).

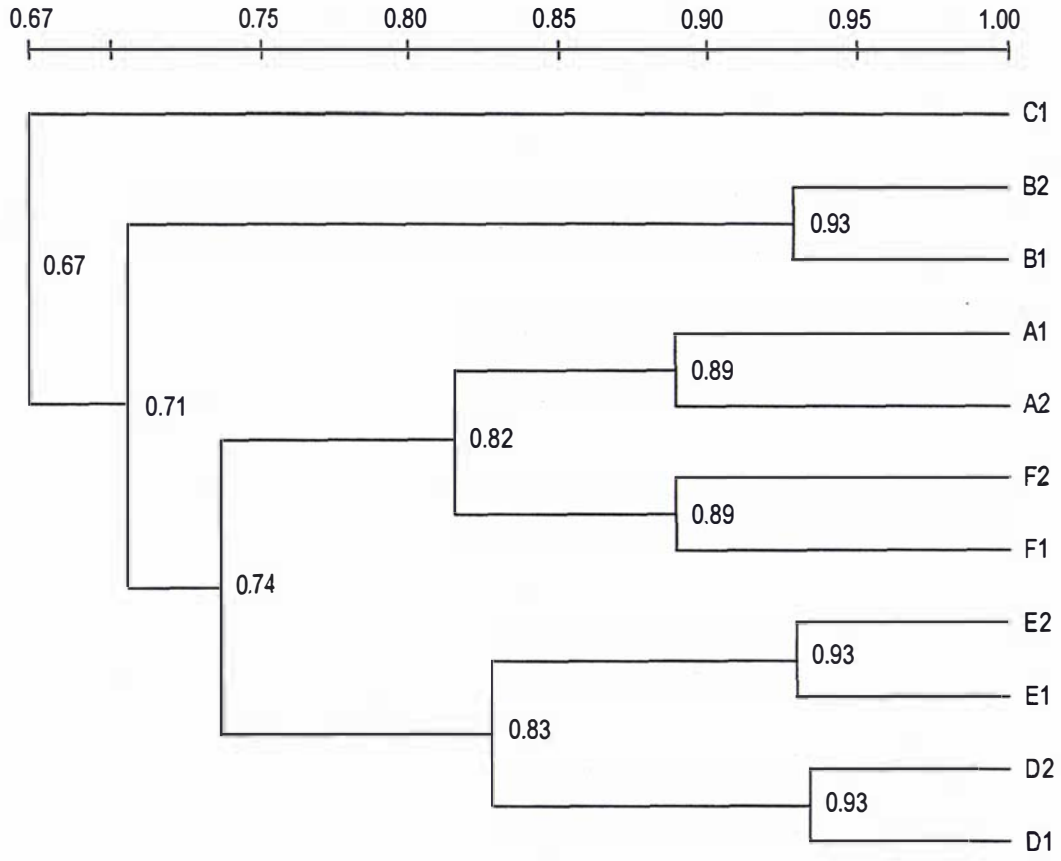


Figure 3.2 Similarity of the PFGE profiles of the *C. fetus* subsp. *fetus* isolates from the Hawke's Bay in 1999 and the Campylovexin® strain (PFGE type A1). This dendrogram was produced using the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis, with PFGE type B1 as the reference. Clusters of PFGE types that were ≥84% similar were classified as belonging to the same PFGE group.

3.3.3 *Campylobacter fetus* subsp. *fetus* vaccine strain

The *C. fetus* subsp. *fetus* Campylovexin® vaccine strain, although not isolated from aborted animals in this study, was most similar to PFGE type A2 at 89% and was named PFGE type A1 (Figures 3.1 and 3.2).

3.3.4 Incidence of PFGE types

The majority of the *C. fetus* subsp. *fetus* isolates (62 out of 81, or 77%) were PFGE type B1 (Table 3.1, Appendix 2). Isolates of this PFGE type were cultured from aborted foetuses from 19 of the 25 farms from which *C. fetus* subsp. *fetus* was isolated. In contrast, types A2, C1, D2, E1, E2, F1 and F2 were each recovered from only one farm. The other types, B2 and D1, were each cultured from foetuses from two farms.

Table 3.1 Number of *C. fetus* subsp. *fetus* isolates of each PFGE type and number of farms from which each of the PFGE types was isolated.

PFGE type	No. isolates	No. farms
A2	1	1
B1	62	19
B2	4	2
C1	1	1
D1	2	2
D2	1	1
E1	1	1
E2	2	1
F1	2	1
F2	5	1
Total:	**81**	**25[a]**

[a]Five farms yielded two different PFGE types each.

On five farms, two distinct *C. fetus* subsp. *fetus* PFGE types were isolated from aborted foetuses from the same flock. The types found on the five farms were B1 and B2; B1 and D1; B1 and F1; D1 and D2; and E1 and E2. Types E1 (one isolate) and E2 (two isolates) were found on the same farm and not on any other property. Within each flock, all of the aborted samples from which these strains were isolated were submitted to the diagnostic laboratories at the same time, with the exception of the flock from which

PFGE types B1 and B2 were isolated. In this flock, the samples from which B2 was isolated were submitted ten days prior to submission of the samples from which B1 was isolated. In contrast, the isolates cultured from another farm from which samples were submitted at different times during the same outbreak were all found to be the same PFGE type.

3.3.5 Vaccination status of affected flocks

Campylobacter fetus subsp. *fetus* was only cultured from flocks that had not been vaccinated with Campylovexin® that season. Two flocks were apparently vaccinated with Campylovexin® more than one year prior to the occurrence of abortions. The PFGE type of isolates from those two flocks was A2 and B1, respectively.

3.4 Discussion

Genotypic analysis of 81 *C. fetus* subsp. *fetus* isolates from sheep abortions on 25 farms in the Hawke's Bay region revealed ten distinct PFGE types. These types were organised by similarity into six different PFGE groups. This compares with the findings of a previous study by de Lisle et al. (1987), who found seven restriction endonuclease analysis (REA) types in a study of 70 isolates from sheep abortions. These studies confirm the genetic diversity of *C. fetus* subsp. *fetus* isolated from abortions in sheep.

In the present study, PFGE type B1 predominated; this type was found on 19 out of 25 farms (76%) from the Hawke's Bay region. It is interesting to note that in the study involving 70 isolates from throughout New Zealand, de Lisle et al. (1987) also found that one type, REA type b, was predominant (38 of 67 farms, 57%). The relationship between this prevalent REA type in 1987 and the prevalent PFGE type found in the present study is investigated in the PFGE analysis of isolates from throughout New Zealand (Chapter 4).

An unexpected finding of the present study was the isolation in five cases of different *C. fetus* subsp. *fetus* PFGE types from aborted foetuses from within the same flock. Campylobacteriosis is an infectious disease and it is commonly assumed that a *Campylobacter* abortion outbreak within a flock is caused by transmission of the same strain between animals. While in one of these flocks there was an interval of ten days between submission of the aborted foetuses from which the different strain types were isolated (B1 and B2), in the other four flocks the isolates were cultured from foetuses submitted on the same day. The isolates from each of these farms fit within the criteria of Tenover et al. (1995) of ≤30 epidemiologically related isolates collected over 1-3 months and for which at least ten DNA bands in the PFGE profile were obtained. Tenover et al. (1995) defined a PFGE band difference as the absence of a band present in the profile of the (other) strain or the presence of a band that is absent in the (other) strain profile. Using this definition, the PFGE profiles of isolates found on three out of the five farms differed by two bands (B1 and B2, D1 and D2, and E1 and E2). According to the criteria of Tenover et al. (1995), these PFGE types differ by one genetic event, can be considered to be closely related, and are probably part of the same

outbreak. These three cases may illustrate the reason for concerns raised about stability of the genome in the epidemiological application of PFGE typing of isolates (Wassenaar et al. 1998; Wassenaar and Newell 2000), and demonstrates that interpretation of typing results is important. In contrast, the PFGE profiles of isolates from two of the farms where samples were submitted at the same time differed by six bands (B1 and F1) and seven bands (B1 and D1) respectively. Six band differences may result from two independent genetic events, thus the isolates are possibly related and possibly part of the same outbreak (Tenover et al. 1995). However, seven band differences between PFGE profiles indicates that there would have to have been three independent genetic events and the isolates are considered to be different and not part of the same outbreak (Tenover et al. 1995). Therefore PFGE typing results from at least one out of these five farms suggest that concurrent abortions in flocks may be associated with more than one *C. fetus* subsp. *fetus* strain.

Campylovexin® is a single-strain vaccine produced from a *C. fetus* subsp. *fetus* isolate which was originally cultured from a sheep abortion in mid-Canterbury, New Zealand in 1978 (Gumbrell 1985b). Although the PFGE type of the vaccine strain, A1, was not found within the 81 *C. fetus* subsp. *fetus* abortion isolates in this Hawke's Bay study, a similar strain type, A2, was found. The relationship between genotypes and serotypes of *C. fetus* subsp. *fetus* has been shown to be complex (de Lisle et al. 1987), therefore it can not be assumed that the diversity of PFGE types necessarily implies lack of vaccine protection in the field. In this study, none of the flocks affected by *C. fetus* subsp. *fetus* abortion had been vaccinated that season. Aspects of Campylovexin® protection are investigated in Chapters 6-8 using the PFGE typing results of *C. fetus* subsp. *fetus* from this study and from the national study (Chapter 4).

3.5 Conclusions

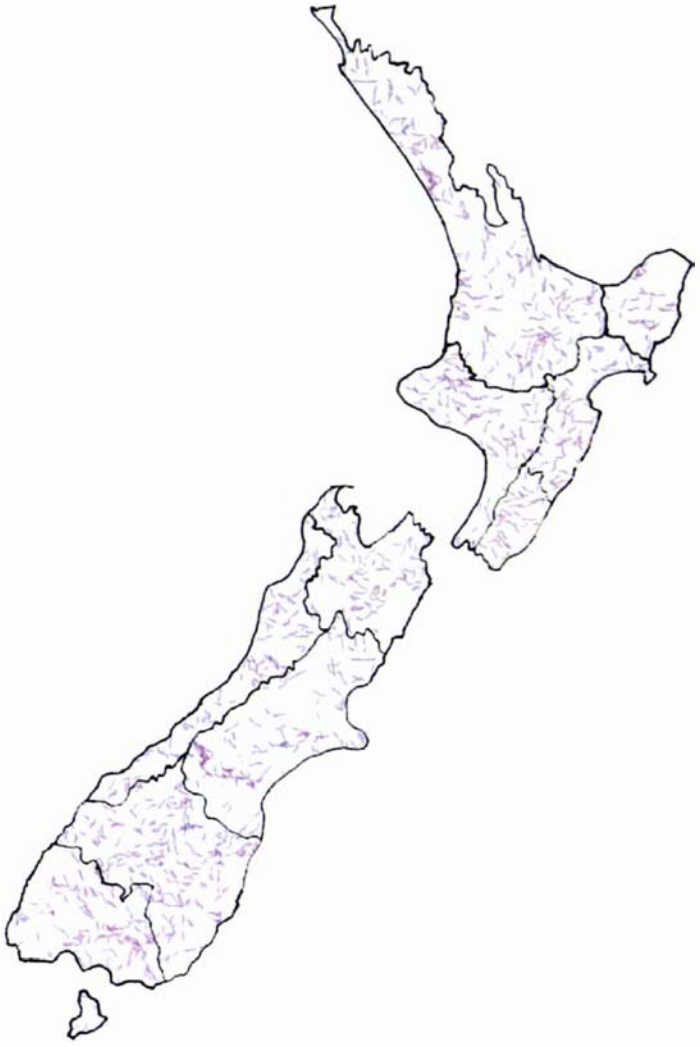
- Ten PFGE types were identified amongst the *C. fetus* subsp. *fetus* sheep abortion isolates from the Hawke's Bay, and one type, B1, was found most frequently.

- Concurrent abortions in flocks may be associated with more than one *C. fetus* subsp. *fetus* strain.

- *Campylobacter fetus* subsp. *fetus* was only isolated from samples from sheep that had not been vaccinated with Campylovexin® that season.

Chapter 4

Typing of *Campylobacter fetus* subsp. *fetus* sheep abortion isolates using pulsed-field gel electrophoresis: a New Zealand study



Aspects of this work are published in:

Mannering S, Fenwick S, Marchant R, Perkins N and West D (2001) *Campylobacter fetus* subsp. *fetus* abortions in sheep: PFGE strain typing of isolates from 1999 and 2000. *Proceedings of the Sheep and Beef Cattle Society of the New Zealand Veterinary Association* 31: 25-30.

4.1 Introduction

Pulsed-field gel electrophoresis typing of *C. fetus* subsp. *fetus* isolates from sheep abortions in the Hawke's Bay region in 1999 showed that there was a variety of types, with the predominance of a single type, B1 (Chapter 3). This investigation of the strain variation of *C. fetus* subsp. *fetus* implicated in sheep abortion was expanded in 2000 to include isolates from throughout New Zealand.

In addition, seven freeze-dried *C. fetus* subsp. *fetus* isolates from the New Zealand Reference Culture Collection representing restriction types a-g found amongst New Zealand sheep abortion isolates in a 1987 study (de Lisle et al. 1987), were typed using PFGE. This chapter describes the results of PFGE typing of *C. fetus* subsp. *fetus* isolates from sheep abortions throughout New Zealand in 2000, and the comparison of these results with those of the earlier studies.

4.2 Materials and Methods

4.2.1 Collection of *Campylobacter* isolates from sheep abortions in 2000

Campylobacter isolates cultured from sheep abortions by the principal veterinary diagnostic laboratories in New Zealand during the 2000 season were collected. The laboratories were:

- LabNet Invermay Ltd., Mosgiel
- LabWorks Animal Health, Lincoln
- Gribbles Veterinary Pathology Animal Health Laboratory, Palmerston North
- Alpha-Scientific Ltd., Hamilton

The approximate geographical location of the farms from which isolates were cultured was supplied by the laboratories. Using this information, the farms were categorised as belonging to the following districts of New Zealand: Waikato, Gisborne, Hawke's Bay, Manawatu, Wairarapa, Marlborough, Canterbury, Otago and Southland (Figure 4.1).

Campylobacter was cultured according to the methods of the individual laboratories. In general, foetal stomach contents were examined microscopically for the presence of *Campylobacter*-like organisms, and *Campylobacter* spp. were cultured on selective media (Skirrows media from Fort Dodge Animal Health or Fort Richard Laboratories Ltd.) under microaerobic conditions (CampyGen sachets, Oxoid Ltd. or BBL® CampyPak Plus, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). Isolates were identified to species level by the laboratories using standard microbiological methods.

Subculturing of the isolates was kept to a minimum and a single colony on selective media was sub-cultured onto a Blood Agar plate (Fort Dodge Animal Health or Fort Richard Laboratories Ltd.) for purity. In the case of Gribbles Veterinary Pathology Animal Health Laboratory (Palmerston North), the selective media and Blood Agar plates were brought to Massey University from the laboratory. A maximum of two sub-cultures from the original growth was performed on Blood Agar before growth from a pure culture was harvested and stored in 15% glycerol at –70°C. In the case of the other

diagnostic laboratories, growth from the purity plate was harvested onto Amies medium (Cultureswab™ Transport Systems, Difco or Copan Diagnostics Inc., Corona, CA, USA) and couriered to Massey University, where fresh Blood Agar plates were inoculated from the swabs, and the growth harvested and stored in 15% glycerol at –70°C.

Figure 4.1 Map of New Zealand showing the districts the farms of the 2000 national study were categorised as belonging to. No *Campylobacter* isolates were obtained from the West Coast.

4.2.2 *Campylobacter fetus* subsp. *fetus* sheep abortion isolates from the New Zealand Reference Culture Collection

Several *C. fetus* subsp. *fetus* isolates were acquired from the New Zealand Reference Culture Collection, Medical Section (NZRM). The *C. fetus* subsp. *fetus* Type strain (NZRM 2398; National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale, London, England 10842) was originally isolated from the brain of an aborted sheep foetus in France in 1952 (Veron and Chatelain 1973).

In addition, seven strains (NZRM 2939-2945) were lodged with the culture collection as representatives of the restriction types a-g, after a New Zealand study in which 70 *C. fetus* subsp. *fetus* sheep abortion isolates from 67 farms were examined using restriction endonuclease analysis (REA) (de Lisle et al. 1987).

- *Campylobacter fetus* subsp. *fetus* Type strain NZRM 2398
- REA type a NZRM 2939
- REA type b NZRM 2940
- REA type c NZRM 2941
- REA type d NZRM 2942
- REA type e NZRM 2943
- REA type f NZRM 2944
- REA type g NZRM 2945

The above freeze-dried *C. fetus* subsp. *fetus* isolates from the NZRM were revived according to accompanying instructions and growth on Blood Agar was harvested and stored in 15% glycerol at –70°C.

4.2.3 Pulsed-field gel electrophoresis of *C. fetus* subsp. *fetus* isolates

Preparation and digestion of genomic DNA and PFGE of *C. fetus* subsp. *fetus* isolates was performed according to the method detailed in Chapter 2.

4.2.4 Analysis of PFGE profiles

The PFGE profiles of the isolates were analysed using BioRad Diversity Database software according to the method detailed in Chapter 2.

4.3 Results

4.3.1 *Campylobacter* sheep abortion isolates

In total, 320 *Campylobacter* isolates were cultured from aborted sheep foetuses from 221 farms. The majority of these were *C. fetus* subsp. *fetus*: 289 isolates from 198 farms. Twenty-three *C. jejuni* isolates were cultured from 20 farms and one *C. coli* isolate was cultured from one farm. From a further two farms, both *C. fetus* subsp. *fetus* and *C. jejuni* isolates were cultured from separate foetuses, which added a further four *C. fetus* subsp. *fetus* and three *C. jejuni* isolates to the collection. This resulted in a total of 293 *C. fetus* subsp. *fetus* isolates from 200 farms, 26 *C. jejuni* isolates from 22 farms and one *C. coli* isolate from one farm for analysis.

The results relating to the *C. jejuni* and *C. coli* isolates are presented in Chapter 5: Pulsed-field gel electrophoresis of *Campylobacter jejuni* sheep abortion isolates.

4.3.2 Pulsed-field gel electrophoresis typing of *C. fetus* subsp. *fetus* sheep abortion isolates from 2000

4.3.2.1 Identification and similarity of PFGE types

Twenty-two distinct PFGE profiles were identified amongst the *C. fetus* subsp. *fetus* isolates from 2000 (Figure 4.2). Six of these profiles were indistinguishable from those found in the Hawke's Bay in 1999: A2, B1, B2, C1, D1, and F2 (Chapter 3). The PFGE profiles consisted of 13-16 DNA bands, which ranged in size between 31.2 and 417.7 kb. Six out of the 13-16 DNA bands were invariable between all the *C. fetus* subsp. *fetus* isolates typed to date. This observation was reflected in the similarity matrix between the PFGE profiles, calculated using the Dice coefficient, where the *C. fetus* subsp. *fetus* PFGE types identified to date were at least 50% similar to each other (Appendix 3). The PFGE types that were ≥84% similar by cluster analysis were considered to belong to the same PFGE group, and there were at least 11 invariable bands in the PFGE profiles within each group. Cluster analysis using the limit of 84% similar resulted in 11 PFGE groups (A-K) (Figure 4.3).

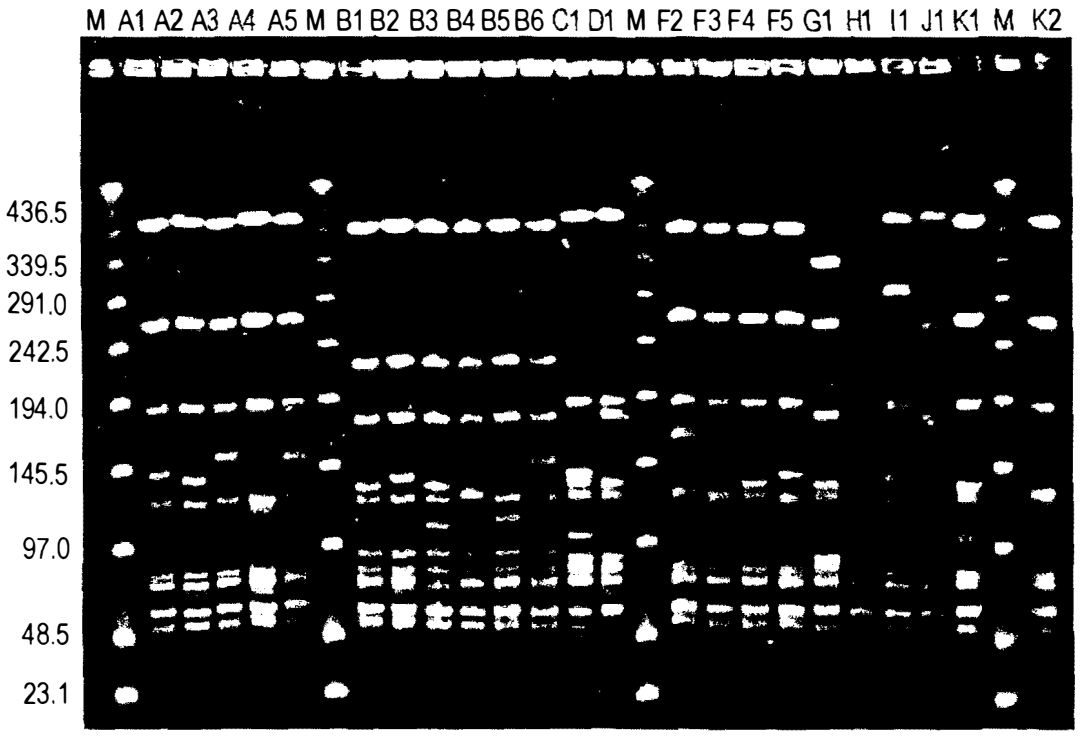


Figure 4.2 Pulsed-field gel electrophoresis using the restriction enzyme *Sma*I of the 22 PFGE types found amongst the 293 *C. fetus* subsp. *fetus* isolates in 2000, and the *C. fetus* subsp. *fetus* Type strain, K2. M = molecular size marker (lambda ladder), DNA standard bands are labelled with sizes (kb).

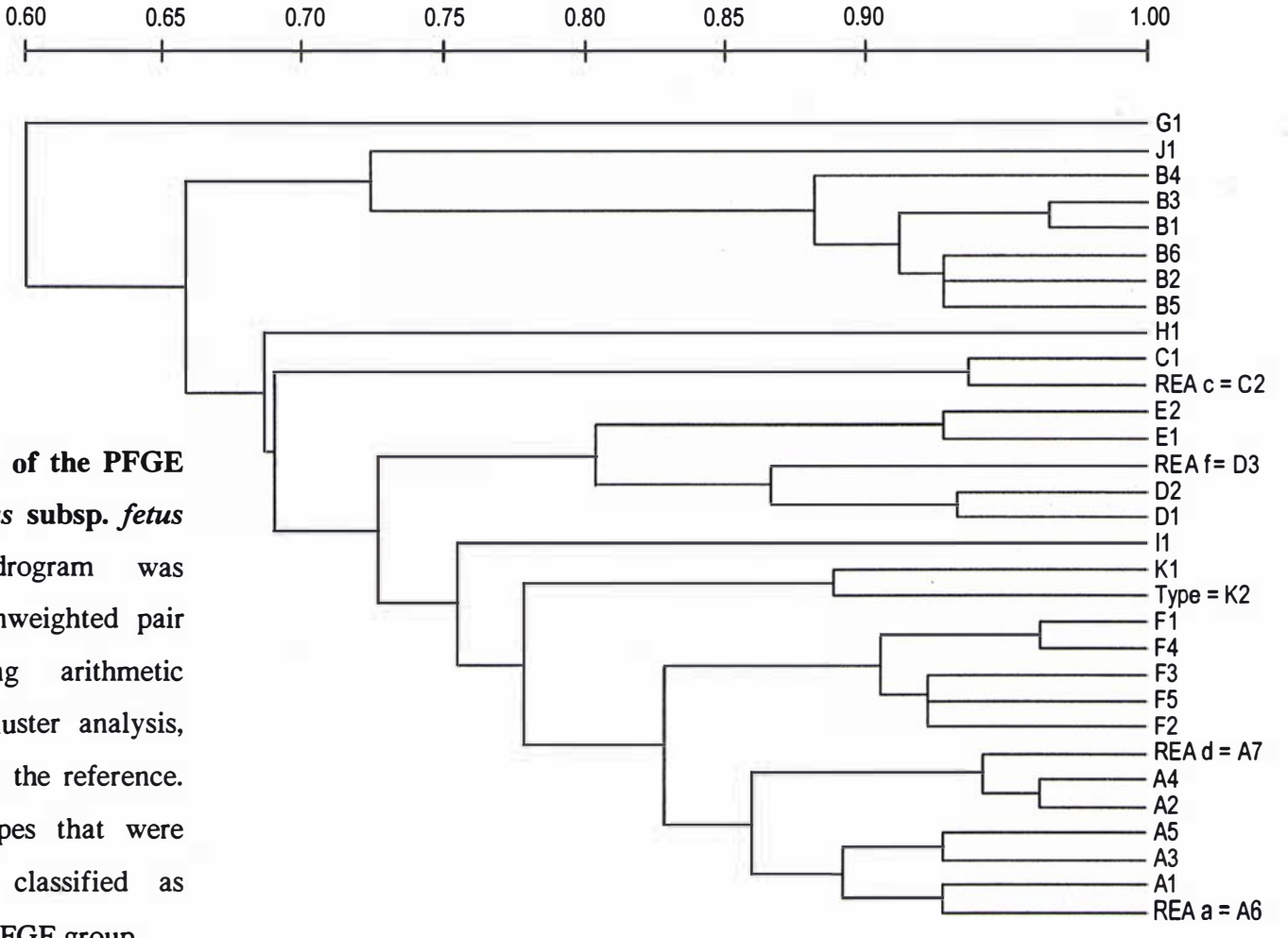


Figure 4.3 Similarity of the PFGE profiles of the *C. fetus* subsp. *fetus* isolates. This dendrogram was produced using the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis, with PFGE type B1 as the reference. Clusters of PFGE types that were ≥84% similar were classified as belonging to the same PFGE group.

4.3.2.2 Frequency of PFGE types

The majority of the *C. fetus* subsp. *fetus* isolates from 2000 belonged to PFGE groups A and B (Table 4.1, Appendix 4). Several PFGE groups were represented by a single isolate from a single farm: G1, H1, I1, and J1. The most common PFGE type was B1, at 69% of the *C. fetus* subsp. *fetus* isolates, and was cultured from 66% of the farms. Seven percent of the *C. fetus* subsp. *fetus* isolates were type B2, 6% were A1, 5% were D1, 3% were A2, and 3% were F4.

Table 4.1 Number of *C. fetus* subsp. *fetus* isolates of each PFGE type and number of farms from which each of the PFGE types was isolated in 2000.

PFGE type	No. isolates		No. farms	
A1	17	(6%)	16	(8%)
A2	8	(3%)	7	(4%)
A3	1		1	
A4	1		1	
A5	1		1	
B1	201	(69%)	132	(66%)
B2	21	(7%)	17	(9%)
B3	2	(1%)	1	
B4	2	(1%)	1	
B5	1		1	
B6	2	(1%)	2	(1%)
C1	2	(1%)	1	
D1	14	(5%)	11	(6%)
F2	3	(1%)	3	(2%)
F3	1		1	
F4	10	(3%)	5	(3%)
F5	1		1	
G1	1		1	
H1	1		1	
I1	1		1	
J1	1		1	
K1	1		1	
Total	**293**		**200**[a]	

[a] Total includes seven farms with more than one PFGE type.

4.3.2.3 Regional distribution of the PFGE types

The *C. fetus* subsp. *fetus* isolates came from 200 farms from eight main regions of New Zealand: Waikato, Hawke's Bay, Manawatu, Wairarapa, Marlborough, Canterbury, Otago and Southland (Table 4.2). Isolates from two Gisborne farms were grouped with those from Waikato. Most of the farms from which *C. fetus* subsp. *fetus* was isolated from sheep abortions were in the regions Hawke's Bay, Manawatu, Canterbury, Otago and Southland.

Pulsed-field type B1 was found on the majority of the farms in each region (Table 4.2). Type D1 was found in seven out of the eight regions, and B2 in six of the regions. The PFGE type of the Campylovexin® vaccine strain, A1, was found on a total of 16 farms located in Canterbury, Otago and Southland. Fourteen out of the 22 PFGE types were found on only one farm in 2000: A3-A5, B3-B5, C1, F3, F5, G1, H1, I1, J1 and K1. The proportion of farms in each region from which the various PFGE types were isolated is illustrated in Figure 4.4.

4.3.2.4 Farms with more than one *C. fetus* subsp. *fetus* PFGE type

On seven farms, two distinct PFGE types of *C. fetus* subsp. *fetus* were isolated from different foetuses. On six of these farms PFGE type B1 was found: three farms had one isolate each of B1 and B2; one farm had one isolate each of B1 and B5; one farm had one isolate each of B1 and F3; and one farm had four B1 and one F4 isolates. The remaining farm had one isolate each of PFGE types A1 and A5.

4.3.2.5 Comparison of Hawke's Bay PFGE typing data between 1999 and 2000

Five PFGE types were found in Hawke's Bay in both 1999 (Chapter 3) and 2000: B1, B2, C1, D1, and F2 (Table 4.3). Five PFGE types (A2, D2, E1, E2 and F1) were found in 1999, but not in 2000 in Hawke's Bay. Three PFGE types (B4, F4, and J1) were found in Hawke's Bay in 2000, but not in 1999. In both years, PFGE type B1 predominated.

Table 4.2 Number of farms in each region from which the various PFGE types of *Campylobacter fetus* subsp. *fetus* were isolated in 2000. The total number of farms from which *C. fetus* subsp. *fetus* was isolated in 2000 was 200.

PFGE type	Waikato[a]	Hawke's Bay	Manawatu	Wairarapa	Marlborough	Canterbury	Otago	Southland
A1						2	7	7
A2				1	2	3	1	
A3								1
A4								1
A5							1	
B1	11	21	24	4	7	21	17	27
B2		4	1	1	1	4		6
B3			1					
B4		1						
B5	1							
B6				1		1		
C1		1						
D1		2	1	1	1	3	2	1
F2		2				1		
F3						1		
F4		1		2		2		
F5							1	
G1								1
H1								1
I1						1		
J1		1						
K1						1		
	11[b]	**32[b]**	**26[b]**	**10**	**11**	**37[b]**	**28[b]**	**45**

[a] Waikato includes two farms from Gisborne.

[b] Total includes farm(s) in the region from which more than one PFGE type of *C. fetus* subsp. *fetus* was isolated.

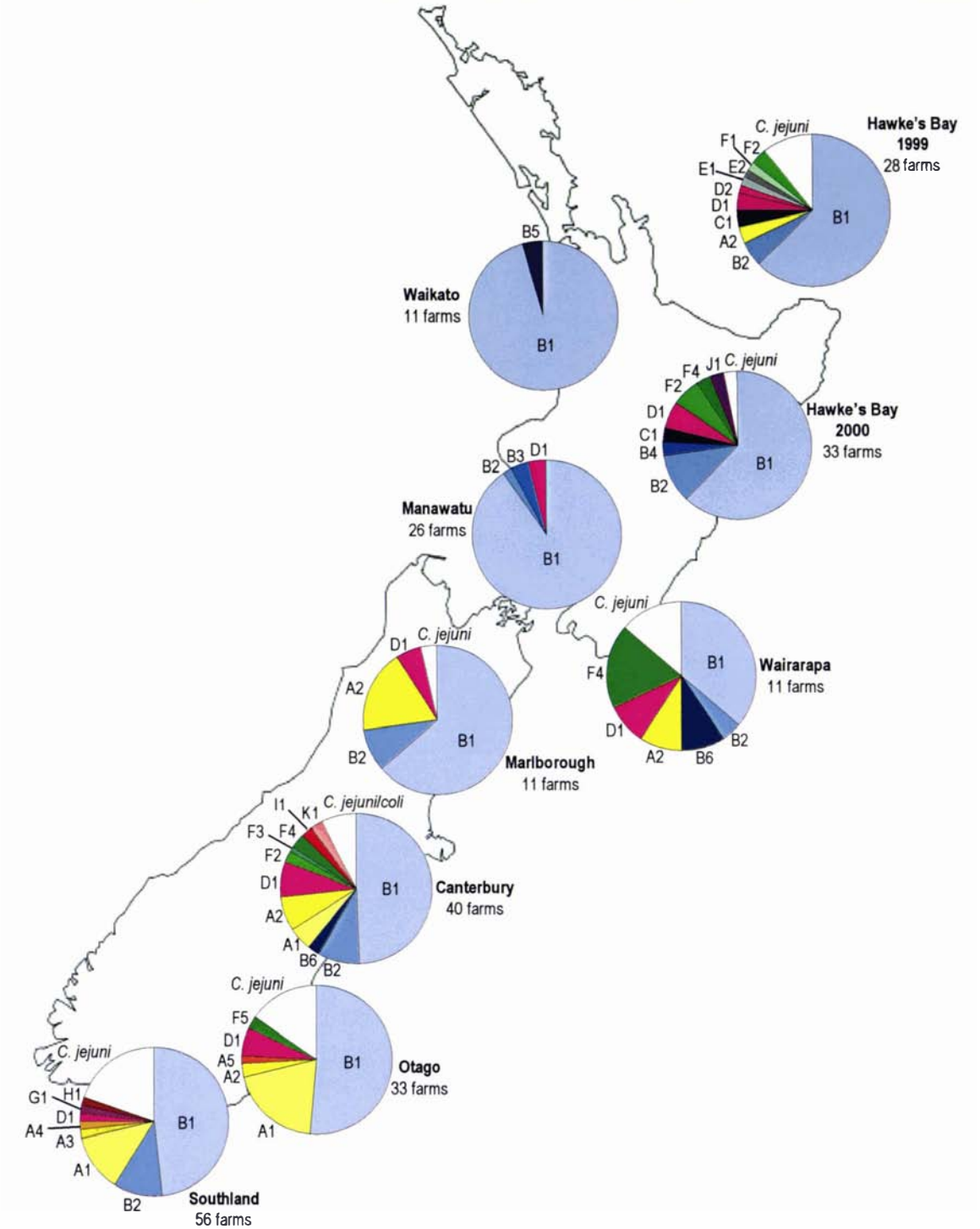


Figure 4.4 Pie charts showing the proportion of farms in each region from which the *Campylobacter fetus* subsp. *fetus* PFGE types were isolated, and from which *C. jejuni* or *C. coli* was isolated.

Table 4.3 Comparison of number of farms in the Hawke's Bay in 1999 and 2000 from which each *C. fetus* subsp. *fetus* PFGE type was found.

	No. Hawke's Bay farms	
PFGE type	**1999**	**2000**
A2	1	-
B1	19	21
B2	2	4
B4	-	1
C1	1	1
D1	2	2
D2	1	-
E1	1	-
E2	1	-
F1	1	-
F2	1	2
F4	-	1
J1	-	1
Total	**25**[a]	**32**[b]

[a] Five farms yielded two different PFGE types each.

[b] One farm yielded two different PFGE types.

4.3.3 Pulsed-field gel electrophoresis typing of REA types a-g

Each of the seven *C. fetus* subsp. *fetus* New Zealand Reference Culture Collection REA types a-g had a different PFGE profile (Figure 4.5). Three REA types had PFGE profiles indistinguishable from profiles already found in the current study; REA b was indistinguishable from PFGE profile B1, REA e was indistinguishable from F4, and REA g was indistinguishable from D1. The other four REA types had PFGE profiles that were unique, but similar to those already found in the current study. The profile of REA a was 93% similar to that of A1 and A5, and was classified as A6 (Figure 4.3, Appendix 3). The profile of REA d was 96% similar to that of A4, and was classified as A7. The profile of REA c was 94% similar to that of C1, and was classified as C2. The profile of REA f was 87% similar to that of D1 and D2, and was classified as D3.

The most common type found in 1987 was REA b (PFGE type B1), which was identified from 38 out of the 67 farms (57%, Table 4.4). The next most frequently found type in 1987 was REA a (PFGE type A6), which was found on 15 farms (22%).

4.3.4 PFGE typing of the *C. fetus* subsp. *fetus* Type strain

The PFGE profile of the *C. fetus* subsp. *fetus* Type strain was 89% similar to PFGE type K1, and was classified as K2 (Figures 4.2 and 4.3, Appendix 3).

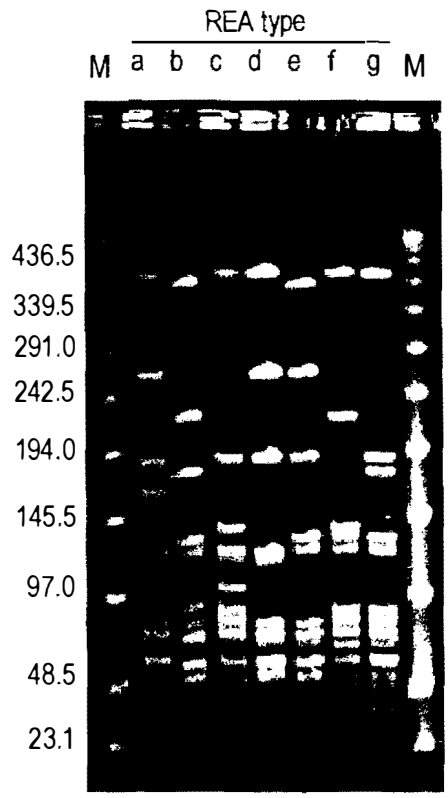


Figure 4.5 PFGE using the restriction enzyme *Sma*I of the *Campylobacter fetus* subsp. *fetus* REA types a-g from the New Zealand Reference Culture Collection. M = molecular size marker (lambda ladder), DNA standard bands are labelled with sizes (kb).

Table 4.4 Pulsed-field gel electrophoresis typing results of the REA types a-g from the New Zealand Reference Culture Collection and the number of farms in 1987 and 2000 from which the types were isolated.

PFGE type	2000 No. farms		1987 No. farms[a]		REA type
A1	16	(8%)	-		
A2	7	(4%)	-		
A3	1		-		
A4	1		-		
A5	1		-		
A6	-		15	(22%)	a
A7	-		4	(6%)	d
Subtotal:	**26**	**(12%)**	**19**	**(28%)**	
B1	132	(66%)	38	(57%)	b
B2	17	(9%)	-		
B3	1		-		
B4	1		-		
B5	1		-		
B6	2	(1%)	-		
Subtotal:	**154**	**(76%)**	**38**	**(57%)**	
C1	1		-		
C2	-		2	(3%)	c
Subtotal:	**1**		**2**	**(3%)**	
D1	11	(6%)	2	(3%)	g
D3	-		1	(1%)	f
Subtotal:	**11**	**(6%)**	**3**	**(4%)**	
F2	3	(2%)	-		
F3	1		-		
F4	5	(3%)	5	(7%)	e
F5	1		-		
Subtotal:	**10**	**(5%)**	**5**	**(7%)**	
G1	1		-		
H1	1		-		
I1	1		-		
J1	1		-		
K1	1		-		
Total:	**200** [b]		**67**		

[a] Figures obtained from de Lisle et al. (1987).

[b] Total includes seven farms with more than one PFGE type.

4.4 Discussion

This study is a genotypic analysis of the most comprehensive collection of *C. fetus* subsp. *fetus* isolates cultured from sheep abortions to date. There was a variety of PFGE types identified, and a single type was found most frequently throughout New Zealand. Pulsed-field type B1 was found on 66% of the farms from which *C. fetus* subsp. *fetus* was cultured in 2000.

It appears that PFGE type B1 has been prevalent for some time, as the most common REA type found in 1987 (REA type b) was found on 57% of farms (de Lisle et al. 1987) and was identified here as PFGE type B1. More recently, in 1999 PFGE type B1 was the most common type in the Hawke's Bay region, found on 76% of farms (Chapter 3).

Seven REA types were identified amongst 70 *C. fetus* subsp. *fetus* sheep abortion isolates from 67 New Zealand farms in 1987 (de Lisle et al. 1987). When representative isolates of these REA types were subjected to PFGE, three types from 1987 were indistinguishable from those found in 2000 and the other four types were very similar to those found in 2000. This suggests the *C. fetus* subsp. *fetus* strains isolated from sheep abortions in New Zealand have remained similar between 1987 and 2000.

As PFGE profiles are simpler and easier to interpret than REA profiles (Arbeit 1995; Newell et al. 2000), it is possible that if PFGE had been performed on all 70 *C. fetus* subsp. *fetus* isolates from 1987, the seven REA types could have been further differentiated. This is illustrated by the classification of the strain used in the manufacture of the New Zealand Campylovexin® vaccine as REA type a (Collins and de Lisle 1985). Pulsed-field gel electrophoresis analysis distinguishes between the isolate lodged in the New Zealand Reference Culture Collection as REA type a (PFGE type A6) and the Campylovexin® vaccine strain (PFGE type A1, Chapter 3). However, REA analysis did differentiate REA type a from type d (PFGE types A6 and A7, respectively), and REA type g from type f (PFGE types D1 and D3, respectively). Therefore, although it is possible that the isolates identified in 1987 as REA type b may not all be PFGE type B1, it is likely that they are at least very similar to PFGE type B1. For this reason, perhaps it is more meaningful to compare the relative frequency of the PFGE *groups* between 1987 and 2000.

In 1987 and 2000, the majority of the isolates belonged to PFGE groups A and B. In 1987, PFGE group A isolates were found on 28% of the farms, but this had apparently dropped to 12% in 2000. Conversely, PFGE group B isolates were found on 57% of farms in 1987, but on 76% of farms in 2000. This indicates the relative prevalence of the most common PFGE groups may have altered slightly over 13 years, yet there has been no major genotypic shift in the population of *C. fetus* subsp. *fetus* implicated in sheep abortion in New Zealand.

Similarly, it is interesting that an isolate was cultured from a farm in Canterbury with a PFGE profile that differed from that of the *C. fetus* subsp. *fetus* Type strain by the position of just three bands (88.9% similar using the Dice coefficient, Appendix 3). The *C. fetus* subsp. *fetus* Type strain (PFGE type K2) was isolated from an aborted sheep foetus in France nearly 50 years prior to the culture of the Canterbury isolate (PFGE type K1). Similarly, On and Harrington (2001) found that a *C. fetus* subsp. *fetus* isolate of unknown origin from Canada was indistinguishable by PFGE to the *C. fetus* subsp. *fetus* Type strain (On and Harrington 2001). That study involved the PFGE analysis of 11 *C. fetus* subsp. *fetus* isolates cultured from humans and aborted sheep and cattle foetuses from countries such as Scotland, Ireland, France, Sweden, Canada and Australia (On and Harrington 2001). Unfortunately, the level of similarity of the PFGE profiles between the other isolates can not be directly compared with that found in the current study, as the respective dendrograms were produced using different cluster analysis and reference strains. The dendrogram of On and Harrington (2001) was produced using the Dice coefficient and Ward's cluster analysis, and showed that the PFGE profiles of all 11 *C. fetus* subsp. *fetus* isolates clustered at 85% similar. In the current study, the dendrogram was produced using the Dice coefficient and UPGMA cluster analysis, and showed that the PFGE profiles of all the *C. fetus* subsp. *fetus* isolates identified so far clustered at 60% similar. As dendrograms are produced using cluster analysis of the similarity of PFGE profiles, there is a discrepancy between the level of similarity between the two least similar PFGE profiles calculated using the Dice coefficient (50% in this study, Appendix 3) and the level of similarity of the *clusters* of PFGE profiles (60% in this study, Figure 4.3). Nevertheless, in the study of On and Harrington (2001) the isolates from Australia and the United Kingdom were clustered together by similarity and the isolates from France, Sweden and Canada were clustered together. On and Harrington (2001) concluded that the isolates from Australia and the

United Kingdom were "somewhat distinct" from the isolates from other European countries and Canada, and suggested that some strains may represent geographically distinct clonal lines (On and Harrington 2001). This is an interesting idea, which would require the typing of many more isolates to explore further. Using this idea, the similarity of the isolate from Canterbury with the Type strain from France groups New Zealand with France, Sweden and Canada and not with the perhaps more likely group of Australia and United Kingdom. Alternatively, it is possible that as a population *C. fetus* subsp. *fetus* is relatively genetically homogenous, regardless of location, and the apparent clustering according to geographical location observed by On and Harrington (2001) is an artefact of the small number of isolates typed in that study. Certainly, in this study in New Zealand *C. fetus* subsp. *fetus* sheep abortion isolates appeared to be less genetically diverse than *C. jejuni* isolates from sheep abortion (Chapter 5).

The relative proportion of farms in Hawke's Bay from which each of the PFGE types was isolated appeared to be quite similar between 1999 (Chapter 3) and 2000. However, with the exception of PFGE type B1, which was found on 76% of farms in 1999 and 66% in 2000, many of the PFGE types were only found on a small number of farms. Therefore it can not be assumed that the apparent appearance or disappearance of uncommon PFGE types is a real phenomenon.

Similarly, the fact that some strain types were not found in a particular region does not preclude the existence of those strain types in that region. In addition, new *C. fetus* subsp. *fetus* PFGE types with the ability to cause abortion in sheep would likely be identified with further strain typing in subsequent years. However, the demonstrated sustained prevalence of PFGE groups A and B suggests that any novel types would probably be minor strains found on a few farms.

In the present study, there were seven cases of different *C. fetus* subsp. *fetus* PFGE types isolated from aborted foetuses from within the same flock. The phenomenon of concurrent abortions in flocks associated with more than one *C. fetus* subsp. *fetus* strain was also discussed when this was identified in the 1999 Hawke's Bay study in Chapter 3. Using the criteria of Tenover et al. (1995) it appears that on five out of these seven farms the PFGE profiles of isolates differed by two bands (A1 and A5, B1 and B5, and three farms with PFGE types B1 and B2). Therefore, the PFGE types are considered to

differ by one genetic event, can be considered to be closely related and are probably part of the same outbreak (Tenover et al. 1995). However, similar to the findings from the Hawke's Bay 1999 study, the PFGE profiles of the isolates from two of the farms differed by five bands (B1 and F4) and seven bands (B1 and F3) respectively. Five band differences may result from two independent genetic events, the isolates are possibly related and possibly part of the same outbreak (Tenover et al. 1995). However, seven band differences between PFGE profiles indicates there would have to have been three independent genetic events and the isolates are considered to be different and not part of the same outbreak (Tenover et al. 1995). Therefore PFGE typing results from at least one farm in the 2000 national study agree with the earlier finding that concurrent abortions in flocks may be associated with more than one *C. fetus* subsp. *fetus* strain, but this may be rarely detected.

The predominance of PFGE type B1 from sheep abortions and the infrequent isolation of PFGE type A1, which is used in the manufacture of the Campylovexin® vaccine, raises the question of cross-protection offered by the vaccine against other *C. fetus* subsp. *fetus* strains. The vaccination status of the affected flocks reported here was not available. However, it appears that these flocks were not vaccinated with Campylovexin®, as there were no reports to Schering-Plough Animal Health Ltd. of *C. fetus* subsp. *fetus* abortions during 2000 in ewes vaccinated with Campylovexin® that season (Roger Marchant, personal communication). This aspect of cross-protection of Campylovexin® against other strains of *C. fetus* subsp. *fetus* is examined in Chapters 6-8.

4.5 Conclusions

- Pulsed-field type B1 was predominant amongst the *C. fetus* subsp. *fetus* isolates cultured from sheep abortions in each region of New Zealand in 2000, as was found in Hawke's Bay in 1999.

- The similarity between PFGE profiles of *C. fetus* subsp. *fetus* sheep abortion isolates from 1987 and 2000, and the relative prevalence of the PFGE groups, suggests that there has been no major genotypic shift in the population of *C. fetus* subsp. *fetus* implicated in sheep abortion in New Zealand during this time.

Chapter 5

Pulsed-field gel electrophoresis of *Campylobacter jejuni* sheep abortion isolates

5.1 Introduction

Campylobacter jejuni can cause abortion in sheep (Diker and Istanbulluoglu 1986; Hedstrom et al. 1987; Delong et al. 1996). However, unlike *C. fetus* subsp. *fetus, C. jejuni* is a common isolate from faecal or rectal samples from sheep, along with *C. coli*, *C. lari*, and *C. hyointestinalis* (Manser and Dalziel 1985; Stanley et al. 1998; Jones et al. 1999).

During the Hawke's Bay study in 1999 and the national study in 2000, 405 *Campylobacter* isolates cultured from aborted sheep foetuses from 249 farms were collected. The majority of these isolates were *C. fetus* subsp. *fetus* and Chapters 3 and 4 describe the PFGE typing results for the *C. fetus* subsp. *fetus* isolates from the Hawke's Bay and national studies, respectively. *Campylobacter jejuni* was cultured from approximately 10% of the farms from which *Campylobacter* spp. were isolated from sheep abortion samples. Four *C. jejuni* isolates were cultured from three farms in Hawke's Bay in 1999. During the national study in 2000, 26 *C. jejuni* isolates were cultured from 22 farms. On two of these farms, both *C. fetus* subsp. *fetus* and *C. jejuni* isolates were cultured from separate foetuses. One *C. coli* isolate was cultured from an abortion sample from one farm in 2000.

Strain typing using the genetic techniques PFGE or amplified fragment length polymorphism (AFLP) has been performed on *C. jejuni* isolates from humans, poultry, cattle, and water (On et al. 1998; Wassenaar et al. 1998; Hudson et al. 1999; Duim et al. 1999 and 2001; Lindstedt et al. 2000). Delong et al. (1996) used the technique restriction endonuclease analysis (REA) to strain type 14 *C. jejuni* sheep abortion isolates from 12 farms in the USA. Five distinct REA types were found amongst the 14 isolates. There were two isolates analysed from each of two farms and in each case, both isolates from the same farm were indistinguishable (Delong et al. 1996).

To date, no genetic typing information has been reported for *C. jejuni* sheep abortion isolates in New Zealand. This chapter describes the genotyping results of the *C. jejuni* and *C. coli* isolates from sheep abortions from the Hawke's Bay study in 1999 and the national study in 2000, using PFGE. The restriction enzymes *Kpn*I and *Sma*I were used

in this analysis. Chang and Taylor (1990) analysed 25 restriction enzymes for their suitability for genome sizing of *C. jejuni* using PFGE. Three enzymes were found to generate a small number of DNA fragments of higher molecular weights that were well resolved after PFGE: *Sma*I, *Kpn*I and *Sal*I (Chang and Taylor 1990). *Sma*I is the restriction enzyme most commonly used when *C. jejuni* isolates are analysed by PFGE (Yan et al. 1991; Wassenaar et al. 1998; Hudson et al. 1999; Lindstedt et al. 2000; de Boer et al. 2000). However, it has been shown that some isolates that are indistinguishable when *Sma*I is used can be further differentiated with the use of *Kpn*I (Gibson et al. 1995; On et al. 1998).

The restriction enzyme *Sma*I has also been used for PFGE analysis of *C. coli*, along with *Sal*I or *Bam*HI (On 1998). Pulsed-field gel electrophoresis using *Sma*I has been suggested as a method to differentiate *C. jejuni* from *C. coli* isolates (Yan et al. 1991). This suggestion was based on the restriction patterns of the species, as *C. jejuni* digested with *Sma*I always produced a DNA band of 400-500 kb, whereas digestion of *C. coli* DNA did not produce a band greater than 250 kb. There were 12 *C. jejuni* isolates and 10 *C. coli* isolates in this study (Yan et al. 1991).

This chapter describes the PFGE analysis of the *C. jejuni* and *C. coli* sheep abortion isolates using the restriction enzymes *Kpn*I and *Sma*I.

5.2 Materials and Methods

5.2.1 *Campylobacter jejuni* and *C. coli* isolates from the 1999 Hawke's Bay and 2000 national studies

The culture and speciation methods for the four *C. jejuni* isolates from three farms in the Hawke's Bay region in 1999 are detailed in Chapter 3. The culture and speciation methods for the 26 *C. jejuni* and one *C. coli* isolates from 23 farms in the national study in 2000 are described in Chapter 4. Hippurate hydrolysis tests on the *C. coli* isolate (as described in Chapter 2) were negative.

5.2.2 *Campylobacter jejuni*, *C. coli*, and *C. fetus* subsp. *fetus* Type strains

The following Type strains from the New Zealand Reference Culture Collection, Medical Section (NZRM) were revived according to accompanying instructions and growth on Blood Agar was harvested and stored in 15% glycerol at –70°C:

- *Campylobacter jejuni* subsp. *jejuni* NZRM 2397 (National Collection of Type Cultures, NCTC, 11351)
- *Campylobacter coli* NZRM 2607 (NCTC 11366)
- *Campylobacter fetus* subsp. *fetus* NZRM 2398 (NCTC 10842)

5.2.3 Pulsed-field gel electrophoresis of isolates

5.2.3.1 *Kpn*I digestion

Preparation of genomic DNA was performed according to the method detailed in Chapter 2. Digestion with the restriction enzyme, *Kpn*I, was performed as follows. One third of each plug was equilibrated for 45 minutes on ice with 1.2× Roche Applied Science Buffer L and 1× BSA (0.1 mg/mL). The plug slices were then equilibrated with 1× Roche Buffer L, 1× BSA (0.1 mg/mL), and 30 units of Roche restriction enzyme *Kpn*I. Digestion reactions were incubated overnight at 37°C. Electrophoresis was performed essentially as detailed in Chapter 2, except electrophoresis was for 21 hours using the switch times 4-20 s. These switch times are normally used for PFGE analysis of *C. jejuni* using *Kpn*I (Gibson et al. 1995 and 1997; On et al. 1998).

5.2.3.2 *Sma*I digestion

Preparation and digestion of genomic DNA and PFGE of the isolates was performed according to the method detailed in Chapter 2.

5.2.4 Analysis of PFGE profiles

5.2.4.1 Analysis of PFGE profiles obtained with *Kpn*I digestion

The *Kpn*I PFGE profiles of the isolates were analysed according to the method used for the *C. fetus* subsp. *fetus* isolates which is detailed in Chapter 2, except with regard to the names assigned to the PFGE profiles. Briefly, BioRad Diversity Database software was used to analyse the PFGE profiles. A similarity matrix between the PFGE profiles was calculated using the Dice coefficient. The dendrogram showing similarity of the PFGE profiles of the isolates was produced using the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis. The *C. jejuni* PFGE type CJ-1 was the reference strain in the production of this dendrogram. Using this analysis, clusters less than 84% similar were considered to belong to different PFGE groups, for example CJ-1, CJ-2, CJ-3, CJ-4 etc. Isolates of the same PFGE group that were not indistinguishable were further organised into types, for example CJ-1, CJ-1a and CJ-1b. The PFGE profiles of the same group that were identified subsequent to the first profile of that group were designated with a letter, for example CJ-1, CJ-1a and CJ-1b. Isolates with indistinguishable PFGE profiles were grouped together as the same type, for example all CJ-2a isolates were indistinguishable by PFGE. The *C. coli* isolate was assigned the name CC-1. The Type strains were identified as such.

5.2.4.2 Analysis of PFGE profiles obtained with *Sma*I digestion

The *Sma*I PFGE profiles of the isolates were analysed according to the method used for *Kpn*I digestion of the *C. jejuni* isolates described above, except with regard to the names assigned to the PFGE profiles. All the isolates that were indistinguishable with *Kpn*I digestion were also indistinguishable with *Sma*I digestion. Therefore, the names of the *Kpn*I PFGE types were assigned to the profiles of the same isolates when digested with *Sma*I. For example, the *Kpn*I PFGE profiles of the isolates OC31 and OC32 were indistinguishable and this PFGE profile was named CJ-1. When OC31 and OC32 were subjected to PFGE after digestion with the enzyme *Sma*I, the profiles of these isolates were still indistinguishable, and this profile was named CJ-1. This means that the PFGE

profiles were named according to the level of similarity obtained after digestion with *Kpn*I, not *Sma*I.

5.3 Results

5.3.1 Pulsed-field gel electrophoresis of *C. jejuni* and *C. coli* isolates from the 1999 Hawke's Bay and 2000 national studies

5.3.1.1 Pulsed-field gel electrophoresis using the restriction enzyme *Kpn*I

Twelve distinct PFGE profiles were identified amongst the 30 *C. jejuni* isolates from 25 farms from the 1999 Hawke's Bay and 2000 national studies (Figure 5.1). The PFGE profiles consisted of 10 to 14 DNA bands, which ranged in size between 9.2 and 429.5 kb. None of the DNA bands were invariable between all the *C. jejuni* isolates. Similarity by cluster analysis of all 12 *C. jejuni* PFGE profiles, using the limit of 84% similar, resulted in seven PFGE groups: CJ-1, CJ-2 ... CJ-7 (Figure 5.2). Two of these groups had subtypes: CJ-1, CJ-1a and CJ-1b and CJ-2, CJ-2a, CJ-2b, and CJ-2c. Twelve out of 14 bands were invariable between the profiles CJ-1, CJ-1a and CJ-1b. Eleven out of the 12 to 14 bands were invariable between the profiles CJ-2, CJ-2a, CJ-2b and CJ-2c. With the exception of the cluster involving CJ-1, CJ-1a and CJ-1b and the cluster involving CJ-2, CJ-2a, CJ-2b, and CJ-2c, the PFGE profiles of the *C. jejuni* isolates were between 0% and 58.3% similar to each other (Figure 5.2, Appendix 5).

The PFGE profile of the *C. coli* isolate (CC-1) was unique and was between 0% and 43% similar to that of the *C. jejuni* isolates (Figures 5.1 and 5.2, Appendix 5).

5.3.1.2 Pulsed-field gel electrophoresis using the restriction enzyme *Sma*I

Nine distinct PFGE profiles were identified amongst the 30 *C. jejuni* isolates, using the enzyme *Sma*I (Figure 5.4). The PFGE profiles consisted of 5 to 10 DNA bands, which ranged in size between 33.4 and 487.5 kb. None of the bands were invariable between all the *C. jejuni* isolates. The isolates of PFGE types CJ-2, CJ-2a, CJ-2b, and CJ-2c distinguished with *Kpn*I were not differentiated by *Sma*I, as digestion of these isolates with *Sma*I invariably resulted in indistinguishable profiles of five DNA bands each (Figure 5.4). Similarity by cluster analysis of the PFGE profiles resulted in one cluster

involving CJ-1, CJ-1a and CJ-1b. Seven out of eight DNA bands were invariable between CJ-1 and CJ-1a and between CJ-1 and CJ-1b. But only six out of eight bands were invariable between CJ-1a and CJ-1b. Therefore, when the similarity was calculated using the Dice coefficient, CJ-1a and CJ-1b were 75% similar, whereas the similarity between CJ-1 and CJ-1a and between CJ-1 and CJ-1b was 88%. With the exception of the cluster involving CJ-1, CJ-1a and CJ-1b the PFGE profiles of the *C. jejuni* isolates were between 0% and 46.2% similar to each other (Figure 5.3, Appendix 6).

The *Sma*I PFGE profile of the *C. coli* isolate (CC-1) was unique and was between 0% and 22.2% similar to that of the C. *jejuni* isolates (Figures 5.3 and 5.4, Appendix 6).

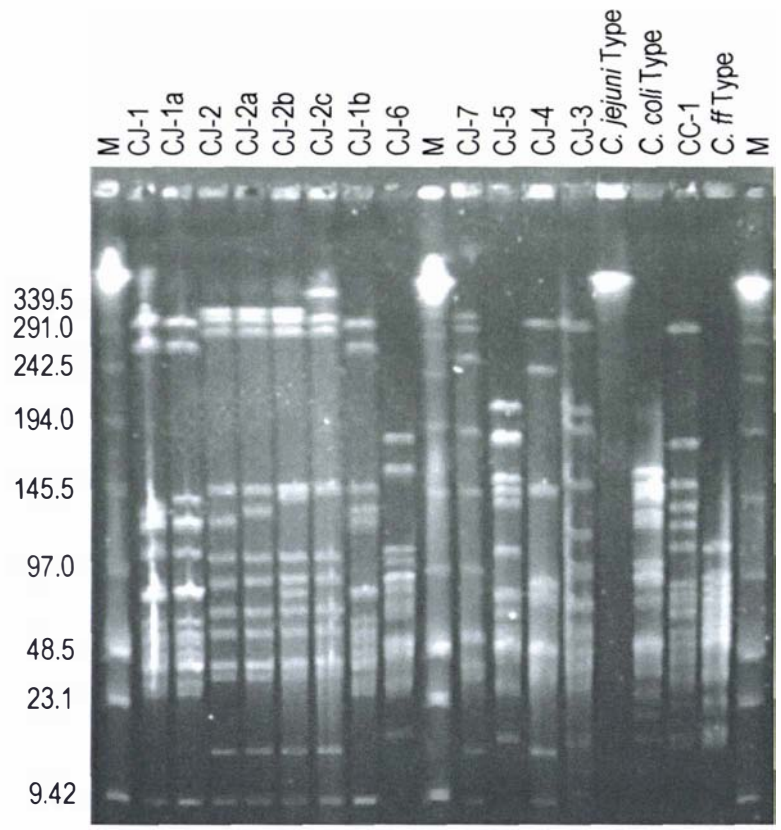


Figure 5.1 Pulsed-field gel electrophoresis using the restriction enzyme *Kpn*I of the 12 PFGE types found amongst the 30 *C. jejuni* isolates from 25 farms in 1999 and 2000 (CJ-1, CJ-1a, CJ-1b...CJ-7); the one *C. coli* isolate (CC-1); and the *C. jejuni*, *C. coli*, and *C. fetus* subsp. *fetus* (*Cff*) Type strains. M = molecular size marker (lambda ladder), DNA standard bands are labelled with sizes (kb).

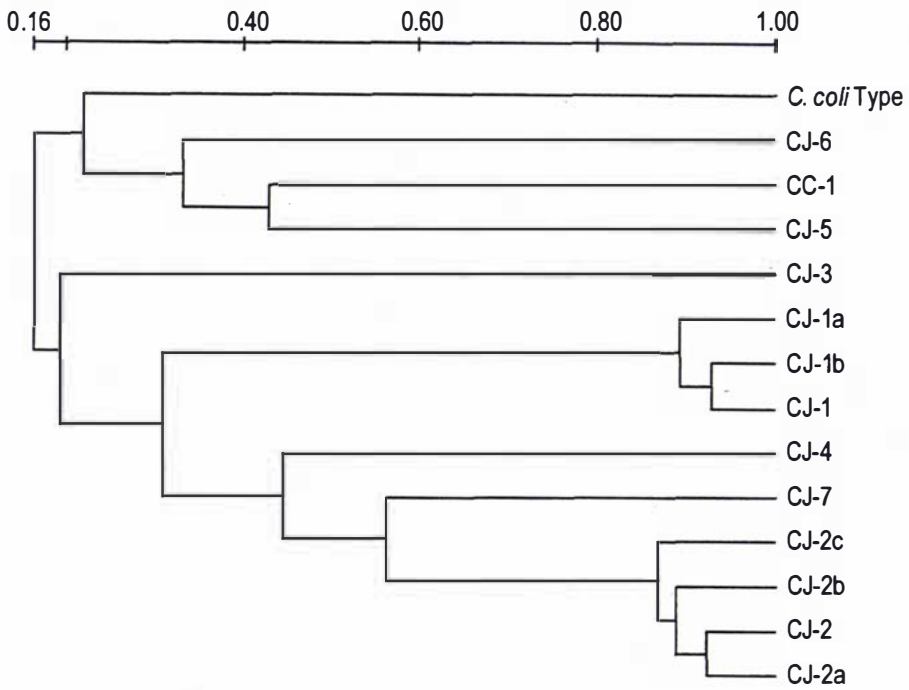


Figure 5.2 Similarity of the *Kpn*I PFGE profiles of the *C. jejuni* and *C. coli* isolates. This dendrogram was produced using the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis, with PFGE type CJ-1 as the reference. Clusters of PFGE types that were ≥84% similar were classified as belonging to the same PFGE group.

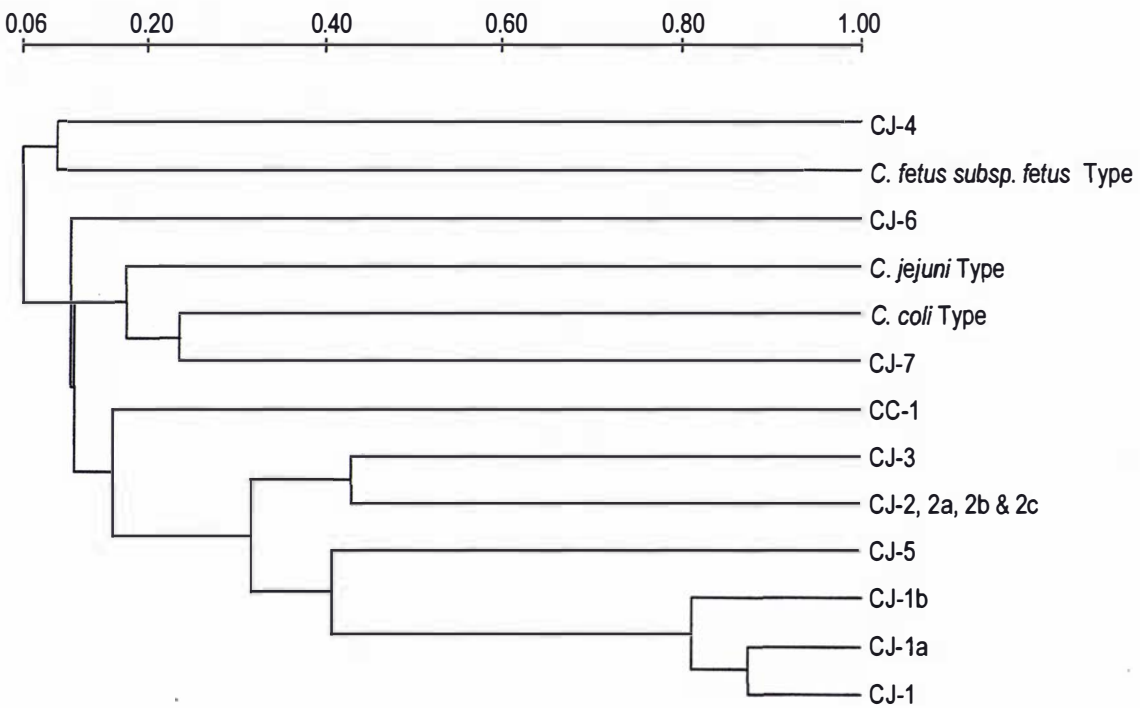


Figure 5.3 Similarity of the *Sma*I PFGE profiles of the *C. jejuni* and *C. coli* isolates. This dendrogram was produced using the UPGMA cluster analysis, with PFGE type CJ-1 as the reference. This *Sma*I dendrogram is labelled with the names of the *Kpn*I PFGE types to facilitate comparison of clustering between the two dendrograms.

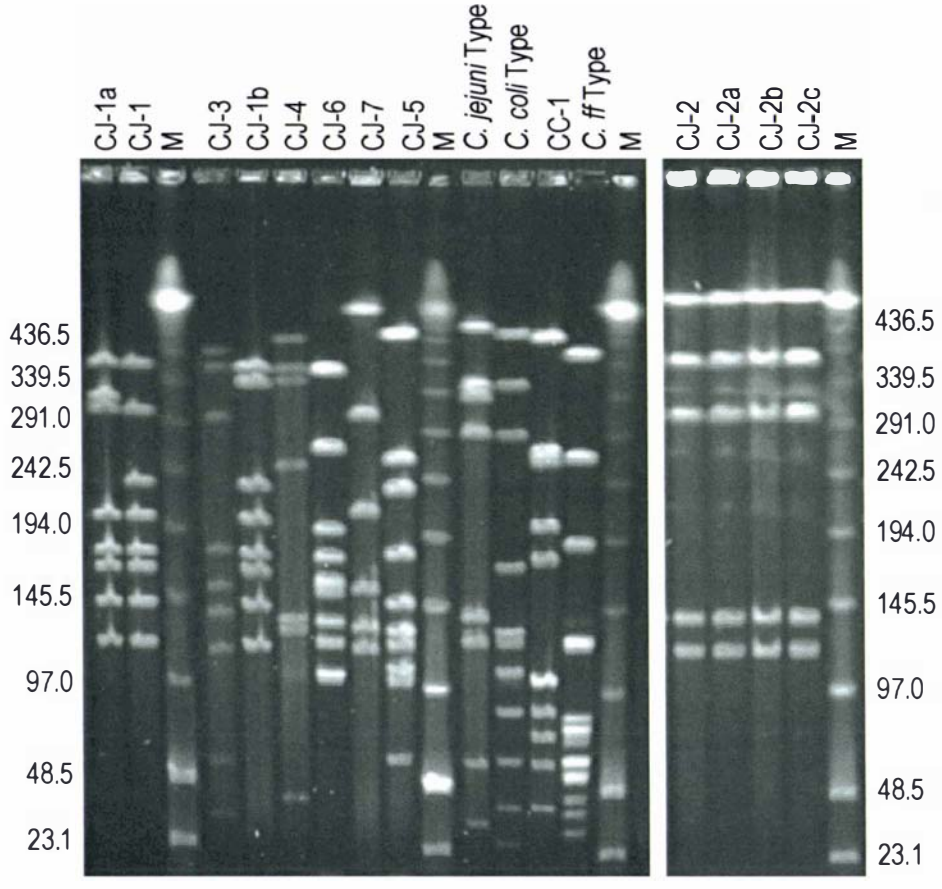


Figure 5.4 Pulsed-field gel electrophoresis using the restriction enzyme *Sma*I of the *C. coli* isolate (CC-1); the *C. jejuni*, *C. coli*, and *C. fetus* subsp. *fetus* (*Cff*) Type strains; and the 12 PFGE types found amongst the 30 *C. jejuni* isolates from 25 farms in 1999 and 2000. The PFGE types CJ-2, CJ-2a, CJ-2b and CJ-2c differentiated by *Kpn*I digestion are indistinguishable by *Sma*I digestion. M = molecular size marker (lambda ladder), DNA standard bands are labelled with sizes (kb).

5.3.2 Frequency of *C. jejuni* PFGE types

Most of the *C. jejuni* isolations from sheep abortion in 2000 were from farms in the South Island (19 out of 22 farms, 86%; Table 5.1). Overall, a total of 140 farms out of 221 from which *Campylobacter* spp. were isolated in 2000 were located in the South Island (63%).

Three *C. jejuni* PFGE types (CJ-1, CJ-1b and CJ-2a) were found most frequently and were each identified from five farms (Table 5.1). The PFGE group consisting of types CJ-1, CJ-1a and CJ-1b was identified from eleven farms (one isolate each of PFGE types CJ-1 and CJ-1a was cultured from the same farm). This includes the identification of PFGE type CJ-1 on two farms in the Hawke's Bay in 1999. Seven PFGE types were represented by only one isolate from one farm: CJ-2, CJ-2b, CJ-2c, CJ-3, CJ-5, CJ-6 and CJ-7.

On one farm, two different *C. jejuni* PFGE types were found. This farm had five isolations of *Campylobacter* spp. from sheep foetuses; three were *C. fetus* subsp. *fetus* PFGE type D1 and the other two were *C. jejuni* CJ-1 and CJ-1a. On one other farm in the national study, both *C. fetus* subsp. *fetus* and *C. jejuni* were isolated. The *C. fetus* subsp. *fetus* isolate was PFGE type B2 and the *C. jejuni* isolate was CJ-3 and this farm is the subject of Section 5.3.4 Investigation of an abortion outbreak attributed to *C. jejuni*.

5.3.3 Pulsed-field gel electrophoresis of Type strains

5.3.3.1 Pulsed-field gel electrophoresis using the restriction enzyme *Kpn*I

A PFGE profile of the *C. jejuni* Type strain was unable to be obtained with the restriction enzyme *Kpn*I, as the DNA of this strain did not digest with this enzyme (Figure 5.1).

The *Kpn*I PFGE profile of the *C. coli* Type strain was unique and was between 0% and 28.6% similar to that of the other isolates including the *C. coli* isolate CC-1 (20% similar) (Figures 5.1 and 5.2, Appendix 5).

The DNA of the *C. fetus* subsp. *fetus* Type strain was digested with the enzyme *Kpn*I but the DNA bands were not considered to be adequately resolved for analysis (Figure 5.1).

5.3.3.2 Pulsed-field gel electrophoresis using the restriction enzyme *Sma*I

Pulsed-field gel electrophoresis profiles of each of the Type strains were obtained with the restriction enzyme *Sma*I (Figure 5.4). There was ≤25% similarity between the PFGE profile of any of the Type strains and that of any of the *C. jejuni* and *C. coli* isolates (Figure 5.3, Appendix 6).

Table 5.1 Number of isolates of each *Campylobacter jejuni* and *C. coli* PFGE type and number and location of farms from which these were isolated in 1999 and 2000.

Type	No. isolates	No. farms	Location
CJ-1	9	5	[a]Hawke's Bay, Wairarapa, Marlborough
CJ-1a	2	2	Marlborough, Southland
CJ-1b	5	5	Otago, Southland
CJ-2	1	1	Hawke's Bay 1999
CJ-2a	5	5	Canterbury, Otago, Southland
CJ-2b	1	1	Southland
CJ-2c	1	1	Otago
CJ-3	1	1	Wairarapa
CJ-4	2	2	Southland
CJ-5	1	1	Southland
CJ-6	1	1	Southland
CJ-7	1	1	Canterbury
Total	**30**	**26** [b]	

[a] Hawke's Bay 1999 and 2000.
[b] Total includes one farm with two distinct PFGE types.

5.3.4 Investigation of an abortion outbreak attributed to *C. jejuni*

In mid-October 2000, we were contacted by a veterinarian concerning a large abortion outbreak on a sheep farm in Wairarapa. *Campylobacter jejuni* had been isolated from the stomach contents of one aborted foetus and was assumed to be the cause of the abortion storm. The veterinarian was aware of our interest in *Campylobacter* sheep abortion and was happy for our involvement in further investigation of the case, as it seemed unusual for *C. jejuni* to cause this number of abortions.

5.3.4.1 Case history

Abortions had begun in July 2000, in a flock of 1900 ewe hoggets which had not been vaccinated with Campylovexin®. The veterinarian was not contacted until the start of lambing in early October, by which time approximately 400 ewes had aborted (21%) and about 30 had died (2%). Samples from three aborted foetuses were submitted to a veterinary diagnostic laboratory for abortion investigation and *C. jejuni* was isolated from the stomach contents of one foetus. However, this isolation was described as light *C. jejuni* growth in light mixed growth, which included faecal coliforms. Culture for *C. fetus* subsp. *fetus* and *Salmonella* Brandenburg was negative, as was toxoplasmosis serology.

5.3.4.2 Case investigation

By mid-October lambing was well under way and aborted foetuses could not be obtained. However, three ewes that had aborted were slaughtered and the uteri collected for culture. The uterine contents were aseptically swabbed (Cultureswab™ Transport Systems, Difco, Detroit, MI, USA) and selective media was inoculated (Blood Agar with Campylobacter Selective Supplement, Oxoid Ltd., Basingstoke, Hampshire, England), and incubated for 48 hours at 37°C in a microaerobic atmosphere (CampyGen sachets, Oxoid Ltd.). The uterus from one ewe contained a substantial amount of thick, purulent material and *C. fetus* subsp. *fetus* was isolated in pure culture from this material. This *C. fetus* subsp. *fetus* isolate was typed using PFGE as B2, and the initial *C. jejuni* isolate cultured from the aborted foetus was PFGE type CJ-3. The other two uteri contained a small amount of purulent material, from which *Campylobacter* spp. were not isolated.

5.4 Discussion

A total of 12 distinct PFGE profiles were found amongst the 30 *C. jejuni* isolates from 25 farms from the national study in 2000 and the Hawke's Bay study in 1999. This genetic diversity of *C. jejuni* isolates from sheep abortions correlates with the findings of Delong et al. (1996), who found five distinct types amongst 14 *C. jejuni* sheep abortion isolates from 12 farms in USA using the technique restriction endonuclease analysis. In the current study, the restriction enzyme *Kpn*I was found to be more discriminating than *Sma*I, as *Sma*I failed to differentiate four PFGE types that were distinguished by *Kpn*I. This difference in the discriminatory power of *Kpn*I and *Sma*I with *C. jejuni* has been found by others (Gibson et al. 1995; On et al. 1998).

It appears that there was not a single *C. jejuni* PFGE type most frequently associated with abortion in sheep, as the three types most frequently found were all identified from five farms. Similarity analysis revealed that the majority of the isolates belonged to two PFGE groups. Delong et al. (1996) also found that there was not a single type identified most frequently, as the three most commonly found types were identified from five, four and three farms out of the total of 12 farms. However, the findings of the current study and of that of Delong et al. (1996) are based on a small number of *C. jejuni* isolates from a small number of farms.

In the current study, four out of the 25 farms over both years had more than one *C. jejuni* isolate cultured from foetuses from that farm. The isolates from three of these farms were all PFGE type CJ-1. From the remaining farm, two distinct *C. jejuni* PFGE types were found: CJ-1 and CJ-1a. The guidelines of Tenover et al. (1995) for interpretation of PFGE profiles were intended for the analysis of a small set of isolates (≤30) that were epidemiologically related in a disease outbreak, collected over a short period of time (1-3 months) and for which at least 10 DNA bands in the PFGE profile were obtained. Digestion of these isolates of different PFGE types from the same farm with the restriction enzyme *Sma*I resulted in PFGE profiles consisting of only eight DNA bands. Digestion with *Kpn*I resulted in PFGE profiles of 14 DNA bands that differed by two bands. Therefore, these PFGE types are considered to differ by one genetic event, can be considered to be closely related and are probably part of the same

outbreak (Tenover et al. 1995). This finding of indistinguishable or similar PFGE types of *C. jejuni* isolates from different foetuses from the same flock is consistent with the findings of Delong et al. (1996). In that study, two *C. jejuni* isolates were cultured from each of two farms and each isolate from the same farm was indistinguishable by REA typing (Delong et al. 1996). This supports the role of *C. jejuni* as an infectious cause of abortion in a flock.

Similarity analysis of the *C. jejuni* PFGE types showed that they were rather dissimilar to each other; apart from the clusters involving CJ-1, CJ-1a, CJ-1b and CJ-2, CJ-2a, CJ-2b, and CJ-2c, the types were between 0% and 58.3% similar. It is difficult to compare this level of genetic diversity of *C. jejuni* sheep abortion isolates with other studies of *C. jejuni* isolates, due to the lack of standardisation of the correlation coefficient, cluster analysis and reference strains used in the production of dendrograms. However, this result may be contrasted with the similarity analysis of the *C. fetus* subsp. *fetus* PFGE types, which showed that they were all at least 50% similar to each other (Chapter 4). It appears that *C. jejuni* sheep abortion isolates are generally more genetically diverse from each other than the *C. fetus* subsp. *fetus* isolates from sheep abortion.

The PFGE profiles of the *C. coli* field isolate, CC-1, and the *C. coli* Type strain were not clearly delineated from that of the *C. jejuni* isolates or the *C. fetus* subsp. *fetus* Type strain. The *C. coli* field isolate was as dissimilar to the *C. coli* Type strain as it was to the *C. fetus* subsp. *fetus* and *C. jejuni* Type strains, at approximately 10% similarity. Pulsed-field gel electrophoresis using *Sma*I has been suggested as a method to differentiate *C. jejuni* from *C. coli* isolates (Yan et al. 1991). This suggestion was based on the restriction patterns of the species, as *C. jejuni* digested with *Sma*I always produced a DNA band of 400-500 kb, whereas digestion of *C. coli* DNA did not produce a band greater than 250 kb (Yan et al. 1991). However in this study, the largest band produced by *Sma*I digestion of four *C. jejuni* types (CJ-1, CJ-1a, CJ-5 and CJ-6) was less than 400 kb at 366.0 kb. In addition, *Sma*I digestion of both the *C. coli* Type strain and the *C. coli* field isolate each produced three DNA bands which were greater than 250 kb. Thus, it appears that *Sma*I PFGE analysis does not reliably distinguish *C. coli* from *C. jejuni* based on band sizes.

One case of resistance to restriction enzyme digestion was found in the course of PFGE typing all of the *C. fetus* subsp. *fetus* and *C. jejuni* isolates in this study. The *C. jejuni* Type strain was resistant to *Kpn*I digestion, but not to *Sma*I digestion. This was the case even when slices from the same DNA plug preparation were subject to digestion with each enzyme, which indicated that sample preparation was not the problem. Resistance to digestion with *Kpn*I has been reported for the *C. jejuni* Type strain before (Gibson et al. 1997). Identified in the report of Gibson et al. (1997) as Penner serotype 23 reference strain from the National Collection of Type Cultures (NCTC), the DNA of the *C. jejuni* Type strain was not cut with *Kpn*I, but was susceptible to *Sma*I digestion (Gibson et al. 1997). Three other *C. jejuni* Penner serotype reference strains were similarly resistant to digestion with *Kpn*I and one of these was resistant to *Sma*I digestion as well (Gibson et al. 1997). *Kpn*I resistance has also been reported for isolates of the *C. sputorum* biovar paraureolyticus (On et al. 1999). Similarly, there is a report of *C. concisus* resistance to *Sma*I digestion, and two out of 53 *C. concisus* isolates in this report were also resistant to digestion with the restriction enzyme *Not*I (Matsheka et al. 2002). Restriction-modification systems have been suggested to be responsible for this enzymatic inactivity (Matsheka et al. 2002), and this appears to be the best explanation for the resistance of the *C. jejuni* Type strain to *Kpn*I digestion.

On two farms in the national study in 2000, both *C. jejuni* and *C. fetus* subsp. *fetus* were isolated. It appears that on one farm, concurrent abortions due to *C. jejuni* and *C. fetus* subsp. *fetus* occurred, as five *Campylobacter* isolates were cultured from sheep foetuses collected at the same time from this farm; three were *C. fetus* subsp. *fetus* PFGE type D1 and the other two were *C. jejuni* CJ-1 and CJ-1a. However, on the other farm, which was the subject of the Wairarapa case study detailed in Section 5.3.4, there was dubious evidence to suggest that concurrent abortions due to *C. jejuni* and *C. fetus* subsp. *fetus* occurred, despite the isolation of both species. Investigation of the initial diagnosis of *C. jejuni* abortion revealed that faecal coliforms were cultured along with light growth of *C. jejuni* from the stomach contents of the aborted foetus. As *C. jejuni* can be found in sheep faeces (Manser and Dalziel 1985; Stanley et al. 1998; Jones et al. 1999), contamination of foetal samples during collection may result in the isolation of *C. jejuni*, even when this was not the cause of foetal death. The presence of mixed bacterial growth including faecal coliforms indicates that contamination of the foetal sample occurred in this case. A diagnosis of *C. jejuni* abortion from this result would be highly

suspect. This case demonstrates that careful interpretation of laboratory results is important in regard to *C. jejuni* abortion diagnoses. The isolation of *C. fetus* subsp. *fetus* in pure culture from the purulent uterus of a ewe that had aborted indicated that this ewe probably aborted due to *C. fetus* subsp. *fetus*. It has been found that *C. fetus* subsp. *fetus* may be excreted from the vagina of the ewe for up to four weeks after abortion (Firehammer and Lovelace 1961). *Campylobacter fetus* subsp. *fetus* was not cultured from the initial submission of samples from three aborted foetuses in early October. However, by this time lambing had begun, and it is possible that the deaths of these lambs were not connected to the infectious agent that caused the abortion storm. It was not possible to determine the cause of the large number of abortions on this farm, largely due to the late involvement of the veterinarian in this case. Despite this, it appears that *C. fetus* subsp. *fetus* was implicated in the abortion of at least one ewe, and is the more likely cause of most of the other abortions in this outbreak.

It appears that the number of *C. jejuni* sheep abortion cases, relative to that of *C. fetus* subsp. *fetus*, is under-represented in this study when compared to the information collated by the Ministry of Agriculture and Forestry (MAF). In the national study in 2000, *Campylobacter* sheep abortion isolates were collected from the veterinary diagnostic laboratories LabNet Invermay Ltd., Mosgiel; LabWorks Animal Health, Lincoln; Gribbles Veterinary Pathology Animal Health Laboratory, Palmerston North; and Alpha-Scientific Ltd., Hamilton. From these laboratories, *C. fetus* subsp. *fetus* was collected from 197 farms and *C. jejuni* or *C. coli* from 22 farms in 2000 (Table 5.2). (Massey University Diagnostic Microbiology Laboratory isolated *C. fetus* subsp. *fetus* from an additional two farms and one *C. fetus* subsp. *fetus* isolate was cultured from the ewe uterus above). However, these figures do not match those collated by MAF from the listed veterinary diagnostic laboratories for 2000; 173 cases of *C. fetus* subsp. *fetus* abortion and 67 cases of "other" *Campylobacter* abortion in sheep (Anon 2001b). It appears that when compared to the information collated by MAF, the number of *C. fetus* subsp. *fetus* abortion cases is over-represented and the number of other *Campylobacter* abortion cases is under-represented in this study. This study indicates that *C. jejuni* was isolated from sheep abortions on approximately 10% of the farms from which *Campylobacter* spp. were cultured in the Hawke's Bay in 1999 (three out of 28) and nationally in 2000 (22 out of 219). The information available from the annual MAF Biosecurity Authority animal health surveillance reports for the last four years indicates

that *C. jejuni* and *C. coli* were isolated from between 26% and 43% of the total number of *Campylobacter* sheep abortion cases (Table 5.2). The true proportion of *Campylobacter* sheep abortion due to *C. jejuni* is not known.

Table 5.2 Number of New Zealand sheep abortion cases of *C. fetus* subsp. *fetus* and other *Campylobacter* species for the years 1995-2001. These figures (except those italicised) were obtained from the annual Ministry of Agriculture and Forestry Biosecurity Authority animal health surveillance reports, which were collated from the veterinary diagnostic laboratories. The italicised entries are the number of the *Campylobacter* spp. obtained in this study from the veterinary diagnostic laboratories during the national study in 2000.

Year	*C. fetus* subsp. *fetus* cases	"other" *Campylobacter* cases	Total *Campylobacter* cases	Reference
1995	87 (85%)	15 (15%)	102	Anon 1996
1996	117 (87%)	18 (13%)	135	Anon 1997
1997	102 (82%)	22 (18%)	124	Anon 1998
1998	86 (60%)	58 (40%)	144	Anon 1999
1999	111 (57%)	85 (43%)	196	Anon 2000
2000	173 (72%)	67 (28%)	240	Anon 2001b
2001	107 (74%)	37 (26%)	144	Poland 2002
2000	*197 (90%)*	*22 (10%)*	*219*	*This study*

The impact on farms of *C. jejuni* compared to farms affected by *C. fetus* subsp. *fetus*, in terms of the numbers of sheep that abort due to each species, is also not known. Most of the farms from which *C. jejuni* was cultured from an aborted sheep foetus had only one isolate cultured. Only four out of the 25 farms (16%) over both years had more than one *C. jejuni* isolate cultured from foetuses from that farm. In contrast, 80 out of the 225 farms that *C. fetus* subsp. *fetus* was isolated from over both years had *C. fetus* subsp. *fetus* diagnosed from more than one foetus (36%). Thus, it appears that *C. fetus* subsp. *fetus* is more likely to be isolated from more than one foetus than *C. jejuni*. This may reflect the number of aborted foetuses available for submission and therefore the number of abortions due to the respective species. Further research on the role of *C. jejuni* as a cause of abortion in sheep flocks in New Zealand is required.

5.5 Conclusions

- Of the 12 distinct PFGE profiles found amongst the 30 *C. jejuni* sheep abortion isolates from 25 farms from the national study in 2000 and the Hawke's Bay study in 1999, it appears that there was not a single *C. jejuni* PFGE type most frequently associated with abortion in sheep.

- Indistinguishable or similar *C. jejuni* PFGE types were identified from different aborted foetuses from the same flock, consistent with the role of *C. jejuni* as an infectious cause of abortion in sheep.

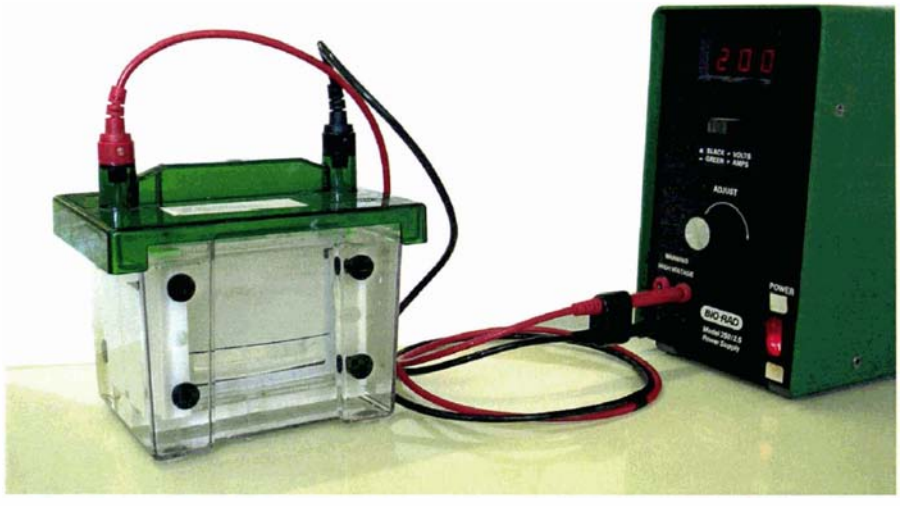
- The *C. jejuni* PFGE types were more dissimilar to each other than the *C. fetus* subsp. *fetus* PFGE types were to each other.

- It appears that DNA band sizes in PFGE analysis does not reliably distinguish *C. coli* from *C. jejuni*.

- Careful interpretation of laboratory results is important in respect to *C. jejuni* abortion diagnoses, as contamination of the foetal sample may result in the isolation of *C. jejuni*, even when this may not have been the cause of foetal death.

Chapter 6

In vitro protein and serological studies of *Campylobacter fetus* subsp. *fetus* strains



6.1 Introduction

Campylobacter fetus cells have an outer capsule made up of a layer of proteins called surface layer proteins (SLPs). These SLPs are most commonly reported with molecular weights of 98-100kDa, 125-127kDa and 149kDa (Winter et al. 1978; Blaser et al. 1987; Pei et al. 1988). They have also been reported with molecular weights of 85kDa, 110kDa, 131-135kDa and 140kDa (Dubreuil et al. 1988 and 1990; Wang et al. 1993). Isolates can spontaneously change the size of the predominant SLP expressed during animal or *in vitro* passage (Fujimoto et al. 1991; Wang et al. 1993).

Surface layer proteins are the predominant protein expressed by *C. fetus* subspecies and are immunodominant protein antigens (Dubreuil et al. 1988). Experimental vaccination of sheep with whole cell *C. fetus* subsp. *fetus* resulted in the production of antibodies against SLPs (Myers et al. 1970; Grogono-Thomas et al. 2003). These vaccinated sheep were protected against abortion upon subsequent artificial challenge with *C. fetus* subsp. *fetus* (Myers et al. 1970; Grogono-Thomas et al. 2003). Sheep that abort due to *C. fetus* subsp. *fetus* also produce antibodies to SLPs (Grogono-Thomas et al. 2000), and this may be an important factor in the formation of immunity following *C. fetus* subsp. *fetus* infection.

Campylovexin® (Schering-Plough Animal Health Ltd.) is a single-strain, killed *C. fetus* subsp. *fetus* vaccine for prevention of abortion in sheep in New Zealand due to *C. fetus* subsp. *fetus*. The aim of this study was to determine whether the single-strain Campylovexin® vaccine elicits the production of antibodies in sheep that are able to recognise the various SLPs of the different PFGE types identified throughout New Zealand (Chapters 3 and 4). This chapter describes the identification of SLPs of the various PFGE types and the interaction of the SLPs with antibodies from ewes vaccinated with Campylovexin®.

6.2 Materials and methods

6.2.1 Identification of SLPs by protein sequencing

Preliminary SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of several *C. fetus* subsp. *fetus* isolates was performed according to the method detailed in Chapter 2.5. The protein profiles obtained from these isolates revealed protein bands which were provisionally identified as SLP bands. This provisional identification was based on the observations that they were the predominant protein expressed (as described by Dubreuil et al. 1988), and they were of the characteristic sizes reported for SLPs of approximately 100kDa, 127kDa and 149kDa (Winter et al. 1978; Blaser et al. 1987; Pei et al. 1988).

Six N-terminal residues of a suspected SLP band of approximately 149kDa expressed by a PFGE type C1 isolate (41972) were sequenced as follows. The protein profile of the isolate was transferred after SDS-PAGE to 0.45 μm BioTrace Polyvinylidene Fluoride (PVDF) membrane (Gelman Laboratory, Pall Corporation, Ann Arbor, MI, USA) according to the Western Blotting method described in Chapter 2.7. After transfer at 100 volts for 1 hour, the membrane was stained with Ponceau S and the protein standards were marked with pencil. The band position of the suspected SLP was marked by cutting the membrane, which was washed overnight in water. The suspected SLP band was cut out of the membrane and submitted to the Protein Microchemistry Facility, Department of Biochemistry, University of Otago for N-terminal protein microsequencing. Protein sequencing was done on an Applied Biosystems Procise Protein Sequencer, Model 492, which performs fully automated chemistry of the Edman Degradation (Hubbard et al. 2000).

The protein sequencing results of the first six residues of the 149kDa suspected SLP were as follows: M I S K S E.

The published sequence of the first six residues of the N-terminus of Type A SLPs (*sapA*) is: M L N K T D (Pei et al. 1988; Blaser and Gotschlich 1990; Dworkin et al. 1995b). The published sequence of the first six residues of the N-terminus of Type B SLPs (*sapB*) is: M I S K S E (Dubreuil et al. 1990; Dworkin et al. 1995b). Therefore, the obtained sequence from the 149kDa protein band was the same as the published

sapB protein sequence. In addition, a National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) protein-protein search using the first six residues of the protein sequence obtained from the 149kDa suspected SLP band uniquely identified this protein as an SLP from *C. fetus* subsp. *fetus* (Appendix 7). Thus, the protein provisionally identified as an SLP was confirmed to be an SLP.

6.2.2 *In vitro* sub-culturing of isolates

It has been shown that *C. fetus* subsp. *fetus* can spontaneously change the predominant SLP expressed during *in vitro* sub-culturing steps (Fujimoto et al. 1991; Wang et al. 1993). In addition, prolonged *in vitro* sub-culturing has also been shown to generate spontaneous mutants lacking SLP expression (Blaser et al. 1987; Fujimoto et al. 1989). To further confirm that the identified protein bands of different isolates were SLPs, continuous sub-culture *in vitro* of six isolates was performed to elicit changes in the size of expressed SLPs or to elicit spontaneous mutants lacking SLP expression.

The six isolates were chosen because each of the sizes commonly reported for SLPs (100kDa, 127kDa, or 149kDa) were expressed by one or more of the isolates. The six isolates also each represented a different PFGE type: A1, A5, B1, B4, C1 and D1 (Table 6.1). Continuous sub-culture *in vitro* of the six isolates was performed by picking a single colony and inoculating a fresh *Campylobacter* blood-free agar plate (*Campylobacter* Blood-free Selective Agar Base, Oxoid) every 2-3 days for a total of 40 passages, according to the method of Fujimoto et al. (1989). Protein samples of the isolates before sub-culturing began and after 18, 34 and 40 passages were prepared, as described in Chapter 2.5.1. Protein samples were analysed by SDS-PAGE as described in Chapter 2.5.2.

In addition, isolates were analysed by PFGE after 33 passages, as described in Chapter 2.4.

Table 6.1 The isolate name, PFGE type and approximate size of the SLP initially expressed of the six isolates selected for continuous sub-culture.

Isolate name	PFGE type	Approx. SLP size (kDa)
5915	A1	100 and 127
NS76	A5	127
CV4	B1	100
OC115	B4	127
NS67	C1	149
CV39	D1	100

6.2.3 Surface layer proteins of the various PFGE types and reactivity of immune sera with SLPs

In total, 385 *C. fetus* subsp. *fetus* isolates of 31 different PFGE types were collected from the Hawke's Bay study (Chapter 3), the national study and the New Zealand Culture Collection (Chapter 4). An investigation was undertaken to determine whether the single-strain Campylovexin® vaccine elicits the production of antibodies in sheep that are able to recognise the various SLPs of the different PFGE types identified throughout New Zealand. The protein profile of at least one *C. fetus* subsp. *fetus* isolate of each of the PFGE types was examined using SDS-PAGE. For many of the PFGE types, additional protein preparations of the same isolate were prepared at a different time in order to investigate reproducibility. The interaction of antibodies in serum from sheep vaccinated with Campylovexin® with these proteins was studied using Western Blotting.

Protein sample preparation and SDS-PAGE was performed as described in Chapter 2.5. Gels were either stained with Coomassie Blue or were used for Western blotting as described in Chapter 2.7.

6.2.3.1 Examination of individual serum samples from 15 ewes

Pre-immune and immune sera were collected from 15 ewes vaccinated with Campylovexin® as described in Chapter 2.6. Initially, antibody binding in the pre-immune and immune serum sample of each sheep was investigated. Individual serum samples were used in Western blotting against the proteins of the strain used in the manufacture of Campylovexin® (strain 5915, PFGE type A1) and against an isolate of PFGE type B1. Protein sample preparation of these isolates was performed as described in Chapter 2.5. 30 µL of the protein sample in a total volume of 190 µL was heated in a boiling water bath for 5 minutes and loaded on a single-lane (preparative-well) BioRad Tris-HCl Ready Gel® precast gel (7.5% resolving gel, 4% stacking gel). Electrophoresis and transfer of the proteins to nitrocellulose membrane was performed as described in Sections 2.5 and 2.7. The membrane was stained with PonceauS and bands of the protein standards were marked with pencil. The membrane was sliced into 22 strips, each containing the *C. fetus* subsp. *fetus* protein profile. PonceauS was washed off with water and the membrane strips were blocked overnight in Tris-buffered saline with milk powder (TBSM). After a brief rinse in fresh TBSM, each membrane strip was incubated with a different individual sheep serum sample diluted 1:250 in TBSM (rocking at room temperature for 2 hours). The membrane strips were washed 5 times for 5 minutes with TBSM and incubated with a 1:30,000 dilution of anti-sheep antibody in TBSM (rocking at room temperature for 2 hours). The membrane strips were washed 5 times for 5 minutes with Tris-buffered saline (TBS). Alkaline phosphatase colour reaction was developed by briefly incubating all the membrane strips together in 33 µL BCIP and 330 µL NBT in 10 mL AP Solution. Colour development was stopped simultaneously for all the membrane strips by rinsing with water.

These experiments demonstrated that most of the individual pre-immune serum samples from the 15 sheep showed no antibody binding to the proteins of *C. fetus* subsp. *fetus*. The pre-immune serum sample from ewes 201, 206, 209, 210, 211, 212, 214 showed some very faintly observed antibody binding to some proteins of *C. fetus* subsp. *fetus*, mostly of molecular weight <66kDa. In contrast, each individual immune serum sample

from the 15 sheep showed similar strong antibody binding to the proteins of *C. fetus* subsp. *fetus*, in particular to the SLP band. Western blotting results of the *C. fetus* subsp. *fetus* strain used in the manufacture of Campylovexin® (5915) with pre-immune and immune serum samples for 11 sheep (ewes 205-215) are shown in Figure 6.1.

For the rest of the Western blotting experiments described in this thesis, an equal volume of pre-immune sera from the 15 sheep was pooled and this was used at a dilution of 1:250, according to the method detailed in Chapter 2. Similarly, an equal volume of immune sera from the 15 sheep was pooled and this was used at a dilution of 1:250.

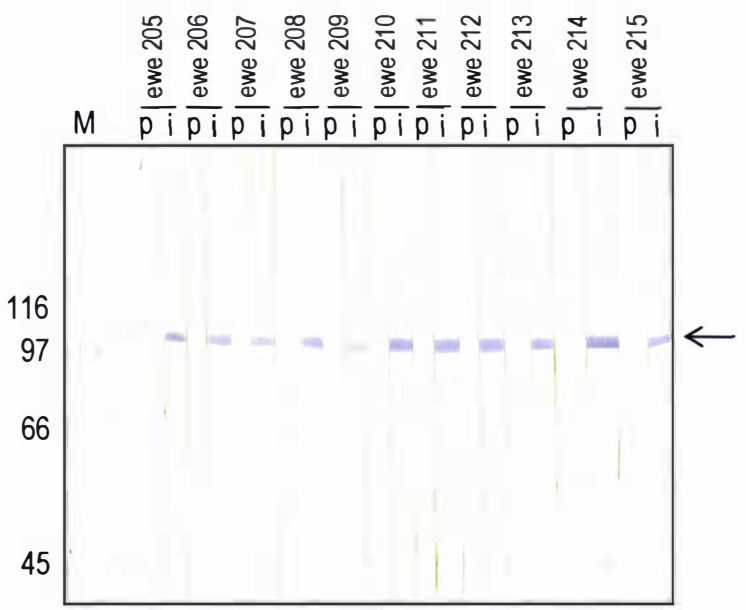


Figure 6.1 Western blot showing antibody binding to the proteins of the Campylovexin® vaccine strain (5915) of pre-immune (p) and immune (i) serum from 11 individual ewes vaccinated with Campylovexin®. The ear tag number of each ewe is shown at the top of the figure. The SLP band is indicated with an arrow. M = molecular weight standards, protein standard bands were labelled with sizes (kDa).

6.3 Results

6.3.1 *In vitro* sub-culturing of isolates

A prominent protein band corresponding to the reported sizes of SLPs was observed in the protein profile of each of the six isolates before continuous sub-culture began (Figure 6.2). The size of the predominantly expressed protein band changed in four out of the six isolates continuously sub-cultured for 40 passages. The isolate of PFGE type A1 initially expressed prominent proteins of approximate molecular weights 100kDa and 127kDa. By 18 passages, only the 127kDa protein was expressed. The predominant protein band of the PFGE type A5 isolate was approximately 100kDa at 18 passages. Initially and at the later sampling points the expressed protein was approximately 127kDa. The PFGE type B4 isolate initially expressed a ~127kDa protein, but by 34 passages the predominant protein expressed was approximately 149kDa. The predominant protein of PFGE type C1 was approximately 100kDa by 18 passages, but was initially approximately 149kDa. There was no detected change in the size of the major protein expressed by the PFGE types B1 and D1. No major change in expression of any other protein band of the isolates was detected.

The PFGE profiles of the isolates before passaging began and after 33 *in vitro* sub-culture steps were indistinguishable (Figure 6.3).

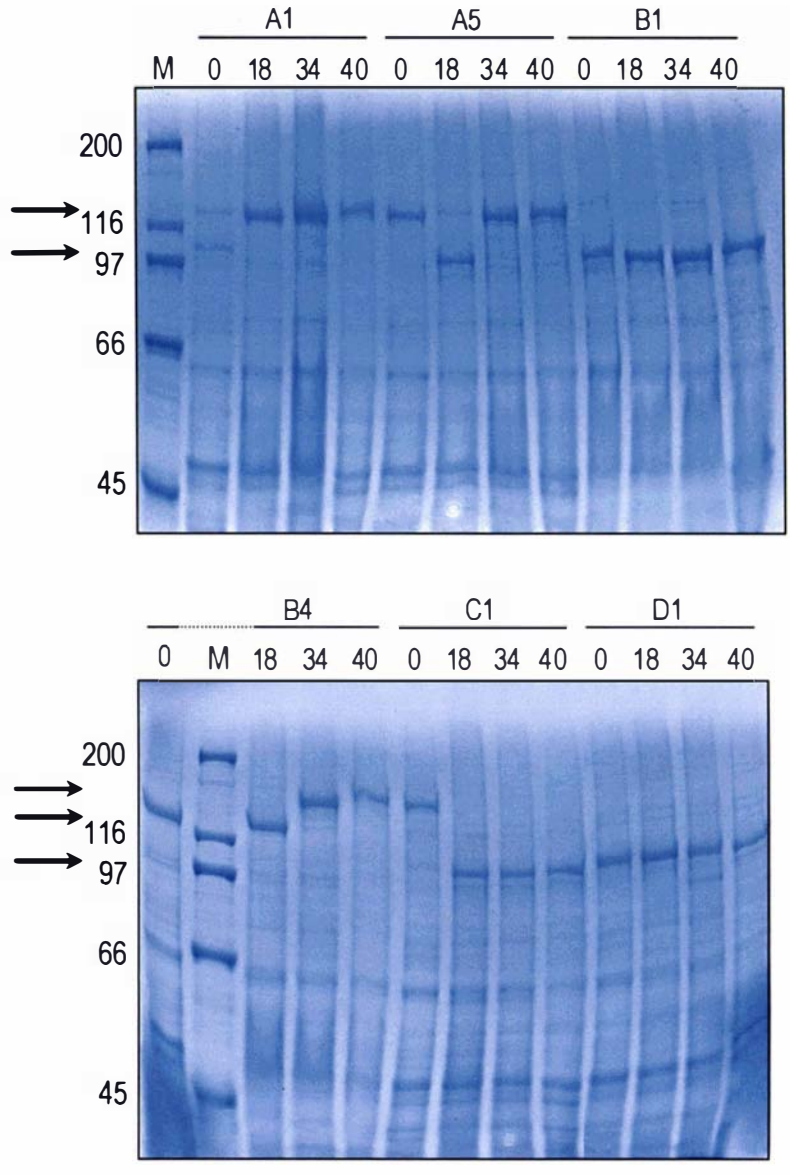


Figure 6.2 Coomassie-stained SDS-PAGE gels of continuously subcultured isolates A1, A5, B1, B4, C1 and D1. A protein sample was prepared for each isolate after 0, 18, 34, and 40 *in vitro* passages. Surface layer protein (SLP) bands were indicated with arrows. M = molecular weight standards, protein standard bands were labelled with sizes (kDa).

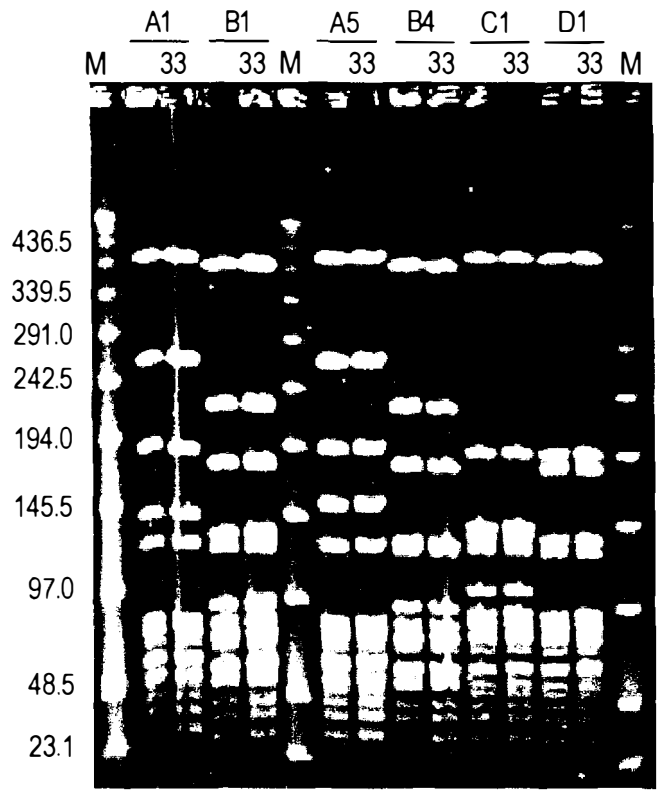


Figure 6.3 PFGE analysis of continuously subcultured isolates using the restriction enzyme *Sma*I. The first lane of each pair was the PFGE profile of the isolate before repeated subculture. The second lane of each pair was that of the same isolate after 33 *in vitro* subculture steps. M = molecular size marker (lambda ladder), DNA standard bands were labelled with sizes (kb).

6.3.2 Surface layer proteins of the various PFGE types

The protein profiles of 56 *C. fetus* subsp. *fetus* isolates, which included at least one isolate from each of the 31 PFGE types, were examined by SDS-PAGE. A prominent protein band corresponding to the reported sizes of SLPs was observed amongst isolates of most of the PFGE types (Figures 6.4-6.6 Panel A). The other proteins in the profile of each isolate appeared similar between isolates.

The following 16 PFGE types expressed a predominant SLP band of approximately 100kDa: A2, A3, A4, B1, B2, B3, B6, D2, E2, F2, F4, F5, G1, I1, J1, and K1 (Table 6.2). Isolates of the PFGE types A5, B4, F1 and H1 expressed a predominant SLP band of approximately 127kDa. The observed expression of these SLPs was reproducible upon the preparation of additional protein samples at a different time.

Pulsed-field gel electrophoresis types A6 and D3 were each represented by a single isolate. In one protein preparation of each isolate two SLPs of equal intensity were expressed of approximate molecular weights 127kDa and 100kDa. In a subsequent protein preparation of each isolate, only the 127kDa SLP was expressed.

The PFGE types A1, C1, and D1 expressed SLPs of different sizes, depending on the actual isolate, or the preparation of the isolate (Table 6.2). These SLP bands were usually approximately 100kDa, 127kDa, or 149kDa, or combinations of these sizes. However. one isolate of PFGE type C1 expressed SLPs of approximately 85kDa and 100kDa. One isolate out of the eight PFGE type A1 isolates examined did not express an SLP.

There was no evidence of SLP expression of the following PFGE types: A7, B5, C2, E1, F3, and K2. Table 6.2 is a summary of the SLP expression of different isolates of the various PFGE types.

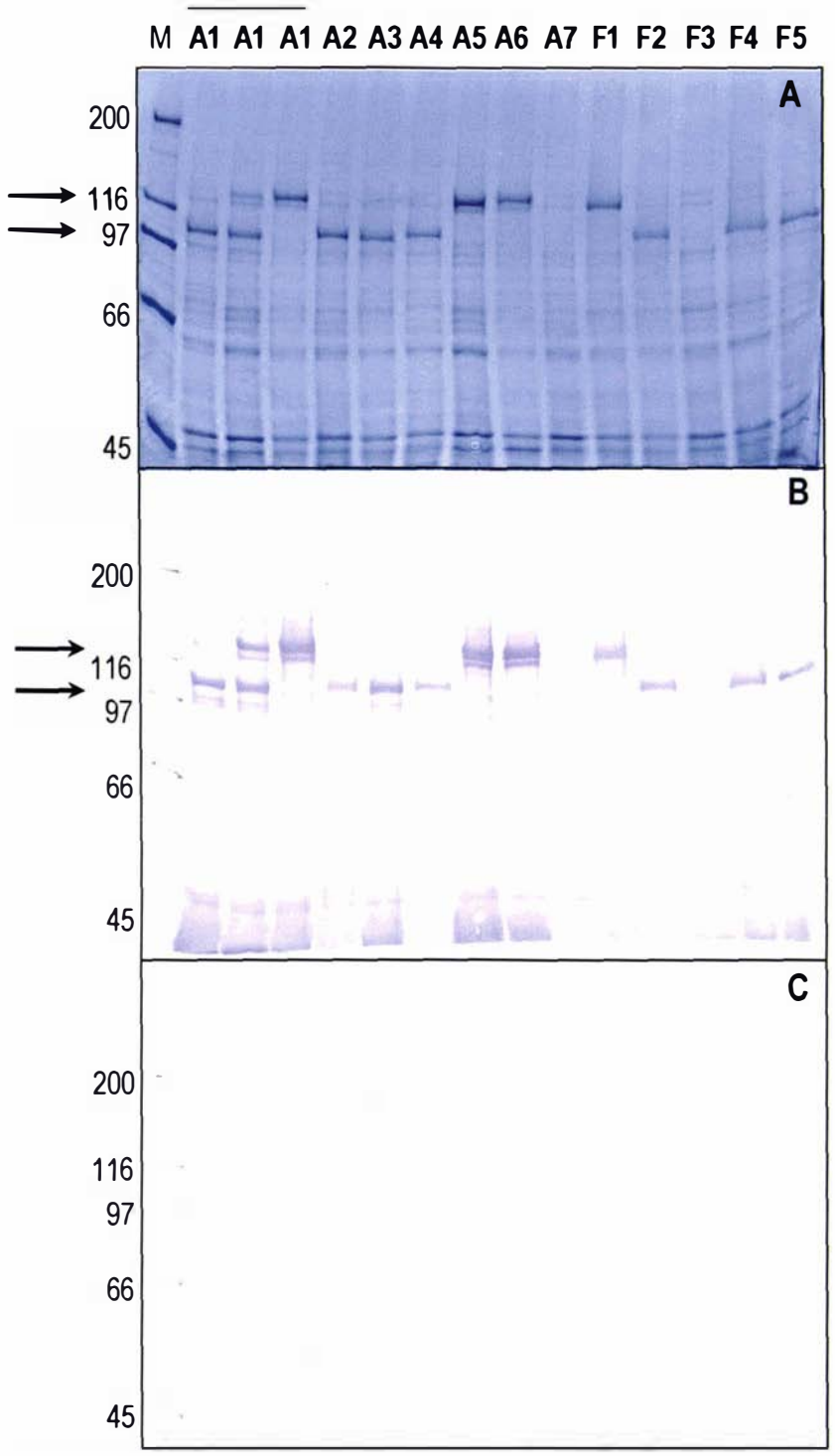


Figure 6.4 SDS-PAGE and Western blotting of PFGE types A1-A7 and F1-F5. Gels were loaded in triplicate: **A** Coomassie-stained SDS-PAGE gel. **B** Western blot using pooled immune sheep sera. **C** Western blot using pooled pre-immune sheep sera. M = molecular weight standards, protein standard bands were labelled with sizes (kDa). SLP bands are indicated with arrows.

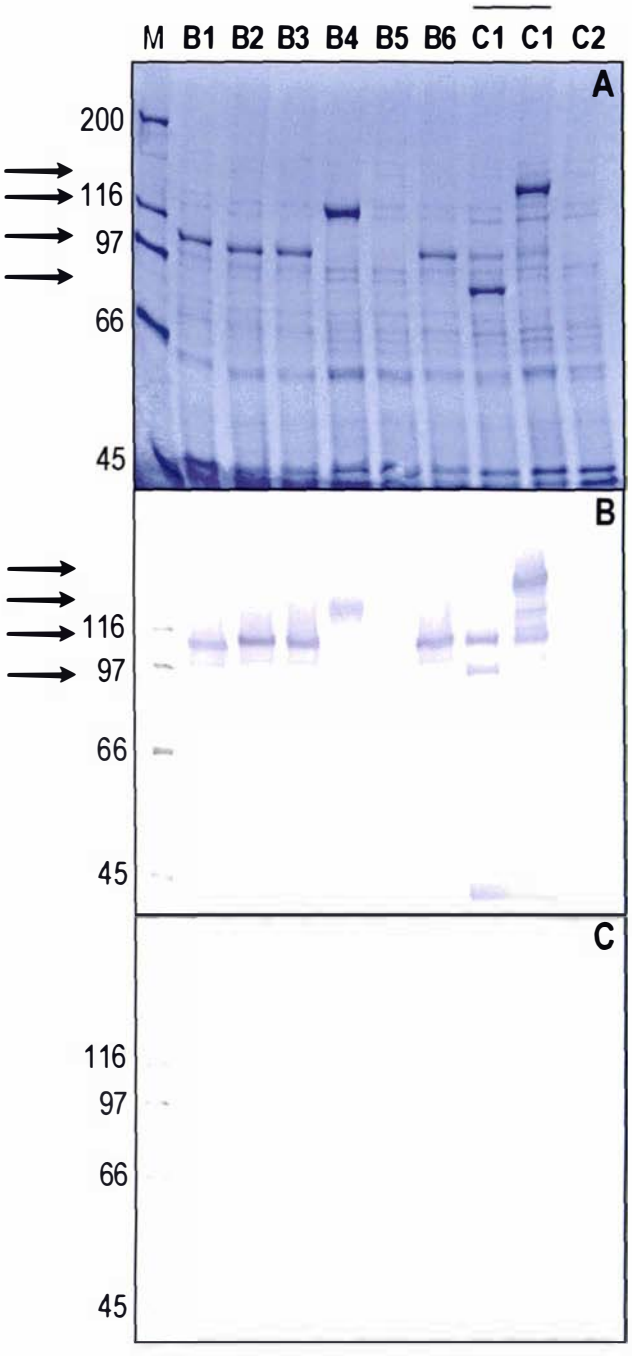


Figure 6.5 SDS-PAGE and Western blotting of PFGE types B1-B6 and C1-C2.
Gels were loaded in triplicate: **A** Coomassie-stained SDS-PAGE gel. **B** Western blot using pooled immune sheep sera. **C** Western blot using pooled pre-immune sheep sera. M = molecular weight standards, protein standard bands were labelled with sizes (kDa). SLP bands are indicated with arrows.

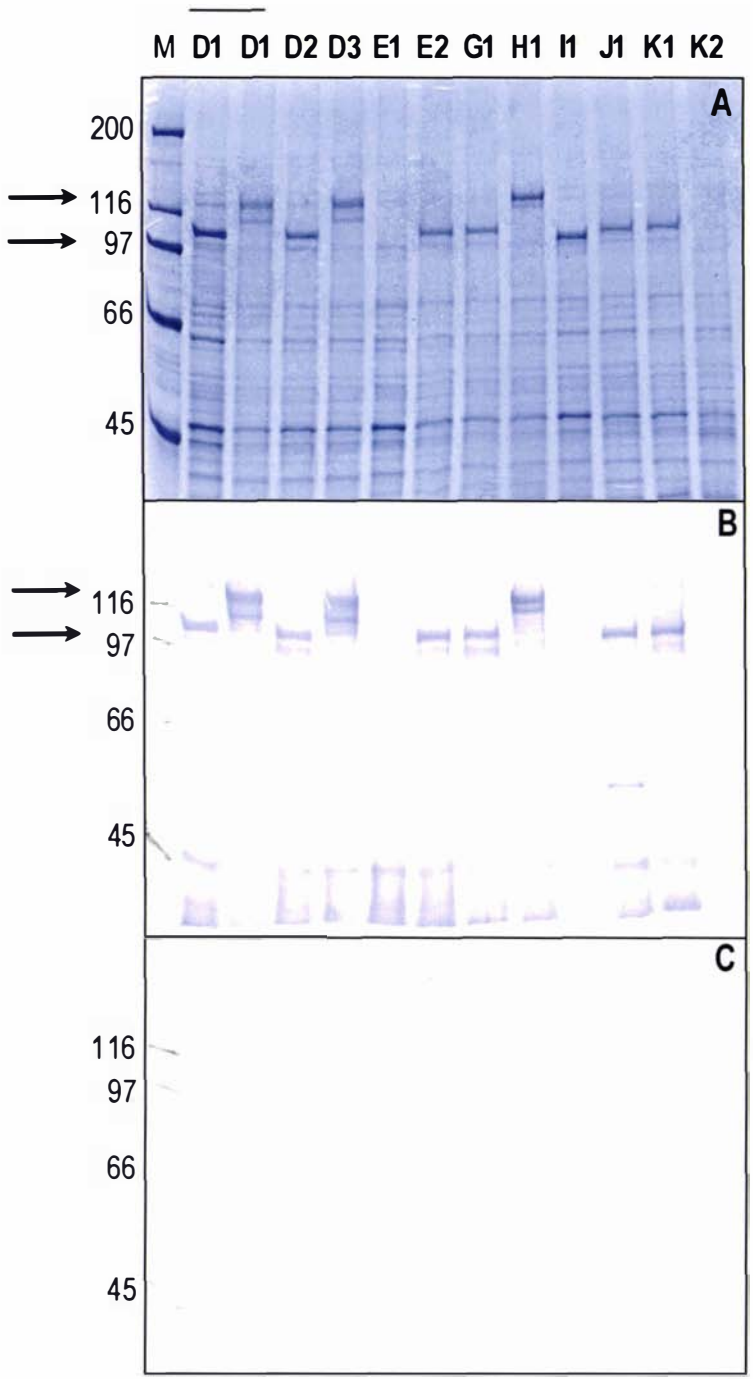


Figure 6.6 SDS-PAGE and Western blotting of PFGE types D1-D3, E1, E2, G1, H1, I1, J1, K1 and K2.

Gels were loaded in triplicate: **A** Coomassie-stained SDS-PAGE gel. **B** Western blot using pooled immune sheep sera. **C** Western blot using pooled pre-immune sheep sera. M = molecular weight standards, protein standard bands were labelled with sizes (kDa). SLP bands are indicated with arrows.

Table 6.2 Summary of SLP expression. The approximate sizes of expressed SLPs of protein preparations of isolates of the various PFGE types are detailed. The number of isolates of each PFGE type in the collection is listed. The number of isolates that were examined by SDS-PAGE is noted, along with the number of protein preparations of the isolates. Notable isolates are identified by name in the final column.

	PFGE type	Approx. SLP size (kDa)	No. isolates available	No. isolates examined	Total no. preps	Isolate name
	A1	100	20		2	5915
	A1	127		Same isolate	1	5915
	A1	100, 127			3	5915
	A1	149, 100		1	1	5915
	A1	100		4	4	
	A1	127		2	2	
	A1	-		1	1	
	A2	100	9	2	4	
	A3	100	1	1	2	
	A4	100	1	1	2	
	A5	127	1	1	2	
	A6	127, 100	1	1	1	REAa
	A6	127			1	
	A7	-	2	2	4	
	B1	100	268	8	13	
	B2	100	25	2	4	
	B3	100	2	1	2	
	B4	127	2	1	3	
	B5	-	1	1	2	
	B6	100	2	1	2	
	C1	85, 100	4	1	2	
	C1	149		1	2	
	C2	-	1	1	2	REAc
	D1	100	18	3	4	
	D1	127		1	2	
	D2	100	1	1	2	
	D3	127, 100	1	1	1	REAf
	D3	127			1	
	E1	-	1	1	2	
	E2	100	2	1	2	
	F1	127	2	1	2	
	F2	100	8	2	3	
	F3	-	1	1	2	
	F4	100	11	4	4	
	F5	100	1	1	2	
	G1	100	1	1	2	
	H1	127	1	1	2	
	I1	100	1	1	2	
	J1	100	1	1	1	
	K1	100	1	1	2	
	K2	-	1	1	3	Type strain
Total:	**31**		**392**	**56**	**101**	

6.3.3 Western blotting using serum from ewes vaccinated with Campylovexin®

Western blotting of protein preparations representative of all the PFGE types, using serum from ewes vaccinated with Campylovexin®, showed antibody binding to the proteins of *C. fetus* subsp. *fetus* (Figures 6.4-6.6 Panel B). Antibodies in the immune serum bound to the expressed SLPs of each of the PFGE types, irrespective of the SLP size. However, antibody binding to the SLP of PFGE type I1 was not as strong as might be expected from the intensity of the protein band in the coomassie stained gel (Figure 6.6, Panels A and B).

In addition to antibody binding of expressed SLP bands, there was antibody binding to other *C. fetus* subsp. *fetus* proteins, in particular to proteins of approximate molecular weights 47kDa and 63kDa, and to proteins in the lower region of the gel <40kDa. There was also antibody binding to these proteins in the isolates that did not express SLP bands.

Western blotting of the PFGE types using pre-immune sheep serum showed some very faintly observed antibody binding to proteins of *C. fetus* subsp. *fetus*, particularly those of approximate molecular weights 63kDa and <45kDa (Figures 6.4-6.6 Panel C).

6.4 Discussion

The sizes of the prominent protein bands in the *C. fetus* subsp. *fetus* protein profiles were consistent with those previously described for *C. fetus* subsp. *fetus* SLPs. The changes in size of the expressed predominant proteins after repeated sub-culturing steps were also consistent with that reported for SLPs. Taken together with the protein sequencing results, the evidence strongly suggests that the identified bands were those of SLPs. No spontaneous mutants lacking SLP expression were generated by continuous sub-culturing.

Pulsed-field gel electrophoresis analysis of the six highly passaged isolates showed that a change in the size of expressed SLPs did not alter the PFGE profile. A change in size of expressed SLP is a function of inversion of the DNA containing the promoter and/or site-specific exchange of SLP gene cassettes (Blaser et al. 1994; Dworkin and Blaser 1996). If the region of DNA involved in the genetic rearrangement does not contain a restriction site, the PFGE profile will remain unaltered, as was the case with the six isolates investigated here.

The characterisation of SLP expression and PFGE type also showed that isolates of different PFGE types may express SLPs of the same size, for example, PFGE types B1 and D1 both expressed SLPs of approximately 100kDa.

Western blotting showed that sheep vaccinated with Campylovexin® produced antibodies that recognised each of the expressed SLPs produced by the PFGE types found in New Zealand. The comparison with Western blotting using pre-immune sera demonstrated that this antibody production was specific to Campylovexin® vaccination. Myers et al. (1970) and Grogono-Thomas et al. (2003) have evidence to suggest that antibodies to SLPs are important in protection against abortion due to *C. fetus* subsp. *fetus*. The results in the current study suggest that vaccination of sheep with Campylovexin® elicits the production of antibodies against all the *C. fetus* subsp. *fetus* strains identified in New Zealand.

The colour intensity of the protein bands on the Western blots suggests that antibody binding was generally strongest with the expressed SLPs compared with the other

proteins. This suggests that SLPs are the immunodominant proteins of the Campylovexin® vaccine and supports previous findings that SLPs are immunodominant proteins *C. fetus* subsp. *fetus* (Dubreuil et al. 1988). However, antibody binding to the PFGE type I1 SLP band was weaker than might be expected from the relative intensity of the SLP band on the stained protein gel. Only one isolate of PFGE type I1 has been identified, so it was not possible to investigate antibody binding with an alternative PFGE type I1 isolate. Antibody binding that was weaker than might be expected from the intensity of the SLP band on stained gels was also detected in one A2 isolate, one B1 isolate and one B2 isolate (data not shown). This may be explained if the vaccine strain was a different serotype to these isolates; either serotype A or B according to an outer membrane lipopolysaccharide. It has been shown that the binding of serum IgG anti-SLP antibodies from sheep challenged with serotype A *C. fetus* subsp. *fetus* was strongest with amino acids 81-100 of the conserved N-terminal region of serotype A SLP (SapA) (Grogono-Thomas et al. 2003). The N-terminal regions of different sized SLPs from serotype A strains are antigenically conserved, and the N-terminal regions of different sized SLPs from serotype B strains are antigenically conserved (Wang et al. 1993). Therefore in the present study, if the Campylovexin® vaccine strain was a different serotype to the isolates for which antibody binding was weaker than might be expected, the reported strong antibody binding with the N-terminal region of SLPs from these isolates would not occur. Nevertheless, the C-terminal regions of SLPs of the same size from serotype A and B strains are antigenically conserved (Wang et al. 1993), and this may explain the (weaker) antibody binding that was observed with the SLPs from these isolates.

Antibodies in serum from vaccinated sheep recognised other *C. fetus* subsp. *fetus* proteins additional to the SLPs. This is consistent with the fact that Campylovexin® is a whole cell vaccine and Western blotting was performed on whole cell protein preparations of *C. fetus* subsp. *fetus*, not purified SLPs. There was antibody binding to proteins corresponding to the reported sizes of major outer membrane proteins (45kDa and 47kDa) and 63kDa flagella proteins (Blaser et al. 1987). There was also antibody binding to proteins in the lower region of the gel, which corresponds to the reported sizes of lipopolysaccharides, 4-20kDa (Varga 1991).

Surface layer proteins of molecular weights 98-100kDa, 125-127kDa and 149kDa are most commonly reported for *C. fetus* (Winter et al. 1978; Blaser et al. 1987; Pei et al. 1988). In this study, the most frequently observed SLP amongst the isolates had an approximate molecular weight of 100kDa. The 127kDa SLP was less commonly found, and SLPs of approximately 149kDa and 85kDa were rarely observed. The relative frequency of the SLP sizes found in this study is the same as that observed by researchers of *C. fetus* subsp. *fetus* sheep abortion isolates in the United Kingdom (Grogono-Thomas et al. 2000).

Occasionally, isolates of the same PFGE type expressed SLPs of different sizes, for example one PFGE type C1 isolate produced SLPs of approximately 85kDa and 100kDa while another isolate expressed a 149kDa SLP. Similarly, different protein preparations of the same 5915 isolate (PFGE type A1) produced SLPs of different approximate sizes; 100kDa, 127kDa, 100 and 127kDa, and 149 and 100kDa. Spontaneous switching of the predominant SLP expressed during *in vitro* passage has been documented previously (Fujimoto et al. 1991; Wang et al. 1993). While these isolates were not subjected to multiple passages once a glycerol stock was prepared, the preparation of protein samples from a glycerol stock necessitates an additional growth step. Each of these SLPs was recognised by antibodies in the immune serum. This is consistent with previous Western Blotting and ELISA experiments which showed that antiserum raised to a purified SLP bound to SLPs of different sizes and from both serotype A and B strains (Pei et al. 1988; Fujimoto et al. 1991; Wang et al. 1993).

There was no evidence of SLP expression in six out of the 31 PFGE types. It has been shown that the S-layer on *C. fetus* subsp. *fetus* cells is essential for producing abortions in sheep (Grogono-Thomas et al. 2000). As each of these *C. fetus* subsp. *fetus* isolates was cultured from an aborted sheep foetus, SLP expression by the isolates may be expected. Five of these PFGE types that appeared not to express an SLP (B5, C2, E1, F3, and K2) were represented by only one isolate in the collection. Therefore, SLP expression from an alternative isolate of the same PFGE type could not be attempted. The remaining PFGE type that appeared to not express an SLP was A7, of which there were two isolates. Neither isolate of PFGE type A7 appeared to express an SLP. The C2, K2 and both A7 isolates were all sourced from the New Zealand Reference Culture Collection, Medical Section (NZRM). One A7 isolate (REA type d) and C2 (REA type

c) had been maintained by sub-culturing from their original isolation until submission to the NZRM (de Lisle et al. 1987). It is possible that the other A7 isolate and the *C. fetus* subsp. *fetus* Type Strain, K2, have also been subjected to multiple sub-culturing steps since their original isolation until submission to the NZRM. Prolonged *in vitro* passaging has been shown to generate spontaneous mutants lacking SLP expression (Blaser et al. 1987). Therefore, the apparent loss of SLP expression in three of the six PFGE types may be due to long-term sub-culturing of these isolates. However, the other three PFGE types that appeared to lack SLP expression (B5, E1, and F3) and the PFGE type A1 isolate that appeared to not express an SLP were sourced from the Hawke's Bay 1999 study and the 2000 national study (Chapters 3 and 4). During the collection of these sheep abortion isolates the sub-culturing steps were kept to a maximum of six. It is possible that spontaneous mutants lacking SLP expression were generated with this number of sub-culture steps, however this has not been reported before. It may also be significant that the isolates of PFGE types B5, C2, E1, F3 and K2 that appeared not to express an SLP were genetically unique in the collection, demonstrated by the fact that they each have a unique PFGE profile. Deletion of the SLP promoter region has been implicated in some spontaneous mutants that do not express SLPs (Tummuru and Blaser 1992; Fujita and Amako 1994). DNA deletion may alter the PFGE profile of an isolate, if the deleted DNA contains a restriction site, or by the reduction in size of a DNA fragment produced after restriction digestion (Tenover et al. 1995). Therefore, these isolates may have lost the ability to produce SLPs through DNA deletion that happened subsequent to the original culture from sheep abortion, and this DNA deletion is reflected in the unique PFGE profile.

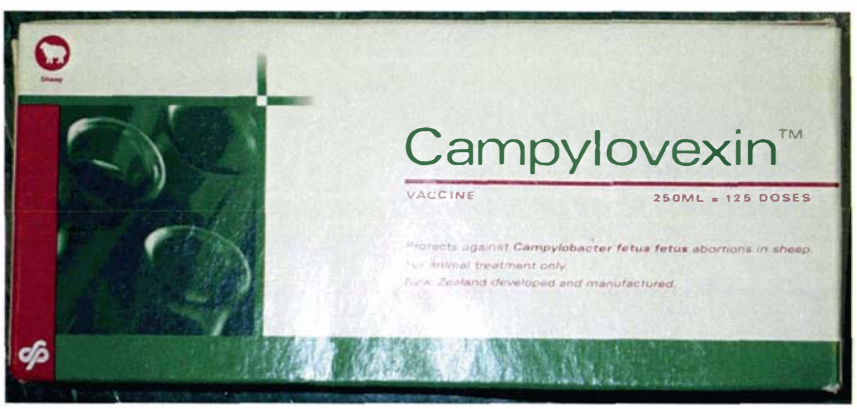
The organisation of the *C. fetus* subsp. *fetus* isolate collection into PFGE types was a convenient way of grouping the isolates. However, as these results show, the relationship between PFGE type and SLP expression is a complex one due to the nebulous nature of SLP expression.

6.5 Conclusion

- Sheep vaccinated with Campylovexin® produced antibodies that recognised expressed SLPs of various sizes produced by each *C. fetus* subsp. *fetus* PFGE type.

Chapter 7

Campylovexin® Breakdown Investigation



Results of this work are published in:

Mannering S, West D and Marchant R (2002) Results of a nation-wide call for suspected cases of Campylovexin® breakdown. *Proceedings of the Sheep and Beef Cattle Society of the New Zealand Veterinary Association* **32** 49-52.

7.1 Introduction

Campylovexin® is the only vaccine available in New Zealand to prevent abortion in sheep due to *C. fetus* subsp. *fetus*. Produced by Schering-Plough Animal Health Ltd., Campylovexin® is a single-strain, killed *C. fetus* subsp. *fetus* vaccine and has been available since the early 1980s. The vaccine strain (strain 5915) was isolated from a field case of sheep abortion in mid-Canterbury (Gumbrell 1985b). It is recommended that unvaccinated ewes be vaccinated before mating, followed by a booster dose 4-8 weeks later. In addition, previously vaccinated ewes should receive an annual booster dose before mating (Schering-Plough Animal Health Ltd. Technical Bulletin).

A number of strains of *C. fetus* subsp. *fetus* have been identified by genotypic and serological studies, which has lead to questions regarding the cross protection offered by the vaccine against other strains (Bird et al. 1984; Collins and de Lisle 1985; de Lisle et al. 1987). For example, the analysis by restriction digestion of 51 *C. fetus* subsp. *fetus* isolates from the 1983 abortion season revealed seven different restriction types (Collins and de Lisle 1985). The most common was REA type b. Of the type b isolates, fourteen isolates reacted with antiserum to the vaccine strain and eighteen did not. Collins and de Lisle (1985) proposed that the type b isolates that were serologically different to the vaccine strain could replace the vaccine strain or be included in the vaccine.

Doubt as to the efficacy of a single-strain vaccine was also prompted in part by the use of serological methods to distinguish strains of *C. fetus* subsp. *fetus* in New Zealand veterinary diagnostic laboratories. Two serotypes were established. One was 5915, the serotype of the vaccine strain and the other serotype was 7438, or "non-vaccine strain" (Jopp 1982). *Campylobacter fetus* subsp. *fetus* isolates from sheep abortions were serotyped as the vaccine strain or non-vaccine strain. In the early 1980s, most of the field isolates were serotyped as the vaccine strain (Pauling 1988). However, by 1987 in the South Island, the percentage of isolates serotyped as the vaccine strain had fallen from 90% to 57-66%. At the same time, the number of isolates serotyped as non-vaccine strain had increased to 24-43% (Pauling 1988).

Despite this concern regarding the efficacy of Campylovexin®, there have been few reports of vaccine failure (Pauling 1988; Marchant 1999). However, in 1996 an apparent vaccine breakdown occurred in the Manawatu region, when fifteen ewes aborted out of a flock of 300 vaccinated two-tooth ewes. *Campylobacter fetus* subsp. *fetus* was isolated from six foetuses, and the isolates were shown to have a different PFGE profile to the vaccine strain. However, it was suggested that the ewes may have been exposed to high *C. fetus* subsp. *fetus* challenge when they were densely stocked each night in a small, "sacrifice" paddock (Fenwick et al. 2000).

The present study was initiated to investigate the significance of vaccine breakdown. Reports of apparent vaccine failure were requested from veterinarians. Campylovexin® is a prescription animal remedy available to farmers only from veterinary practices. Therefore, veterinarians were considered to be the best source of apparent breakdown cases, as farmers that experienced abortions in vaccinated ewes were likely to seek veterinary assistance. This chapter describes the detection and investigation of cases of *C. fetus* subsp. *fetus* abortion in ewes vaccinated with Campylovexin®.

7.2 Materials and Methods

7.2.1 Notification to veterinarians of the Campylovexin® breakdown investigation

In 2001, veterinarians that practice in sheep farming areas were asked to participate in a nation-wide survey for suspected cases of Campylovexin® breakdown. Veterinarians were asked to notify Associate Professor Dave West (IVABS, Massey University) or Dr Roger Marchant (Schering-Plough Animal Health Ltd.) if they became aware of *C. fetus* subsp. *fetus* abortions in sheep that had been vaccinated with Campylovexin®.

The veterinarians were contacted in three ways:

- In May 2001, at the Sheep and Beef Cattle Veterinarians Conference in Christchurch, the conference participants were informed of the Campylovexin® breakdown investigation.
- A notice was placed in the June 2001 newsletter for the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association
- In late June 2001, 187 veterinary practices throughout New Zealand that serviced sheep farmers were contacted by mail.

7.2.2 Investigation of suspected Campylovexin® breakdown cases

Once alerted to a case of suspected Campylovexin® breakdown, the farmer was interviewed by telephone and details of the affected flock were collected.

- Sheep breed, age, number in the flock, and number aborted.
- Campylovexin® vaccination programme on the farm.
- Progression of abortions in the flock.
- Flock management details including stocking rate, grazing system, and lambing date.

If abortions were still occurring in the flock, the aborted foetuses were submitted to the nearest veterinary diagnostic laboratory for a full abortion examination.

7.2.3 *Campylobacter fetus* subsp. *fetus* isolates

The *C. fetus* subsp. *fetus* isolates that were cultured by the veterinary diagnostic laboratories from foetal samples from the suspected Campylovexin® breakdown farms were collected. Subculturing of the isolates was kept to a minimum: a single colony on the initial selective media was sub-cultured onto a Blood Agar plate for purity. Growth from the purity plate was harvested with an Amies with Charcoal Swab and couriered to Massey University, where fresh Blood Agar plates were inoculated from the swabs, and the growth harvested and stored in 15% glycerol at –70°C.

7.2.4 Pulsed-field gel electrophoresis typing of the isolates

Pulsed-field gel electrophoresis was used to type the *C. fetus* subsp. *fetus* isolates, as described in Chapter 2.4: Pulsed-field gel electrophoresis.

7.2.5 Protein profile and serological analysis of the isolates

The protein profiles of the *C. fetus* subsp. *fetus* isolates were examined as described in Chapter 2.5: SDS-PAGE gel electrophoresis.

Pooled sera from the 15 ewes vaccinated with Campylovexin® described in Chapter 2.6, was used in Western blotting, as described in Chapter 2.7. This was to examine the recognition of antibodies in the sera with the proteins of each isolate cultured from flocks with suspected Campylovexin® breakdown.

7.2.6 Isolates from the apparent 1996 Campylovexin® breakdown case

Four *C. fetus* subsp. *fetus* isolates from the apparent Campylovexin® breakdown case in the Manawatu region in 1996 (Fenwick et al. 2000) were obtained. These isolates were also typed using PFGE, the protein profile studied, and the binding of antibodies from ewes vaccinated with Campylovexin® with the proteins of the isolates was examined.

7.3 Results

7.3.1 Three cases of suspected Campylovexin® breakdown were reported

7.3.1.1 Case 1: North Canterbury

In mid-August 2001 a North Canterbury farmer running 1100 Romney ewes on flat to easy rolling country reported abortions in his ewes, some of which had been vaccinated with Campylovexin®. The first abortion was noticed on 12th July, approximately six weeks before lambing was due to start. Abortions continued until lambing started on 25th August, and some lambs were born dead at full-term. Approximately 40 ewes aborted and the farmer thought the number of affected dead lambs was approximately 60, as a number of the ewes were carrying twins. Intensive rotational grazing of the ewes on river flats over winter meant that there was a high stocking density preceding and during the initial abortions.

Campylobacter fetus subsp. *fetus* abortions had been diagnosed on the farm in 1998, so in 1999, 2000 and 2001 the two-tooth ewes had been vaccinated with two doses of Campylovexin® given pre- and post-mating. In 2001, the 291 two-tooth ewes were vaccinated on 30th March and then six weeks later on 14th May. There was no annual booster vaccination administered to previously vaccinated ewes in subsequent years. All 1100 ewes were run together, so the mob consisted of unvaccinated older ewes, six-tooth (four-year-old) ewes vaccinated in 1999, four-tooth (three-year-old) ewes vaccinated in 2000 and two-tooth (two-year-old) ewes vaccinated in 2001.

The abortions occurred across all age groups. The farmer thought that amongst the ewes that aborted, four were two-tooth ewes, six were four-tooth ewes, and five were six-tooth ewes. The remaining ewes that aborted were either older than six-tooth or of unknown age. On 25th August two foetuses that were considered to have come from two-tooth ewes were submitted to the veterinary diagnostic laboratory, Labworks Animal Health. *Campylobacter fetus* subsp. *fetus* was cultured from one foetus and this isolate was sent to Massey University for PFGE typing and serological analysis.

7.3.1.2 Case 2: Southland

In early August 2001, approximately 12 ewes aborted from a flock of 800 two-tooth and mixed-age Coopworth ewes on a farm in Southland. Abortions began about one month before the start of lambing on 4th September, and there were approximately two abortions each day for a week. The ewes were intensively grazed, with a stocking density of 800 ewes per 0.5 ha, and break fed with a daily shift which included supplementation with hay.

Since 1999 the ewe hoggets had been vaccinated with two doses of Campylovexin®, the first before mating and the second at ram withdrawal 6-8 weeks later. Prior to 1999, the two-tooth ewes were vaccinated annually with two doses. The affected mob of 800 ewes consisted of two-tooth ewes vaccinated in 2000 (as hoggets), four-tooth ewes vaccinated in 1999 (as hoggets), and older ewes (vaccinated as two-tooth ewes). No annual booster vaccination was performed in subsequent years. Of the 12 ewes that aborted, the farmer thought that most were two-tooth ewes.

Two aborted foetuses were submitted to the veterinary diagnostic laboratory, LabNet Invermay Ltd. on 7th August. One foetus had circular necrotic lesions on the surface of the liver, consistent with *Campylobacter* abortion (Hartley and Kater 1964; Gill and Clark 2000), and *C. fetus* subsp. *fetus* was cultured from the liver of this foetus. *Campylobacter fetus* subsp. *fetus* was cultured from the stomach contents of the other aborted foetus. This isolate was sent to Massey University for PFGE typing and serological analysis.

7.3.1.3 Case 3: Southland

During August 2001, approximately 15 abortions occurred in a flock of 950 mixed-age Romney ewes on a Southland farm. On 7th August, four abortions were noticed in the flock and there was an additional abortion every day for the next 3-4 days, followed by an occasional abortion. This was about three weeks prior to the start of lambing on 1st September. The ewes were grazed intensively at a density of 950 ewes per 0.5 ha on daily breaks on pasture.

The two-tooth ewes had been vaccinated with two doses of Campylovexin given pre- and post-mating and this policy had been in place for some years. Thus all ewes would

have been vaccinated as two-tooth ewes but this had not been boosted subsequently. It was not known whether any of the ewes that aborted were two-tooth ewes.
Two foetuses were submitted to the veterinary diagnostic laboratory, LabNet Invermay Ltd. on 7th August. *Campylobacter fetus* subsp. *fetus* was isolated from the stomach contents of one foetus. This isolate was sent to Massey University for PFGE typing and serological analysis.

In the continuing investigation of this case, an aborted foetus submitted to LabNet Invermay Ltd. on 31st August resulted in the isolation of *Salmonella* Brandenburg. Approximately half of all the ewes that aborted in this flock died within two days of aborting, which suggests that *Salmonella* Brandenburg may have been a significant cause of abortion in this flock.

7.3.2 Pulsed-field gel electrophoresis typing of the *C. fetus* subsp. *fetus* isolates from the case farms

One *C. fetus* subsp. *fetus* isolate was collected from each of the three farms with suspected Campylovexin® breakdown abortions. The isolate from Case 1 was PFGE type C1. The isolates from Cases 2 and 3 were both PFGE type A1, the same type as the vaccine strain (Figure 7.1).

7.3.3 Protein and serological analysis of the *C. fetus* subsp. *fetus* isolates from the case farms

The protein profile of the *C. fetus* subsp. *fetus* isolates obtained from the three case farms showed that each of the isolates had a dominant Surface Layer Protein (SLP) band of approximately 100kDa (Figure 7.2A). When sera collected from 15 ewes vaccinated with Campylovexin® was used in Western blotting, there was antibody binding to the proteins of the *C. fetus* subsp. *fetus* isolates including the SLP bands (Figure 7.2B). In addition, Western blotting revealed minor SLP bands of approximately 127 and 149kDa in the protein profile of the Case 1 isolate, a minor 149kDa SLP band in the protein profile of the Case 2 isolate, and a minor 127kDa SLP band in the profile of the Case 3 isolate. In contrast, Western blotting of the three isolates using pre-immune sheep serum showed no antibody binding to the proteins of *C. fetus* subsp. *fetus* (Figure 7.2C).

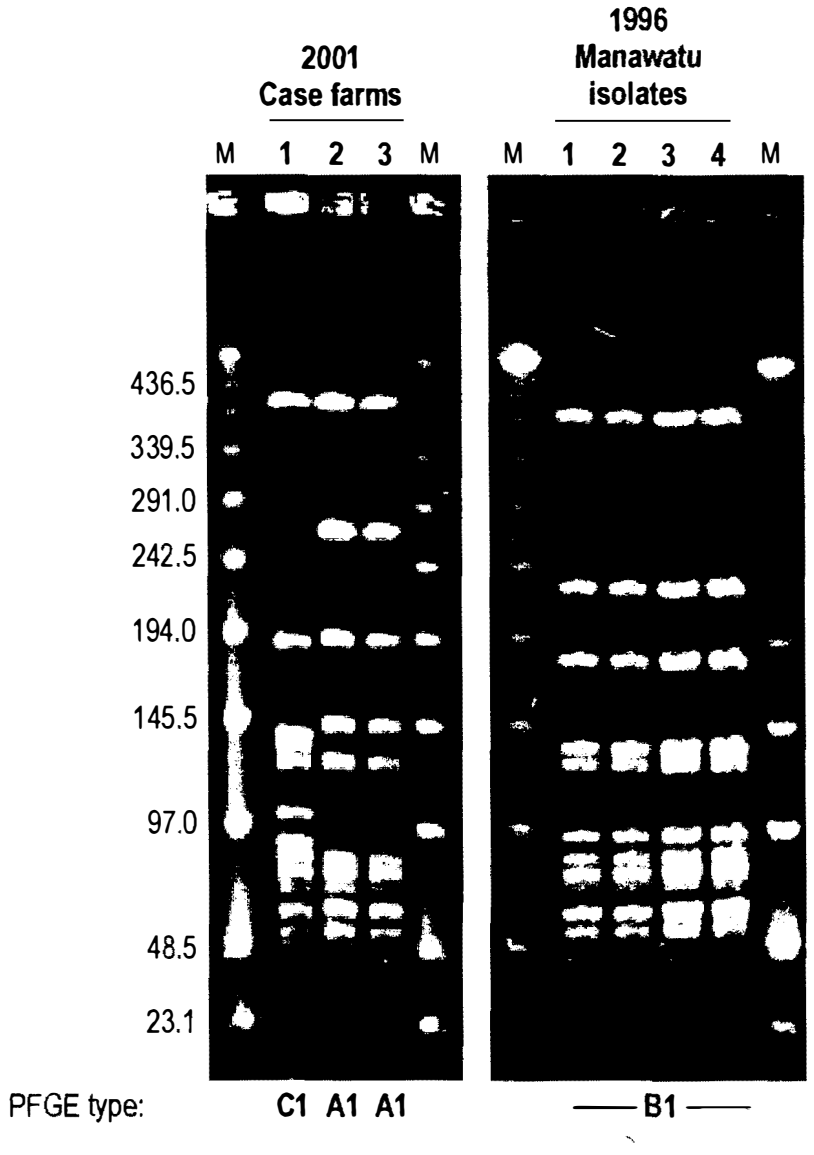


Figure 7.1 Pulsed-field gel electrophoresis using the restriction enzyme *Sma*I of the *C. fetus* subsp. *fetus* isolates from Cases 1-3 from 2001 and the isolates from the 1996 Manawatu case (Fenwick et al. 2000). M = molecular size marker (lambda ladder), DNA standard bands are labelled with sizes (kb).

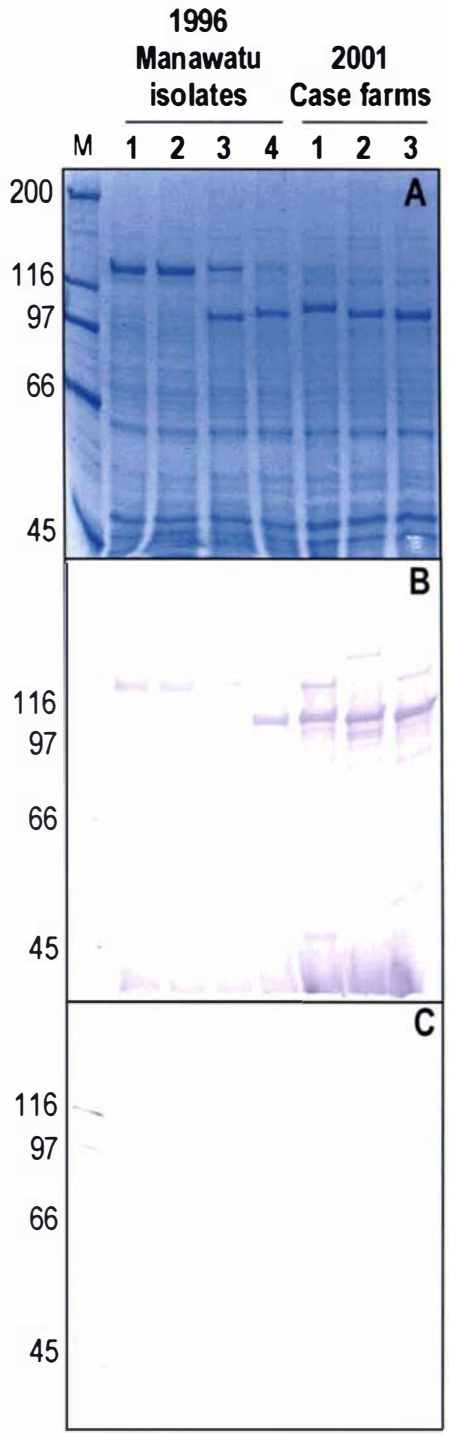


Figure 7.2 Protein profile and serological analysis of the isolates from Cases 1-3 from 2001 and the isolates from the 1996 Manawatu case (Fenwick et al. 2000). Gels were loaded in triplicate: **A** Coomassie-stained SDS-PAGE gel. **B** Western blot using pooled immune sheep sera. **C** Western blot using pooled pre-immune sheep sera. M = molecular weight standards, protein standard bands were labelled with sizes (kDa).

7.3.4 Pulsed-field gel electrophoresis and protein results of the isolates from the apparent 1996 Campylovexin® breakdown case

The four *C. fetus* subsp. *fetus* isolates from the apparent 1996 Campylovexin® breakdown case (Fenwick et al. 2000) were all PFGE type B1 (Figure 7.1).

The protein profile of these four isolates showed that two of the isolates had a major SLP band of approximately 127kDa. The third isolate had two main SLP bands of approximately 100kDa and 127kDa. The fourth isolate had a major SLP band of approximately 100kDa and a weaker SLP band of approximately 127kDa (Figure 7.2A).

When sera from ewes vaccinated with Campylovexin® was used in Western blotting, there was antibody binding to the proteins of each isolate, including the SLP bands (Figure 7.2B). Antibody binding to the two SLP bands of isolate 3 was not as strong as might be expected from the intensity of the protein bands in the coomassie stained gel. Western blotting of the four isolates using pre-immune sheep serum showed no antibody binding to the proteins of *C. fetus* subsp. *fetus* (Figure 7.2C).

7.4 Discussion

A large effort was made in 2001 to alert New Zealand veterinarians involved with the sheep industry of the investigation into potential Campylovexin® breakdowns. Given the widespread use of the Campylovexin® vaccine, it is significant that only three suspected cases were reported.

Of the three cases, Case 1 in North Canterbury was perhaps the most significant because it involved ewes that had been vaccinated with Campylovexin® that year. Although abortions occurred in all age groups, the farmer believed that four two-tooth ewes were amongst the 40 ewes that aborted. The 291 two-tooth ewes had been vaccinated with two doses of Campylovexin® that season. *Campylobacter fetus* subsp. *fetus* was cultured from an aborted foetus believed to have come from a two-tooth ewe. Similar to the report of Fenwick et al. (2000), the ewes were stocked at a high density on a daily break, a practice which has been shown to be a risk factor for abortion (Quinlivan and Jopp 1982). In this case vaccinated two-tooth ewes were stocked at a high density with unvaccinated older ewes. Under these circumstances the challenge to vaccinated ewes may have been high due to close confinement with aborting unvaccinated ewes. It is possible that in these circumstances, without vaccination, many more ewes would have aborted.

Cases 2 and 3 from Southland both involved abortion in ewes that had not been vaccinated in the current year. Case 2 involved about 12 out of 800 two-tooth and mixed-age ewes that had been vaccinated as hoggets. Case 3 involved about 15 out of 950 mixed-age ewes that had been vaccinated in previous years as two-tooth ewes. The manufacturer recommends annual boosting to provide solid protection, and this had not been done in these two cases. As in Case 1, there may have been a high challenge due to the high stocking density. It appears that high stocking density may overwhelm immunity to *Campylobacter* disease. This may be due to increased social and food stresses, potentially greater faecal shedding of *C. fetus* subsp. *fetus* due to stress, and the exposure of a large number of ewes to any aborted foetuses due to the close proximity of the animals. Combined with the slight immune suppression necessary in pregnancy, it appears that high stocking density may be an important factor in the production of *C.*

fetus subsp. *fetus* abortions in vaccinated ewes. The incidence of ewe death and the diagnosis of *Salmonella* Brandenburg in Case 3 indicated that this was an additional cause of abortion in this flock.

The *C. fetus* subsp. *fetus* isolates from the Southland cases (Cases 2 and 3) were PFGE type A1, the same type as the vaccine strain. This PFGE type was commonly found on Southland farms in the national survey in 2000 (Chapter 4), when it was isolated from seven out of 45 (16%) farms with *C. fetus* subsp. *fetus* abortions. Pulsed-field type C1 found in Case 1 from Canterbury has been isolated less frequently and has only previously been detected in the Hawke's Bay (Chapter 4). Pulsed-field type C1 was isolated from one out of 30 (3%) Hawke's Bay farms with *C. fetus* subsp. *fetus* abortions in 1999 and one out of 32 Hawke's Bay farms in 2000. The fact that this PFGE type has now been identified from a farm in Canterbury is not likely to be significant or indicate spread of this type, given the relatively small number of *C. fetus* subsp. *fetus* isolates that have been typed in each region. The isolates from the apparent 1996 Campylovexin® breakdown case in the Manawatu (Fenwick et al. 2000) were PFGE type B1. This was the most common PFGE type found in each region in the national survey, and was found on 24 out of 26 (92%) Manawatu farms with *C. fetus* subsp. *fetus* abortions in 2000 (Chapter 4). It would appear that there was not a single *C. fetus* subsp. *fetus* PFGE type associated with Campylovexin® breakdown. In addition, with the exception of the Canterbury case, the PFGE types isolated from *C. fetus* subsp. *fetus* abortions in vaccinated ewes were known to be common in the region.

The protein profiles of the isolates from apparent vaccine breakdown appeared similar to the protein profiles obtained for other *C. fetus* subsp. *fetus* isolates (Chapter 6). The SLP bands were of the same approximate molecular weights that have been commonly reported: 100, 127 and 149kDa (Chapter 6; Winter et al. 1978; Blaser et al. 1987; Pei et al. 1988; Grogono-Thomas et al. 2000). The PFGE type A1 isolates from Cases 2 and 3 both had major SLP bands of 100kDa, and Western blotting revealed minor SLP bands of 149 and 127kDa respectively. This SLP expression has been observed previously in other PFGE type A1 isolates (Chapter 6). However, the Case 1 isolate of PFGE type C1 also expressed a dominant SLP band of 100kDa, with minor SLP bands of both 127 and 149kDa. This SLP expression was not observed in either of the two PFGE type C1 isolates previously examined (Chapter 6). Instead, one of these isolates expressed a

major SLP band of 149kDa and minor bands of 100 and 127kDa, and the other expressed two SLP bands of 85 and 100kDa (Chapter 6). Similarly, three of the PFGE type B1 isolates from the apparent 1996 Campylovexin® breakdown case in the Manawatu (Fenwick et al. 2000) expressed dominant SLP bands of atypical sizes for this PFGE type; two isolates expressed a dominant SLP of approximately 127kDa and the third expressed two SLP bands of 100 and 127kDa. In contrast, the eight PFGE type B1 isolates examined in Chapter 6 all expressed a dominant SLP band of approximately 100kDa. It is not known why some of the isolates from apparent vaccine breakdown had SLP expression atypical to that previously observed for the respective PFGE type. One explanation could be that the protein profile of only a small number of isolates of each PFGE type has been examined, and more variability would be observed with the examination of more isolates. This may be the case for the PFGE type C1 isolates at least, as both isolates examined in Chapter 6 had different SLP expression, which differed again from that of the Case 1 isolate. Alternatively, as these isolates were obtained from the foetuses of apparently vaccinated ewes, it is possible that the SLP expression of these strains had changed as a result of the ewes' immune response. Nevertheless, just as there was not a single PFGE type associated with *C. fetus* subsp. *fetus* abortions in vaccinated ewes, there was not an SLP of a particular size expressed by isolates associated with these abortions. This suggests that despite the potential selective pressure of a vaccine on the *C. fetus* subsp. *fetus* population, there has not been the emergence of a strain of *C. fetus* subsp. *fetus* that expresses an SLP able to evade the immunity of vaccinated ewes. This is supported by the Western blotting data using pooled sera from Campylovexin® vaccinated ewes. Antibodies in the sera recognised the major protein bands of each of the isolates from the apparent vaccine breakdowns, including the SLP bands. These antibodies were developed after vaccination, as Western blotting using pre-immune sera shows no antibody binding to the isolates' proteins. This suggests that the proteins, including the SLPs, of these breakdown isolates were able to be recognised by immune animals. The studies of Myers et al. (1970) and Grogono-Thomas et al. (2003) presented evidence to suggest that antibodies to SLPs are important in protection against abortion due to *C. fetus* subsp. *fetus*.

Nevertheless, it seems that in a small number of ewes on a few farms, the immunity of the ewes against *C. fetus* subsp. *fetus* abortion appears to have been overcome. The importance of high stocking density in overwhelming the immunity to disease has

already been discussed. In addition, although the ewes had apparently been initially vaccinated by the farmers according to the manufacturer's recommendations, it can not be verified that all ewes received both doses of the vaccine administered correctly. The combination of incomplete immunity in some ewes with factors such as high stocking density may have the potential to allow abortions in an apparently vaccinated flock.

Ideally, when investigating abortion in vaccinated flocks, the cause of each abortion in the flock should be examined. Due to the time delay between the start of abortions and the diagnosis of *C. fetus* subsp. *fetus*, and the further time delay between diagnosis and notification from the veterinarian, this was not possible. The diagnosis of *C. fetus* subsp. *fetus* abortion can only be assigned with any degree of certainty to the actual foetuses from which *C. fetus* subsp. *fetus* was isolated. This is illustrated by the late diagnosis of *Salmonella* Brandenburg from the Case 3 flock. It is impossible to know now how many of the 15 abortions in this flock were associated with *C. fetus* subsp. *fetus* infection and how many were associated with *S.* Brandenburg. However, as approximately half of the ewes died soon after abortion, and as ewe death is not a normal outcome of *C. fetus* subsp. *fetus* abortion but is often associated with *S.* Brandenburg abortion (Roe 1999), it is reasonable to assume that *S.* Brandenburg was responsible for a significant proportion of the abortions on this farm.

7.5 Conclusions

- It appears that despite the widespread occurrence of distinct strains of *C. fetus* subsp. *fetus* (Chapter 4), abortion due to *C. fetus* subsp. *fetus* is a rare event in ewes vaccinated with Campylovexin®.

- Despite efforts to solicit reports of Campylovexin® failure, only one case was reported in which ewes that had been apparently vaccinated in the 2001 season subsequently aborted due to *C. fetus* subsp. *fetus*. The management of this flock meant that there was likely to have been a high challenge to vaccinated ewes from unvaccinated aborting ewes.

- It appears that there is not a single *C. fetus* subsp. *fetus* PFGE type associated with abortions in ewes vaccinated with Campylovexin®. In two of the three cases from 2001, the PFGE type of the isolated *C. fetus* subsp. *fetus* was the same as the vaccine strain.

- There was not a particular SLP type expressed by *C. fetus* subsp. *fetus* isolates from abortions in ewes vaccinated with Campylovexin®. The SLPs of the breakdown isolates were recognised by antibodies in pooled immune sera, indicating that the SLPs of these breakdown isolates were able to be recognised by immune animals.

- Some flocks operated with high risk management procedures such as high stocking density may require annual boosting of immunity, as recommended by the manufacturer.

Chapter 8

Campylovexin® efficacy in the pregnant guinea pig model

8.1 Introduction

A number of PFGE types were identified from the Hawke's Bay and national surveys (Chapters 3 and 4), and the most common type was found to be B1. This PFGE type was also associated with a report of abortions in ewes that had apparently been vaccinated with Campylovexin® (Fenwick et al. 2000; Chapter 7), which raised concerns regarding the cross-protection offered by the vaccine against the B1 strain. *In vitro* studies using serum from vaccinated ewes resulted in consistent recognition of SLPs of each PFGE type (Chapter 6), but it was considered necessary to support this finding with an *in vivo* challenge experiment. It would perhaps have been ideal to investigate the protection offered by Campylovexin® against each of the *C. fetus* subsp. *fetus* PFGE types found in New Zealand, however, animal ethics considerations and the practicalities of animal work determined that the vaccine strain and one other strain be selected for testing. Pulsed-field gel electrophoresis type B1 was chosen because of its prevalence and the report of abortions in vaccinated ewes in 1996.

Despite the occurrence of *Campylobacter* abortions in sheep, this species may not be an ideal model for investigation of *Campylobacter* abortion and vaccine protection. Challenge trials using sheep have not given reproducible results, as two *C. fetus* subsp. *fetus* challenge trials of unvaccinated sheep that differed only by three weeks of gestation in the ewes produced abortion rates of 91% and 20% (Grogono-Thomas et al. 2000). Similarly, other subcutaneous challenge trials of unvaccinated sheep resulted in 50-63% abortions (Grogono-Thomas and Woodland 1996) and 20% abortions (Grogono-Thomas et al. 2003). In addition, sheep are generally seasonal breeders with oestrus activity occurring in autumn and sheep have a relatively long gestation period.

A *C. fetus* subsp. *fetus* challenge model was reported in mice in 1990 (Pei and Blaser 1990), however this model was restricted to the comparison of strain virulence and has not been established as a model to investigate vaccine efficacy. Although rabbits are frequently used for the production of antiserum for *Campylobacter* research (Marsh and Firehammer 1953; Williams et al. 1976; Bryner et al. 1978; Bird et al. 1984; Yrios and Balish 1986b; de Lisle et al. 1987; Pei and Blaser 1990; Diker and Turutoglu 1995;

Grogono-Thomas et al. 2000), they have not been established as a model of *C. fetus* subsp. *fetus* disease.

The first detailed study using pregnant guinea pigs as a model for *Campylobacter* abortions was in 1953 (Ristic and Morse 1953). Since then, abortion in pregnant guinea pigs has been the principal animal model used for testing the efficacy of *Campylobacter* vaccines (Bryner et al. 1978, 1979, and 1988; Diker and Turutoglu 1995) or the virulence of strains (SultanDosa et al. 1983; Taylor and Bryner 1984; Coid et al. 1987). As guinea pigs have a short gestation time (63-69 days), are susceptible to *C. fetus* subsp. *fetus* abortions, can be effectively immunised against abortion, and a vaccine can be evaluated in guinea pigs in three months, they are a useful model for testing *C. fetus* subsp. *fetus* vaccine efficacy (Bryner et al. 1978 and 1979).

Typically, using this model guinea pigs are 3-5 months old and weigh 600-770g when mated. Challenge with *C. fetus* subsp. *fetus* is performed in the third or fourth week of pregnancy. An intraperitoneal injection of 100×Minimum Abortive $Dose_{50}$ ($100\times MAD_{50}$) has been the standard challenge used for assessing the efficacy of a vaccine, where the MAD_{50} is the minimum number of viable cells needed to produce abortion in 50% of unvaccinated guinea pigs (Bryner et al. 1988; Diker and Turutoglu 1995). Guinea pig challenge studies using various *C. fetus* subsp. *fetus* strains have determined MAD_{50} values of between 100-1000 colony forming units (145 cfu by Bryner et al. 1979; 1000 cfu by Bryner et al. 1978; 100-1000 cfu by Bryner et al. 1988; and 1000 cfu by Diker and Turutoglu 1995). Abortion is preceded by vaginal bleeding and takes place 1-12 days post-challenge. In order to ensure detection of abortion, guinea pigs should be weighed daily and lose on average ≥50g upon abortion (Bryner et al. 1978 and 1979; SultanDosa et al. 1983; Diker and Turutoglu 1995).

This chapter describes the use of pregnant guinea pigs as a model, to determine the *in vivo* protection offered by the Campylovexin® vaccine against PFGE type B1 compared to the vaccine strain.

8.2 Materials and Methods

8.2.1 Animals

Female Duncan Hartley pigmented and albino guinea pigs were sourced from University of Otago Laboratory Animal Sciences. Male Duncan Hartley albino guinea pigs were sourced from the Schering-Plough Animal Health Ltd. (SPAH) Animal Breeding Unit. The same four males were used in each experiment and at the time of mating in the first experiment were four months old. All guinea pigs were individually identified (natural markings or raddle spray if necessary).

8.2.2 Animal Ethics

Approval for this work was gained from the Massey University Animal Ethics Committee. Protocol number: 01/99.

8.2.3 Housing and husbandry

Guinea pigs were housed at the Massey University Small Animal Production Unit according to conditions specified by The Universities Federation for Animal Welfare (UFAW) Handbook on the Care and Management of Laboratory Animals (Poole 1986) and The Biology of the Guinea Pig (Wagner and Manning 1976). The guinea pigs were housed in a room with the temperature set at 21°C and a light-dark cycle of 12 hours. Each cage floor measured 116 × 44 cm, which could house a maximum of eight animals according to the specifications of Wagner and Manning (1976). The cages were plastic on five sides with wire doors. The floor had drainage holes and beneath each cage were drip trays with sawdust to collect and absorb waste. Each cage had plastic tunnels for guinea pigs to hide in (Figure 8.1). Cages were washed with Virkon® disinfectant (Antec International Ltd., Suffolk, UK) each week. Guinea pigs had *ad libitum* access to water and guinea pig feed sourced from SPAH. Hay was excluded in case of possible *Campylobacter* contamination.

8.2.4 Guinea pig bodyweight monitoring

Guinea pigs in all experiments were weighed at least weekly until challenge to monitor health; thereafter they were weighed daily. Weight was measured using a Navigator™ Balance (Ohaus Corporation, Florham Park, NJ, USA), using the Animal Weighing function set at the activity level of 2 (range is 0-3, where 3 is very active).

The normal daily fluctuation of guinea pig bodyweight was measured by weighing all 25 guinea pigs in the first experiment daily for 36 consecutive days prior to pregnancy detection (Figure 8.2). This was to establish the ordinary weight fluctuation of guinea pigs, as one of the reported abortion indicators was an average sudden weight loss of ≥50g (Bryner et al. 1978 and 1979; SultanDosa et al. 1983; Diker and Turutoglu 1995). It was found that guinea pig weight fluctuated markedly from one day to the next. On four occasions out of 900 (36 days × 25 guinea pigs) as much as 40g was lost in 24 hours by healthy, growing guinea pigs (Figure 8.3).

Figure 8.1 Guinea pig cage, showing feed hoppers and drink bottles, tunnels, plastic floor with drainage holes and drip trays.

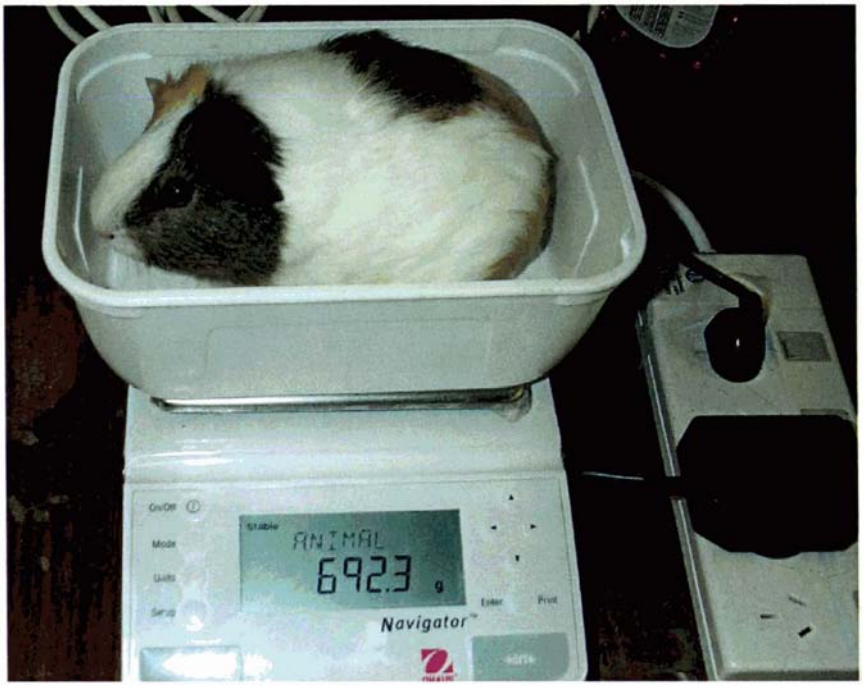


Figure 8.2 Guinea pig weighing.

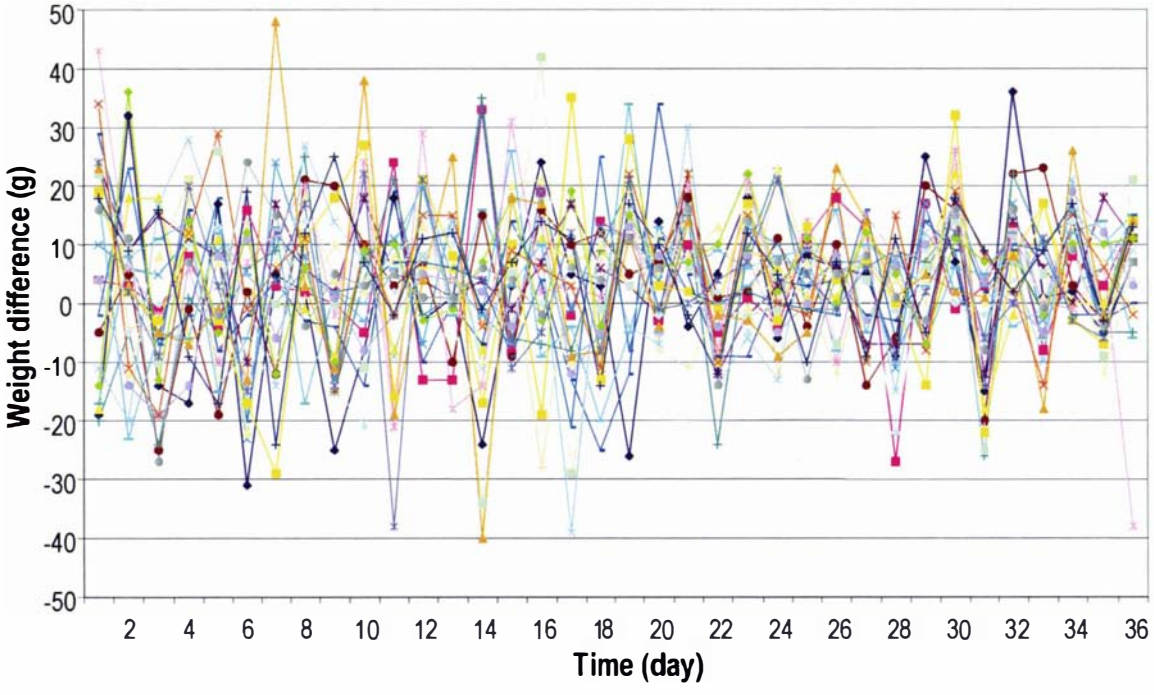


Figure 8.3 Difference in the daily weight of 25 guinea pigs for 36 consecutive days. On four occasions out of 900 (days 11, 14, 17 and 36) approximately 40g was lost in 24 hours by healthy, growing guinea pigs.

8.3 Determination of MAD_{50} for A1 and B1

Initially, the dose of the *C. fetus* subsp. *fetus* vaccine strain (PFGE type A1) that induced abortion in half of the guinea pigs (MAD_{50}) was determined. With this information, challenge of vaccinated and unvaccinated guinea pigs was undertaken, using the same strain (PFGE type A1) at a dose rate of $100\times MAD_{50}$ (Section 8.4).

The same two experiments were performed with PFGE type B1 as the challenge strain. Firstly, the MAD_{50} was determined for PFGE type B1. Then challenge of the vaccinated and unvaccinated guinea pigs was undertaken with $100\times MAD_{50}$ PFGE type B1 (Section 8.4).

8.3.1 Determination of MAD_{50} for A1

8.3.1.1 Animals

Twenty-five 2-4 month old female and four male guinea pigs.

8.3.1.2 *Campylobacter fetus* subsp. *fetus* strain

Pulsed-field gel electrophoresis type A1, Campylovexin® vaccine strain 5915 sourced from SPAH.

8.3.1.3 Method

8.3.1.3.1 *Mating and pregnancy detection*

After a period of 28 days to acclimatise and individually identify the guinea pigs, the 25 female guinea pigs were randomly assigned to one of four mating groups (Figure 8.4). Each of the males was randomly assigned to one mating group and housed with that group for 14 days. Pregnancy was detected after a further 18-21 days by ultrasound (Ultramark 9 Ultrasound System, Advanced Technology Laboratory Australia Pty. Ltd., Dee Why, NSW, Australia) using a Linear Array 5-10 probe. For this, the abdomen of each guinea pig was shaved while the guinea pigs were held. Lubricating gel was applied to the shaved area and the probe was moved across the abdomen (Figure 8.6). Generally, placentae, foetuses and foetal heart beats could be readily detected in pregnant guinea pigs (Figure 8.7). The pregnant guinea pigs were randomly assigned to one of three challenge groups (A, B, and C).

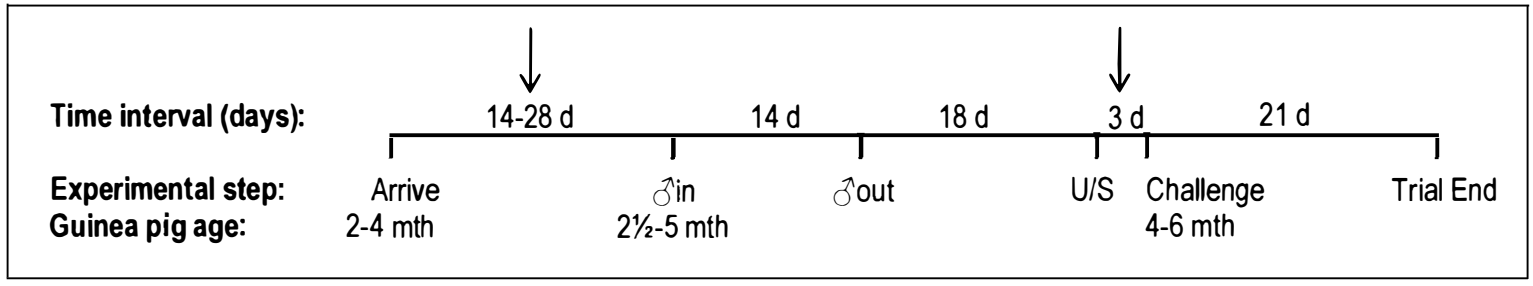


Figure 8.4 Timeline for both the A1 and B1 MAD_{50} determination experiments. The time interval between each step in the experiment is shown in days. The ages of the guinea pigs at arrival, at mating and at challenge are indicated in months. U/S designates ultrasound detection of pregnancy. The arrows indicate the stages that guinea pigs were randomised into different groups.

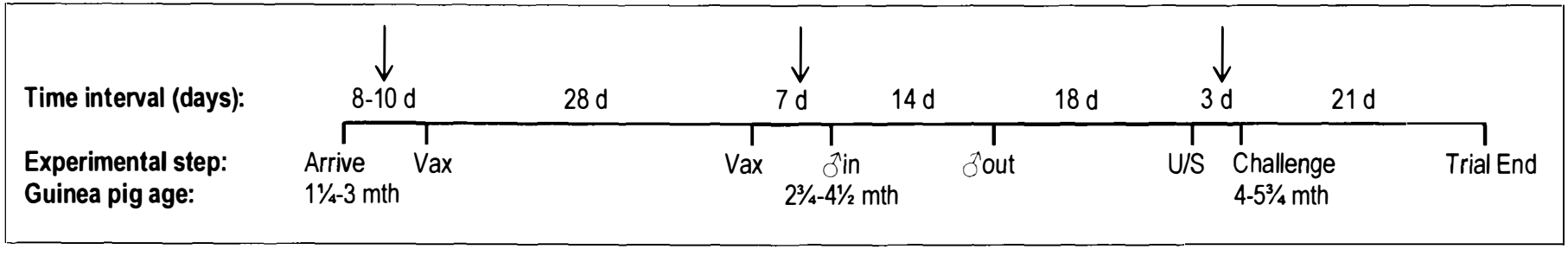


Figure 8.5 Timeline for both the A1 and B1 vaccine challenge experiments. The time interval between each step in the experiment is shown in days. Vax designates vaccination with Campylovexin. The ages of the guinea pigs at arrival, at mating and at challenge are indicated in months. U/S designates ultrasound detection of pregnancy. The arrows indicate the stages that guinea pigs were randomised into different groups. The difference between this timeline and that for the MAD_{50} determination experiments (Figure 8.4) is the initial period during which half the guinea pigs were vaccinated.

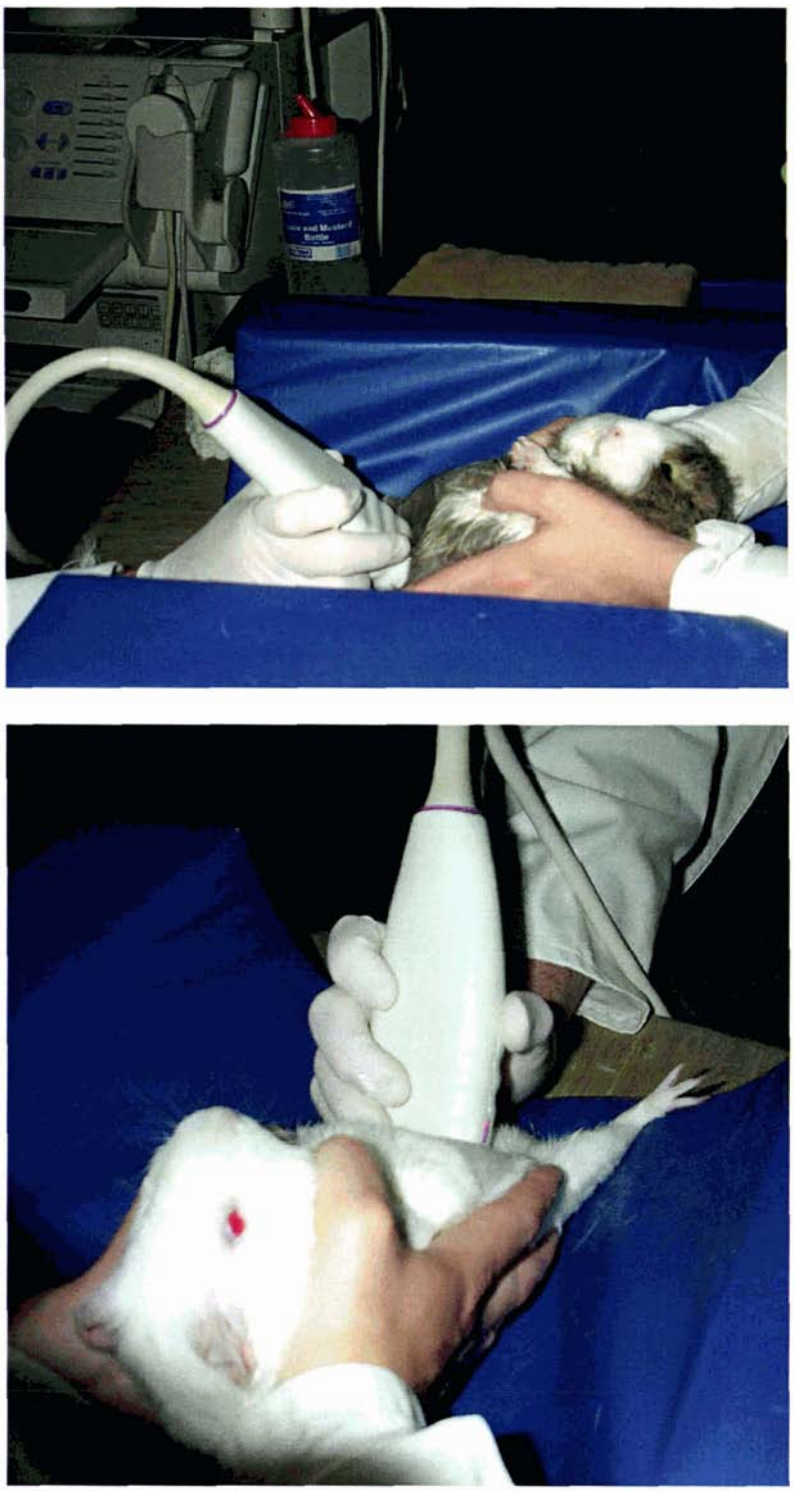


Figure 8.6 Ultrasound scanning of guinea pigs to detect pregnancy using a Linear Array 5-10 probe with the Ultramark 9 Ultrasound System (Advanced Technology Laboratory Australia Pty. Ltd., Dee Why, NSW, Australia). At 18-21 days from the end of the mating period, the abdomen of each guinea pig was shaved, lubricating gel was applied to the shaved area, and the probe was moved across the abdomen while the guinea pigs were held. Generally, placentae, foetuses and foetal heart beats could be readily detected in pregnant guinea pigs.

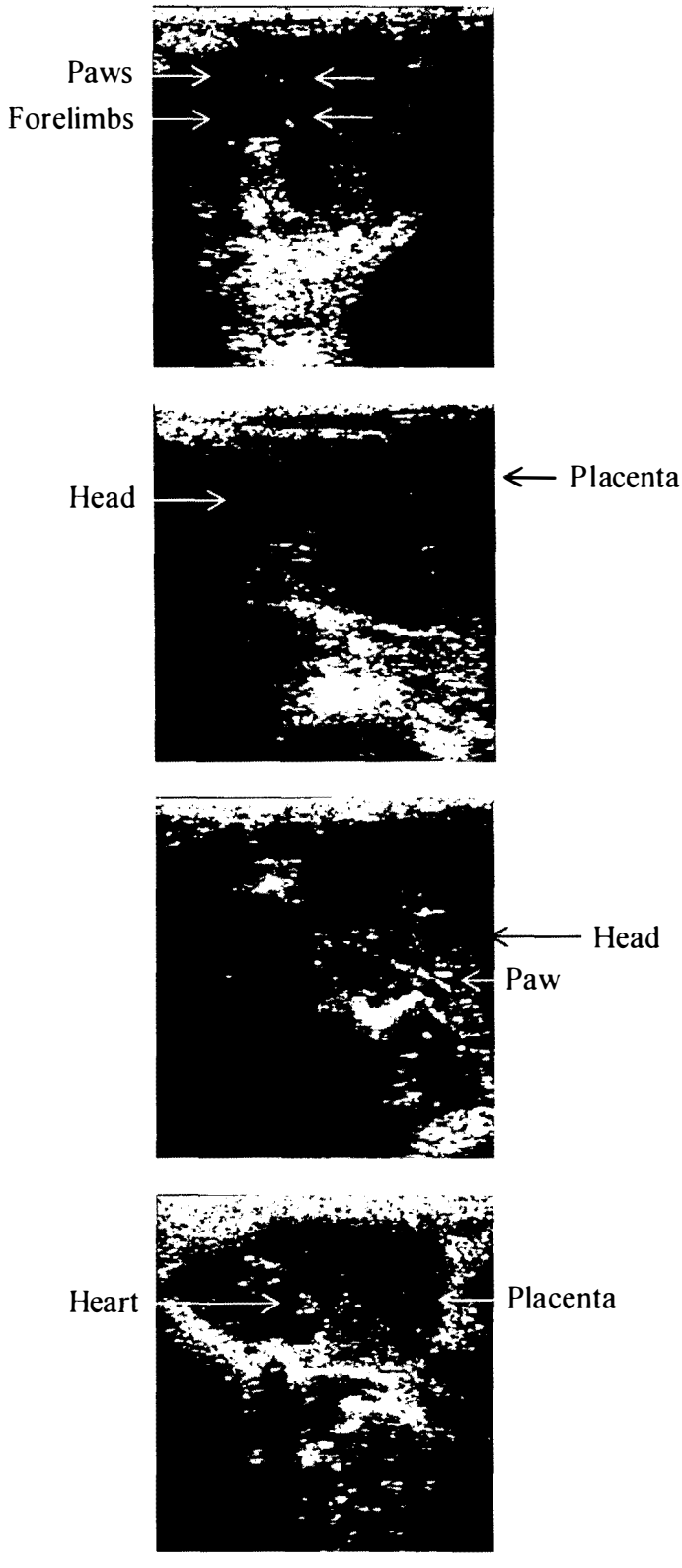


Figure 8.7 Ultrasound photographs of guinea pig foetuses and placentae after 3-5 weeks gestation.

8.3.1.3.2 *Preparation of challenge inoculum*

Preparation of the challenge inocula started four days prior to the day of challenge. The challenge strain was grown on blood agar from the frozen glycerol stock, then sub-cultured once onto fresh blood agar to ensure purity. Growth after 48 hours on blood agar was harvested into 10 mL Heart Infusion Broth to form a suspension.

The number of bacteria in the suspension was calculated with a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) as follows. The suspension was diluted $^{1}/_{100}$ with saline and the counting chamber was filled with this diluted suspension. The grid of the counting chamber was located using a light microscope at 1000× magnification. The *C. fetus* subsp. *fetus* organisms in at least 20 squares of the counting chamber were counted. The average number of *C. fetus* subsp. *fetus* organisms per square was multiplied by the dilution factor (100) and by 2×10^{7} to obtain the number of organisms per mL (Directions for the Use of Petroff-Hausser and Helber Counting Chambers, Hausser Scientific).

Using the calculated number of bacteria per mL, the suspension was serially diluted to the required densities for the inocula: 10^{4} cfu/mL, 10^{3} cfu/mL, and 10^{2} cfu/mL. A 1mL dose with these densities encompassed the range of MAD_{50} found in previous guinea pig challenge studies using other *C. fetus* subsp. *fetus* strains (Bryner et al. 1978, 1979 and 1988; Diker and Turutoglu 1995).

Dilution plating of the inocula was performed to confirm the calculated number of bacteria in the inocula. For this, 100 µL and 200 µL aliquots of the inocula and of serial 10-fold dilutions of the inocula were spread onto blood agar plates in triplicate. After incubation in a microaerobic atmosphere for 48 hours, the number of colonies on each plate was counted. The average number of colonies on the triplicate plates which contained between 50-300 colonies per plate was calculated. This was multiplied by the dilution factor and the inverse of the volume plated to give the cfu/mL of the inocula. As the dilution plating method relies on the growth of *C. fetus* subsp. *fetus*, results were not available until two days after inoculation. The concentration of *C. fetus* subsp. *fetus* in the challenge inocula (determined by dilution plating) was 65-80% of the concentration calculated by the Petroff-Hausser method (Table 8.1).

Table 8.1 The challenge doses for both PFGE type A1 and B1 MAD_{50} determination experiments, as calculated by the Petroff-Hausser method and by dilution plating. Pregnant guinea pigs were randomly assigned into dose groups A, B or C. Guinea pigs in group A received the highest dose, those in group B the intermediate dose, and guinea pigs in group C the lowest dose.

		Challenge dose (cfu)	
MAD_{50} determination	**Dose group**	**Petroff-Hausser**	**Dilution plating**
A1	A	10,000	6,500
	B	1,000	800
	C	100	~80
B1	A	10,000	6,575
	B	1,000	720
	C	100	~73

8.3.1.3.3 *Protein profile and serological analysis of challenge inoculum*

A protein sample of the isolate used as the challenge strain was prepared as described in Chapter 2: Protein sample preparation.

In addition, a protein sample of the challenge inoculum was prepared as follows. The density of bacteria in an aliquot of the initial suspension was estimated by measurement of OD_{600nm}. Bacteria in a 1 mL aliquot of this suspension were pelleted by centrifugation at 13,000 rpm for 5 min, and the pellet was resuspended in a volume equivalent to $200 \times OD_{600nm}$ Sample Buffer and stored at –70°C. The protein profile was examined as described in Chapter 2: Protein electrophoresis. Gels were either stained with Coomassie Blue or used for Western blotting as described in Chapter 2: Western blotting.

8.3.1.3.4 *Challenge*

Twenty-one days from the end of the mating period (third-fourth week of gestation), each guinea pig randomly assigned to Group A was inoculated by intraperitoneal injection with 1 mL Heart Infusion Broth containing 6,500 cfu/mL. In the same way, the Group B guinea pigs were inoculated with 800 cfu/mL, and the Group C guinea pigs with 80 cfu/mL (Table 8.1).

8.3.1.3.5 *Post-challenge*

Each morning for 21 days after challenge, the guinea pigs were weighed and observed for signs of abortion, such as vaginal bleeding or the presence of foetal material. Each evening, the guinea pigs were observed for signs of abortion and any guinea pigs suspected to have aborted were weighed. Aborting animals were euthanased promptly with increasing CO_2 and, following necropsy, the uterine contents were cultured for *C. fetus* subsp. *fetus* as described in Section 8.3.1.3.6 (Figure 8.8 and 8.9). Animals that had not aborted 21 days after challenge were euthanased by an intraperitoneal injection of 2 mL sodium pentobarbitone (Pentobarb 500, Chemstock Animal Health Ltd., Christchurch, NZ) and the uterine contents cultured for *C. fetus* subsp. *fetus* (Figure 8.10). Pregnancy of guinea pigs that had not aborted was confirmed at necropsy.

8.3.1.3.6 *Culture of Campylobacter fetus subsp. fetus from guinea pig organs*

Uteri and placentae (and in some cases liver) were collected for culture from each challenged guinea pig. Organs were aseptically swabbed and swabs were used to inoculate blood agar plates, which were streaked for single colonies. Extent of growth on these primary plates was scored after 48 hours. A single colony was picked and streaked onto fresh blood agar plates for Gram stain and species confirmation.

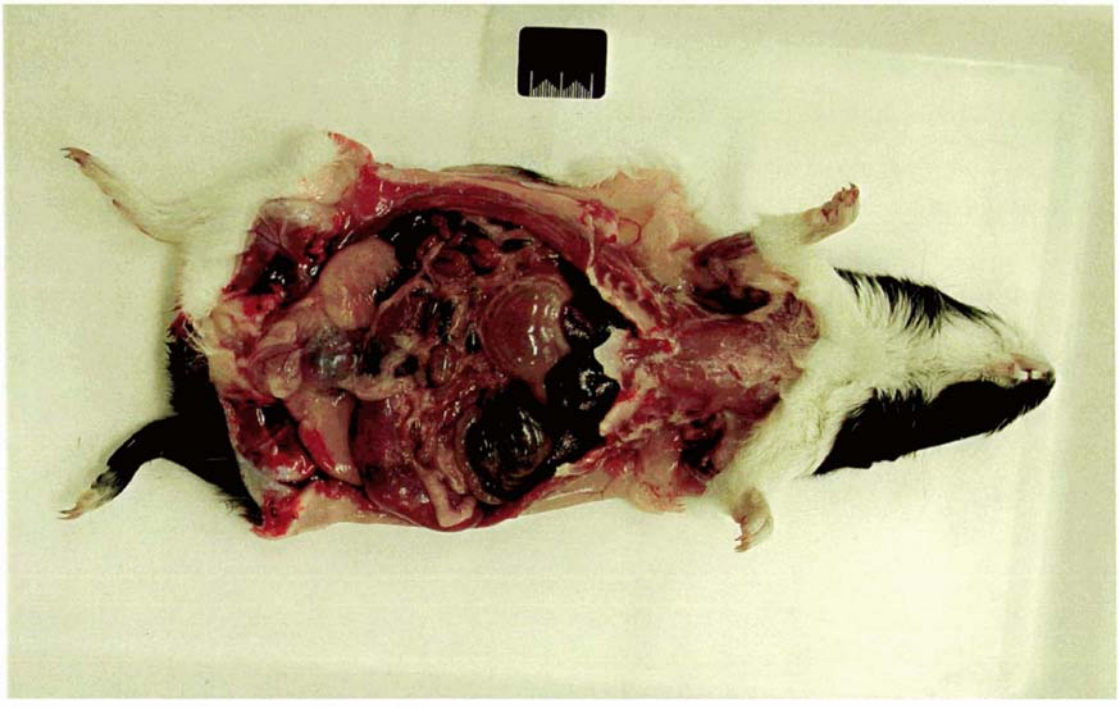


Figure 8.8 Necropsy of an aborting guinea pig.

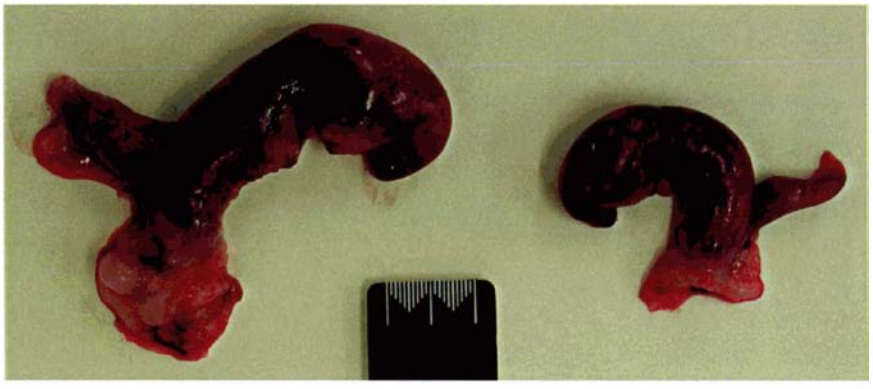


Figure 8.9 Haemorrhagic uteri from aborting guinea pigs in the fifth to sixth week of gestation.

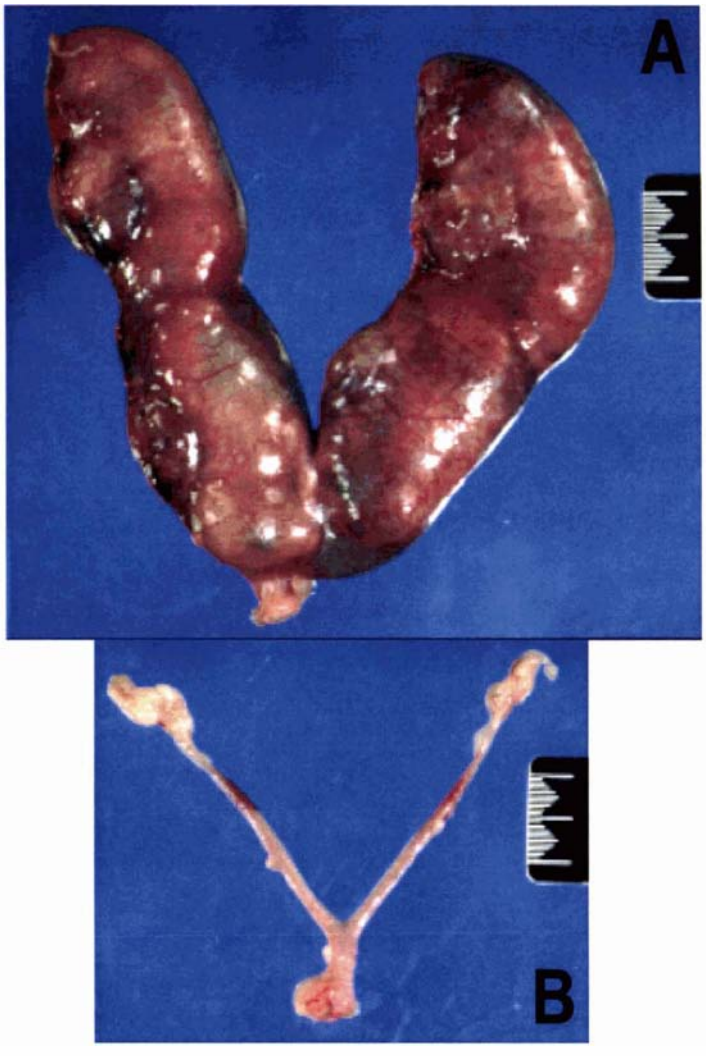


Figure 8.10 Guinea pig uteri. A uterus from a pregnant guinea pig, in the seventh to eighth week of gestation, 21 days after challenge. **B** uterus from a non-pregnant guinea pig.

8.3.2 Determination of MAD_{50} for B1

8.3.2.1 Animals

Twenty-five 2-3 month old female and four male guinea pigs.

8.3.2.2 *Campylobacter fetus* subsp. *fetus* strain

Pulsed-field gel electrophoresis type B1, isolate NS41 sourced from a sheep abortion case in the Manawatu in 2000. A large number of ewes from this flock aborted (90 out of 330 ewes). Three isolates were cultured from this flock and all were PFGE type B1. This isolate came from AgriQuality Farm Network Animal Health Laboratory (Palmerston North) and was stored in 15% glycerol at –70°C after two passages on blood agar.

8.3.2.3 Method

The same experimental protocol was used as for the determination of MAD_{50} of PFGE type A1. Care was taken to ensure consistent conditions for the guinea pigs between the two experiments (Figure 8.4).

8.3.3 Results of MAD_{50} determination for A1

8.3.3.1 Number of aborting guinea pigs in the A1 MAD_{50} determination experiment

Twelve out of 21 guinea pigs aborted in the A1 MAD_{50} determination experiment. Six guinea pigs aborted from the high dose group, four from the medium dose group, and two from the low dose group (Table 8.2). Abortions occurred from the 2^{nd} –15^{th} day after challenge. The weight loss of the guinea pigs upon abortion ranged from 15g to 167g (Figure 8.11), and the average weight loss of aborting guinea pigs was 70g. Four out of the 12 guinea pigs that aborted in this experiment lost less than 50g upon abortion. The average number of foetuses was 3.4. Figure 8.11 summarises the information from this experiment.

8.3.3.2 Calculation of A1 MAD_{50}

A chi-squared test for trend was performed and the data were consistent with a linear relationship between dose and response. Using this relationship, the dose at which half

the guinea pigs would abort was calculated to be 431 cfu (Table 8.2 and Figure 8.12). At a proportion of 0.5, the 95% confidence interval for the mean log dose ranged between 2.33-2.89 (Figure 8.12). These values equated to 214-776 cfu.

Table 8.2 Proportion of guinea pigs (GP) that aborted after each challenge dose in the experiment to determine the minimum dose at which half of the unvaccinated guinea pigs abort (Minimum Abortive $Dose_{50}$, MAD_{50}) for vaccine strain 5915 (PFGE type A1). This table was used in conjunction with the equation from Figure 8.12 below to calculate the MAD_{50} for PFGE type A1.

	No. GP challenged	No. GP aborted	Proportion GP aborted	Log_{10}(dose)	Dose (cfu)
	7	6	0.86	3.81	6500
	7	4	0.57	2.90	800
	7	2	0.29	1.90	80
Calculated MAD_{50}	7	3.5	0.5	2.63	**431**

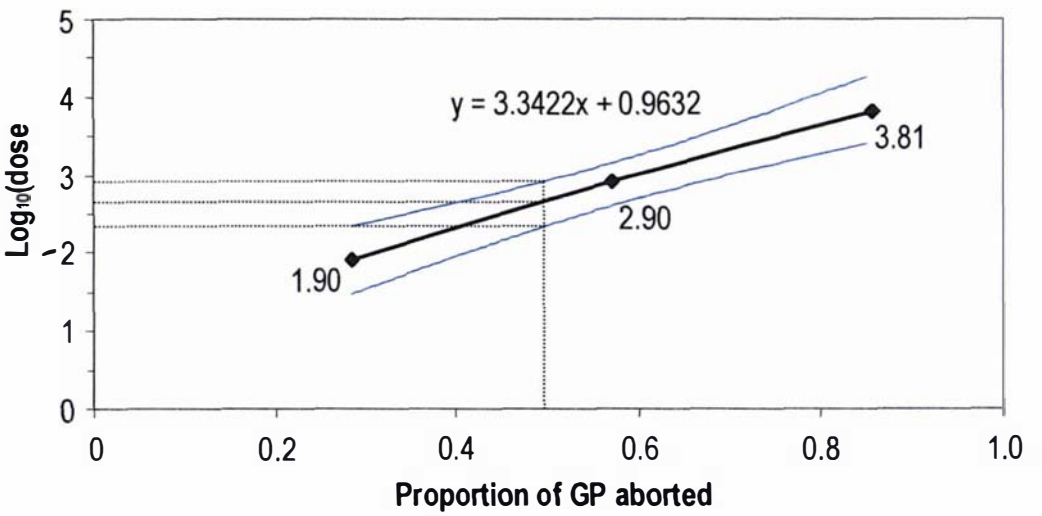
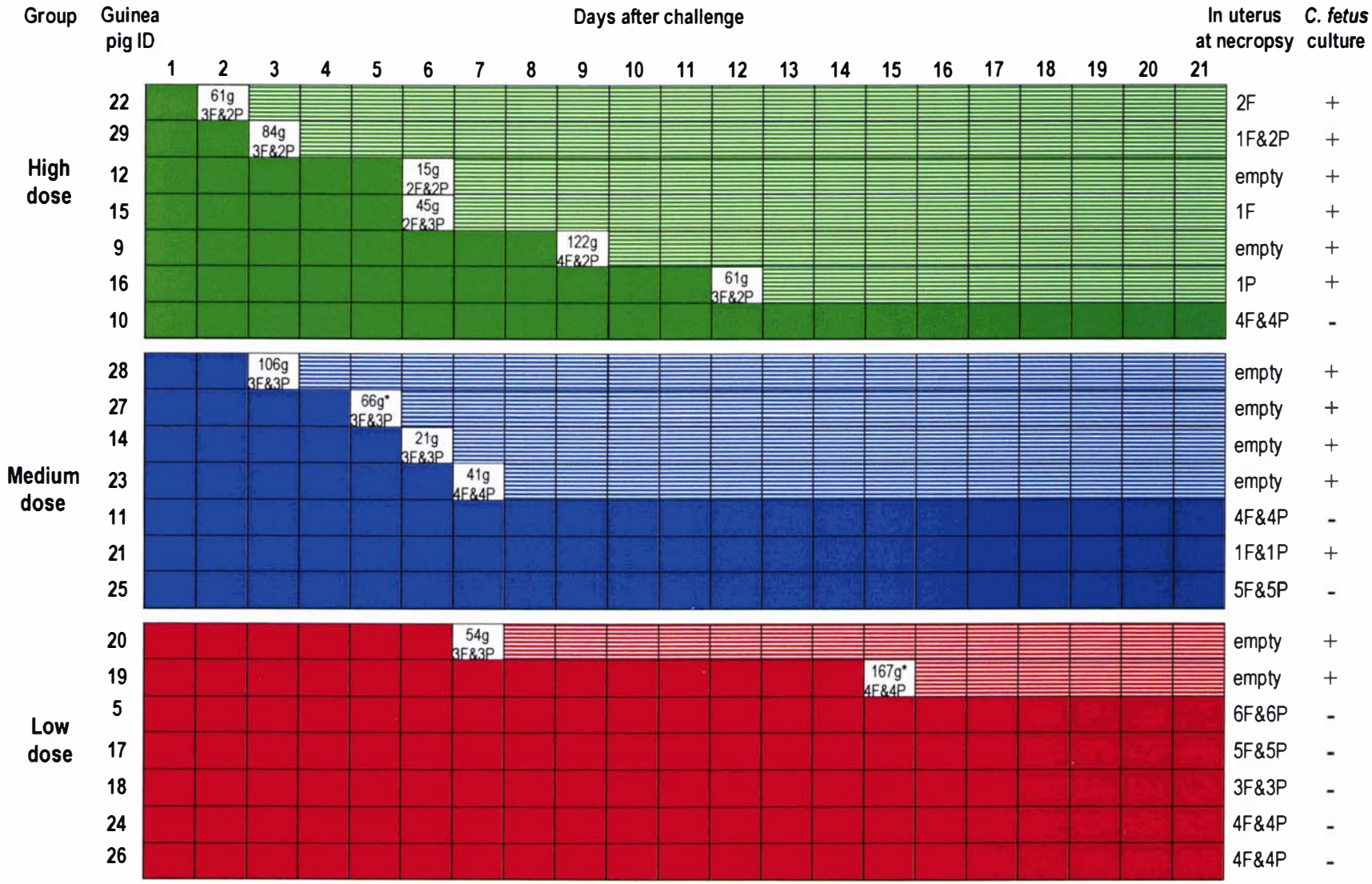


Figure 8.12 Proportion of guinea pigs (GP) that aborted plotted against log_{10} inoculum dose in the experiment to determine the minimum dose at which half of the unvaccinated guinea pigs abort (Minimum Abortive $Dose_{50}$, MAD_{50}) for vaccine strain 5915 (PFGE type A1). The MAD_{50} can be calculated from the equation of the line. The 95% confidence intervals are shown (blue lines). The broken lines indicate the log_{10} dose at a proportion of half of the guinea pigs aborting.

Figure 8.11 Summary of guinea pig abortion data in the experiment to determine the MAD_{50} for A1. The individually identified guinea pigs were housed according to the challenge dose received. The data for the guinea pigs that received the high dose are in green, the medium dose in blue, and the low dose in red. The guinea pigs were weighed each day after challenge for 21 days. Solid colour indicates the daily change in weight was within the normal range (as determined in Section 8.2.4). The presence of foetal material in the cage indicated abortion and the aborting guinea pig was identified by vaginal bleeding and weight loss (g), and euthanased. The number of foetuses (F) and placentae (P) in the cage and in the uterus at necropsy was noted. All abortions were detected in the morning, except those indicated with *, which were detected in the afternoon. Guinea pigs that did not abort by 21 days after challenge were euthanased and necropsied and the number of foetuses and placentae in the uterus is noted. + or – indicates whether *C. fetus* subsp. *fetus* was cultured from the uterus of each guinea pig.


Group
Guinea pig ID
Days after challenge
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
In uterus at necropsy
C. fetus culture
High dose
22 61g 3F&2P 2F +
29 84g 3F&2P 1F&2P +
12 15g 2F&2P empty +
15 45g 2F&3P 1F +
9 122g 4F&2P empty +
16 61g 3F&2P 1P +
10 4F&4P -
Medium dose
28 106g 3F&3P empty +
27 66g* 3F&3P empty +
14 21g 3F&3P empty +
23 41g 4F&4P empty +
11 4F&4P -
21 1F&1P +
25 5F&5P -
Low dose
20 54g 3F&3P empty +
19 167g* 4F&4P empty +
5 6F&6P -
17 5F&5P -
18 3F&3P -
24 4F&4P -
26 4F&4P -

8.3.3.3 *Campylobacter fetus* subsp. *fetus* culture results for the A1 MAD_{50} determination experiment

Campylobacter fetus subsp. *fetus* was cultured from the uterus of each of the 12 aborting guinea pigs in the A1 MAD_{50} determination experiment (Table 8.3). Growth of *C. fetus* subsp. *fetus* from the uteri was usually a heavy growth in pure culture. *Campylobacter fetus* subsp. *fetus* was also cultured as moderate growth from the placentae of all but two of these guinea pigs. In one of these cases, placentae were not recovered from the uterus and culture was not pursued due to overgrowth of contaminants. In the other case, placentae could not be obtained for certain from this guinea pig due to an empty uterus and another guinea pig aborting in the same cage at the same time (Figure 8.11). *Campylobacter fetus* subsp. *fetus* was cultured from the liver of two guinea pigs, and culture from the livers of a further two guinea pigs was unsuccessful. Growth was sparse in comparison with that typically obtained from the uterus and placentae, so in subsequent experiments culture from the liver was not routinely attempted.

Campylobacter fetus subsp. *fetus* was cultured from the uterus, placenta and liver of one guinea pig that did not abort (Table 8.3). Culture of *C. fetus* subsp. *fetus* was unsuccessful from the uterus, placenta, and liver from the other eight guinea pigs that did not abort.

Table 8.3 Number of challenged guinea pigs (GP) in each MAD_{50} determination experiment that were culture positive for *C. fetus* subsp. *fetus*.

MAD_{50} determination	No. GP challenged	No. GP aborted	Culture positive GP
A1	7	6	6
	7	4	5
	7	2	2
B1	7	5	6
	7	5	6
	6	1	1

8.3.4 Results of MAD_{50} determination for B1

8.3.4.1 Number of aborting guinea pigs in the B1 MAD_{50} determination experiment

Eleven out of 20 guinea pigs aborted in the B1 MAD_{50} determination experiment, comprising five from the high dose group, five from the medium dose group, and one from the low dose group (Table 8.4). Most abortions occurred between the 3rd and 11th day after challenge. However, one guinea pig from the medium dose group aborted 20 days after challenge. The weight loss of the guinea pigs upon abortion ranged from 29g to 200g (Figure 8.13), and the average weight loss of aborting guinea pigs was 64g. Six out of the 11 guinea pigs that aborted in this experiment lost less than 50g upon abortion. The average number of foetuses was 3.1. Figure 8.13 summarises the guinea pig information from this experiment.

8.3.4.2 Calculation of B1 MAD_{50}

Using the relationship between dose and response, the dose of B1 at which half of the guinea pigs aborted was calculated to be 488 cfu (Table 8.4 and Figure 8.14). However, at a proportion of 0.5, the 95% confidence interval for the mean log dose ranged between –0.90 and 6.25 (Figure 8.15). These values equate to between 0.8 cfu and 1,778,279 cfu.

8.3.4.3 *Campylobacter fetus* subsp. *fetus* culture results for the B1 MAD_{50} determination experiment

Campylobacter fetus subsp. *fetus* was cultured from the uterus of each of the 11 aborting guinea pigs in the B1 MAD_{50} determination experiment, usually as heavy growth in pure culture (Table 8.3). *Campylobacter fetus* subsp. *fetus* was also cultured as a moderate growth from the placentae of all of these guinea pigs.

Campylobacter fetus subsp. *fetus* was cultured from the uterus and placenta of two guinea pigs that did not abort (Table 8.3). Culture of *C. fetus* subsp. *fetus* was unsuccessful from the uterus and placenta from the other seven guinea pigs that did not abort.

Figure 8.13 Summary of guinea pig abortion data in the experiment to determine the MAD_{50} for B1. The individually identified guinea pigs were housed according to the challenge dose received. The data for the guinea pigs that received the high dose are in green, the medium dose in blue, and the low dose in red. The guinea pigs were weighed each day after challenge for 21 days. Solid colour indicates the daily change in weight was within the normal range (as determined in Section 8.2.4). The presence of foetal material in the cage indicated abortion and the aborting guinea pig was identified by vaginal bleeding and weight loss (g), and euthanased. The number of foetuses (F) and placentae (P) in the cage and in the uterus at necropsy was noted. All abortions were detected in the morning, except those indicated with *, which were detected in the afternoon. Guinea pigs that did not abort by 21 days after challenge were euthanased and necropsied and the number of foetuses and placentae in the uterus is noted. + or – indicates whether *C. fetus* subsp. *fetus* was cultured from the uterus of each guinea pig.

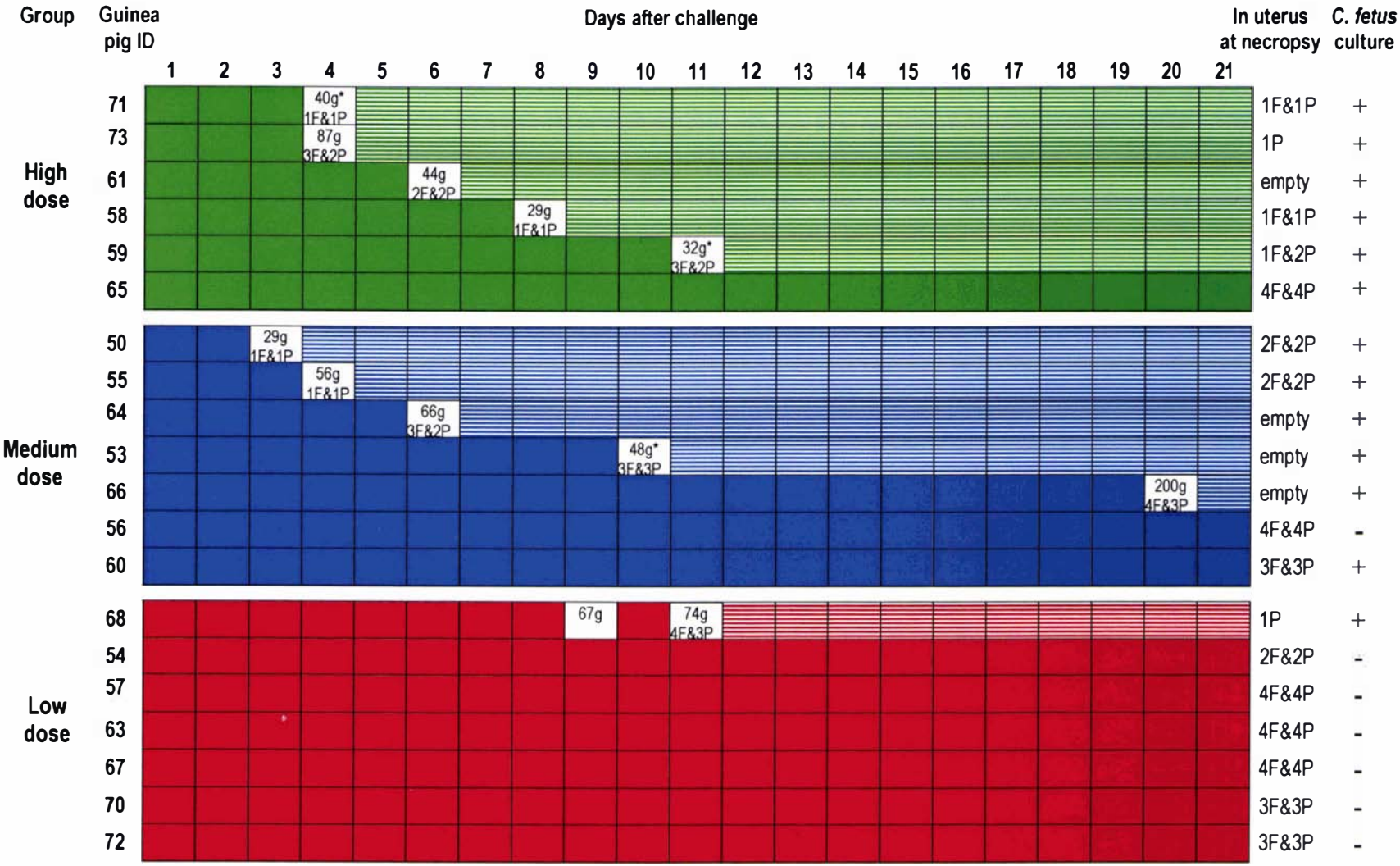

Group
Guinea pig ID
Days after challenge
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
In uterus at necropsy
C. fetus culture
High dose
71 40g* 1F&1P 1F&1P +
73 87g 3F&2P 1P +
61 44g 2F&2P empty +
58 29g 1F&1P 1F&1P +
59 32g* 3F&2P 1F&2P +
65 4F&4P +
Medium dose
50 29g 1F&1P 2F&2P +
55 56g 1F&1P 2F&2P +
64 66g 3F&2P empty +
53 48g* 3F&3P empty +
66 200g 4F&3P empty +
56 4F&4P -
60 3F&3P +
Low dose
68 67g 74g 4F&3P 1P +
54 2F&2P -
57 4F&4P -
63 4F&4P -
67 4F&4P -
70 3F&3P -
72 3F&3P -

Table 8.4 Proportion of guinea pigs (GP) that aborted after each challenge dose in the experiment to determine the minimum dose at which half of the unvaccinated guinea pigs abort (Minimum Abortive $Dose_{50}$, MAD_{50}) for PFGE type B1. This table was used in conjunction with the equation from Figure 8.14 below to calculate the MAD_{50} for PFGE type B1.

	No. GP challenged	No. GP aborted	Proportion GP aborted	Log_{10}(dose)	Dose (cfu)
	6	5	0.83	3.82	6575
	7	5	0.71	2.86	720
	7	1	0.14	1.86	73
Calculated MAD_{50}	7	3.5	0.5	2.69	**488**

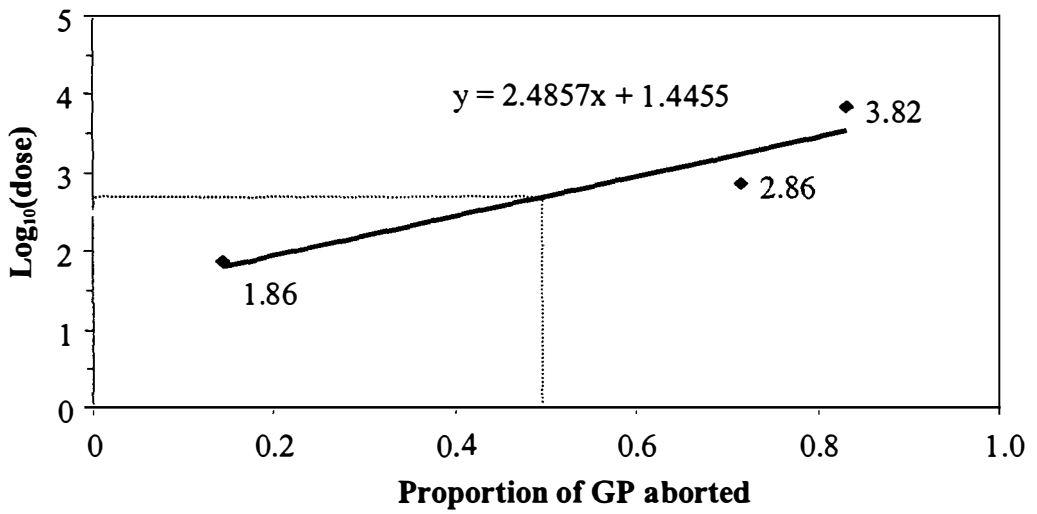


Figure 8.14 Proportion of guinea pigs (GP) that aborted plotted against log_{10} inoculum dose in the experiment to determine the minimum dose at which half of the unvaccinated guinea pigs abort (Minimum Abortive $Dose_{50}$, MAD_{50}) for PFGE type B1. The MAD_{50} can be calculated from the equation of the line. The broken lines indicate the log_{10} dose at a proportion of half of the guinea pigs aborting.

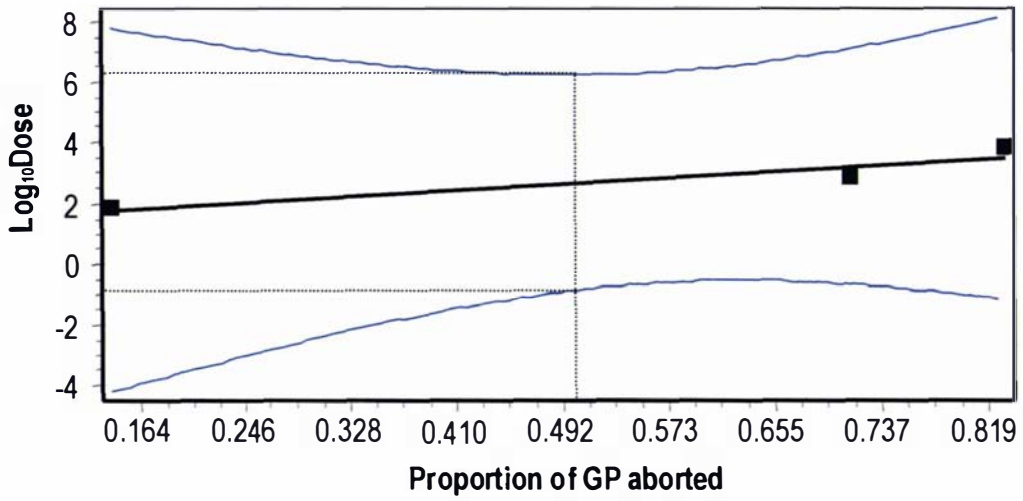


Figure 8.15 Proportion of guinea pigs (GP) that aborted plotted against log_{10} inoculum dose in the experiment to determine the minimum dose at which half of the unvaccinated guinea pigs abort (Minimum Abortive $Dose_{50}$, MAD_{50}) for PFGE type B1, showing the 95% confidence intervals (blue lines). The broken lines indicate the log_{10} dose of confidence intervals at a proportion of half of the guinea pigs aborting.

8.3.5 Discussion

The data from the A1 MAD_{50} determination experiment were consistent between dose and response, as an additional two animals aborted for each step up in challenge dose. The MAD_{50} obtained, 431 cfu, was within the range reported from guinea pig challenge studies using other *C. fetus* subsp. *fetus* strains (Bryner et al. 1979 and 1988; Diker and Turutoglu 1995). The 95% confidence interval of the MAD_{50} equated to a range of 214-776 cfu. The purpose of the elucidation of the MAD_{50} dose was to determine the dose for the vaccine challenge experiment, of $100\times MAD_{50}$ (2 logs). Therefore, if the true MAD_{50} value was at either end of the 95% confidence interval, the dose for the vaccine challenge experiment would be in error by $^1/_3$ log. This was not considered significant as the vaccine challenge dose was in the order of 4 logs, at approximately 4×10^4 cfu.

In contrast, the determination of the MAD_{50} of PFGE type B1 yielded data that were not as consistent between dose and response. This was largely due to the similar proportion of animals that aborted in the medium and high dose groups (0.71 and 0.83, respectively). The late abortion of a medium dose guinea pig, just one day before the end of the experiment and considerably later than the other affected animals in this experiment, contributed to this. One or two guinea pigs had the potential to make a substantial difference to the overall result, due to the small number of animals in the experiments. The inconsistency between dose and proportion aborting resulted in the 95% confidence interval of the MAD_{50} equating to a range of 0.8 cfu to 1,778,279 cfu. However, the calculated MAD_{50} (488 cfu) was very similar to that obtained for the vaccine strain (431 cfu). Therefore, the two calculated MAD_{50} doses were well within a log of each other, and the arbitrarily-defined standard vaccine challenge dose of $100\times MAD_{50}$ equated to approximately 4×10^4 cfu for each strain. As the experiment to determine the MAD_{50} of A1 yielded data that were consistent between dose and response, and the results for the determination of B1 MAD_{50} were close to that of A1 but not as robust, it was decided to use the same challenge dose of each strain for the two vaccine challenge experiments. Therefore, the B1 challenge dose in the vaccine challenge experiment was the same as that calculated for PFGE type A1.

Campylobacter fetus subsp. *fetus* was cultured from the uterus of each guinea pig that aborted, as has been found in a previous study using guinea pigs as a model for *C. fetus*

subsp. *fetus* disease (SultanDosa et al. 1983). In addition, a total of three guinea pigs that did not abort had *C. fetus* subsp. *fetus* cultured from the uteri post-mortem. One of these culture-positive non-aborting guinea pigs was from the medium dose group of the A1 MAD_{50} determination experiment. In the B1 MAD_{50} determination experiment, the only guinea pig that did not abort in the high dose group and one of the two non-aborting guinea pigs from the medium dose group were positive for *C. fetus* subsp. *fetus*. However, culture-positive non-aborting guinea pigs were not considered in the final tally of number of abortions. If they had been, the MAD_{50} of the vaccine strain becomes 318 cfu and the MAD_{50} of B1 becomes 327 cfu. It is uncertain whether, given more time, these guinea pigs would have gone on to completely eliminate the infection or to abort. Animal ethics considerations necessitated the termination of the experiment at 21 days after challenge.

The average weight loss of the aborting guinea pigs in each MAD_{50} determination experiment was greater than 50g (70g and 64g respectively), as has been described previously (Bryner et al. 1978 and 1979; SultanDosa et al. 1983; Diker and Turutoglu 1995). However on an individual basis, 10 out of the 23 guinea pigs that aborted in these experiments lost less than 50g upon abortion. Six of these guinea pigs lost ≤40g upon abortion, which was within the range of weight fluctuation for healthy guinea pigs determined in Section 8.2.4. Fortunately, weight loss was not the only indicator of abortion. The presence of blood and foetal material in the cage clearly indicated that a guinea pig housed in that cage was aborting or had aborted. Vaginal bleeding specified which particular guinea pig had aborted and weight loss supported this determination. The range of weight loss displayed by aborting guinea pigs may be due to the number and size of foetuses carried. The total number of foetuses carried by guinea pigs that aborted in these MAD_{50} determination experiments ranged from two to five. The size of the foetuses varied between the guinea pigs, as there was a potential difference in gestation time of up to 14 days (the length of the mating period). In addition, there was variation in the number of foetuses that had been expelled at the time of discovery of abortion, as a proportion of the total number of foetuses carried.

8.4 Vaccine challenge experiments

Having determined the MAD_{50} for the challenge strains PFGE types A1 and B1, this section details the challenge of vaccinated and unvaccinated guinea pigs with the same strains at a dose rate of $100 \times MAD_{50}$.

8.4.1 Challenge of vaccinated animals with $100 \times MAD_{50}$ A1

8.4.1.1 Animals

Twenty 1¼-2 month old female and four male guinea pigs.

8.4.1.2 *Campylobacter fetus* subsp. *fetus* strain

The same glycerol stock of the vaccine strain 5915, PFGE type A1, was used for this experiment as that used for the A1 MAD_{50} determination experiment.

8.4.1.3 Method

8.4.1.3.1 *Vaccination*

After a period of ten days to acclimatise and individually identify the guinea pigs, the female guinea pigs were randomly assigned to one of four vaccination groups, Groups A-D (Figure 8.5). The ten guinea pigs in Groups A and B were vaccinated with Campylovexin® by 2 mL subcutaneous injection over the shoulder (Batch 533A, Exp. 08/2002). Twenty-eight days later the same guinea pigs were given a second vaccination by 2 mL subcutaneous injection over the other shoulder, taken from the same vaccine pack stored at 4°C.

Swelling at the site of vaccination within ten days of vaccination was common amongst the guinea pigs, and injection site nodules have been reported before in vaccinated guinea pigs (Bryner et al. 1979). Swelling had typically subsided to be small or undetectable within 28 days of vaccination. Five guinea pigs developed a vaccination site lesion that ruptured. Of these, three guinea pigs developed this lesion after the first vaccination and two after the second vaccination. No guinea pig developed a lesion that ruptured after both vaccinations. The hair around each ruptured lesion was clipped to keep the area clean, but otherwise the lesions were left untreated. No adverse symptoms

were observed in guinea pigs with ruptured lesions, such as weight loss or inappetence. The vaccination site lesions resolved rapidly and were typically healed within 21 days.

8.4.1.3.2 *Mating and pregnancy detection*

Seven days after the second vaccination, the female guinea pigs were randomly assigned to one of four mating groups. Each of the males was randomly assigned to one mating group, and housed with that group for 14 days. Pregnancy was detected by ultrasound after a further 18-20 days and the pregnant guinea pigs were randomly assigned to one of two challenge groups. Twenty-one days from the end of the mating period (third-fourth week of gestation), each pregnant guinea pig was inoculated by intraperitoneal injection with 1 mL Heart Infusion Broth containing $100\times MAD_{50}$ cfu *C. fetus* subsp. *fetus*.

8.4.1.3.3 *Preparation of challenge inoculum*

The challenge inoculum was prepared in the same way as for the MAD_{50} determination experiments (Section 8.3.1.3.2). Briefly, once revived from the glycerol stock, the challenge strain was sub-cultured once onto fresh blood agar to ensure purity. Growth after 48 hours on blood agar was harvested into 10 mL Heart Infusion Broth. The number of bacteria in the suspension was calculated with a Petroff-Hausser counting chamber, and this suspension was serially diluted to the required concentration for the inoculum.

Dilution plating of the initial suspension and the inoculum was performed to confirm the calculated number of bacteria in the inoculum. The number of viable bacteria in the initial suspension (determined by dilution plating) was 65-70% of that calculated by the Petroff-Hausser method. The concentration of *C. fetus* subsp. *fetus* in the challenge inoculum (determined by dilution plating) was 81-92% of the concentration calculated by the Petroff-Hausser method (Table 8.5).

Table 8.5 The challenge doses for both PFGE type A1 and B1 vaccine challenge experiments, as calculated by the Petroff-Hausser method and by dilution plating.

	Challenge dose (cfu)	
Challenge Strain	**Petroff-Hausser**	**Dilution plating**
A1	43,100	39,600
B1	43,100	35,033

8.4.1.3.4 *Protein profile and serological analysis of challenge inoculum*

A protein sample of the challenge inoculum was prepared as described for the MAD_{50} determination experiments. SDS-PAGE and Western blotting were performed as described in Chapter 2.

8.4.1.3.5 *Post-challenge*

The protocol for the 21 day period following challenge was the same as for the MAD_{50} determination experiments.

8.4.1.3.6 *Campylobacter fetus subsp. fetus culture from guinea pig organs*

Culture of *C. fetus* subsp. *fetus* from uteri and placentae of all challenged guinea pigs was the same as for the MAD_{50} determination experiments.

8.4.2 Challenge of vaccinated animals with 100×MAD_{50} strain B1

8.4.2.1 Animals

Eighteen 2-3 month old female and four male guinea pigs.

8.4.2.2 *Campylobacter fetus* subsp. *fetus* strain

The same glycerol stock of PFGE type B1 (isolate NS41) was used as for the B1 MAD_{50} determination experiment.

8.4.2.3 Method

The same experimental protocol was used as for the vaccine challenge experiment with PFGE type A1 (Figure 8.5), and care was taken to ensure consistent conditions for the guinea pigs between these two experiments. The same vaccine pack was used for all Campylovexin® injections of the guinea pigs in the vaccine challenge experiments, and was stored at 4°C.

8.4.3 Results of challenge of vaccinated animals with 100×MAD_{50} A1

Seven vaccinated and eight unvaccinated pregnant guinea pigs were challenged with 39,600 cfu A1. None of the vaccinated guinea pigs aborted by experiment end, 21 days after challenge. Six unvaccinated guinea pigs aborted (Table 8.6). Fisher's exact test was used to assess the association between vaccination status and abortion with the resulting P-value (P = 0.007) being significant. Abortions occurred between the 2nd and the 6th day after challenge. The weight loss of the guinea pigs upon abortion ranged from 26g to 108g (Figure 8.16), and the average weight loss of aborting guinea pigs was 63g. Three out of the 6 guinea pigs that aborted in this experiment lost less than 50g upon abortion. The average number of foetuses was 3.3. Figure 8.16 summarises the guinea pig information from this experiment.

Campylobacter fetus subsp. *fetus* was cultured from the uterus of each guinea pig that aborted. *Campylobacter fetus* subsp. *fetus* was also cultured from the available placentae of the aborting guinea pigs. In contrast, *C. fetus* subsp. *fetus* was not cultured from the uterus or placentae of any of the guinea pigs that did not abort.

8.4.4 Results of challenge of vaccinated animals with 100×MAD_{50} B1

Nine vaccinated and seven unvaccinated pregnant guinea pigs were challenged with 35,000 cfu B1. Three of the vaccinated guinea pigs aborted by experiment end and all of the unvaccinated guinea pigs aborted (Table 8.7). Fisher's exact test was used to assess the association between vaccination status and abortion with the resulting P-value (P = 0.0114) being significant. Abortions occurred between the 2nd and the 7th day after challenge. The weight loss of the guinea pigs upon abortion ranged from 1g to 78g (Figure 8.16), and the average weight loss of aborting guinea pigs was 42g. Seven out of the 10 guinea pigs that aborted in this experiment lost less than 50g upon abortion. The average number of foetuses was 3.4. Figure 8.17 summarises the guinea pig information from this experiment.

Campylobacter fetus subsp. *fetus* was isolated from the uterus and the available placentae of each guinea pig that aborted. However, *C. fetus* subsp. *fetus* was not cultured from the uterus or placentae of any of the guinea pigs that did not abort.

Table 8.6 Numbers of vaccinated and unvaccinated guinea pigs that were mated, were found to be pregnant and challenged, and subsequently aborted in the vaccine challenge experiment with 100×MAD_{50} A1.

	Vaccine challenge with 100×MAD_{50} A1		
	Vaccinated	**Non-vaccinated**	**Total**
Mated	10	10	20
Pregnant & challenged	7	8	15
Aborted	0	6	6

Table 8.7 Numbers of vaccinated and unvaccinated guinea pigs that were mated, were found to be pregnant and challenged, and subsequently aborted in the vaccine challenge experiment with 100×MAD_{50} B1.

	Vaccine challenge with 100×MAD_{50} B1		
	Vaccinated	**Non-vaccinated**	**Total**
Mated	9	9	18
Pregnant & challenged	9	7	16
Aborted	3	7	10

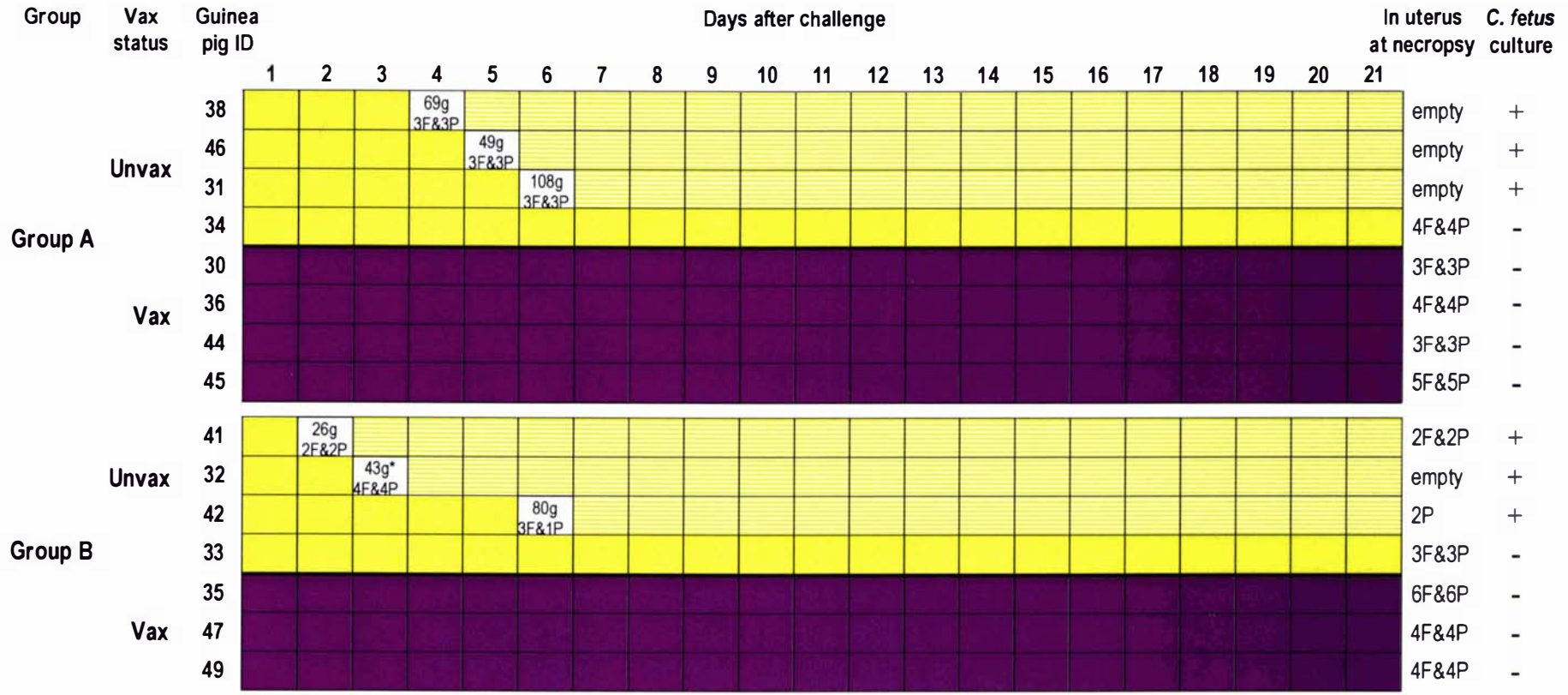


Figure 8.16 Summary of guinea pig abortion data in the vaccine challenge experiment with A1. The individually identified guinea pigs were housed in two groups, with unvaccinated (unvax) and vaccinated (vax) animals together. The data for the unvaccinated guinea pigs are in yellow and for the vaccinated guinea pigs, violet. The guinea pigs were weighed each day after challenge for 21 days. Solid colour indicates the daily change in weight was within the normal range (as determined in Section 8.2.4). The presence of foetal material in the cage indicated abortion and the aborting guinea pig was identified by vaginal bleeding and weight loss (g), and euthanased. The number of foetuses (F) and placentae (P) in the cage and in the uterus at necropsy was noted. All abortions were detected in the morning, except those indicated with *, which were detected in the afternoon. Guinea pigs that did not abort by 21 days after challenge were euthanased and necropsied and the number of foetuses and placentae in the uterus is noted. + or – indicates whether *C. fetus* subsp. *fetus* was cultured from the uterus of each guinea pig.

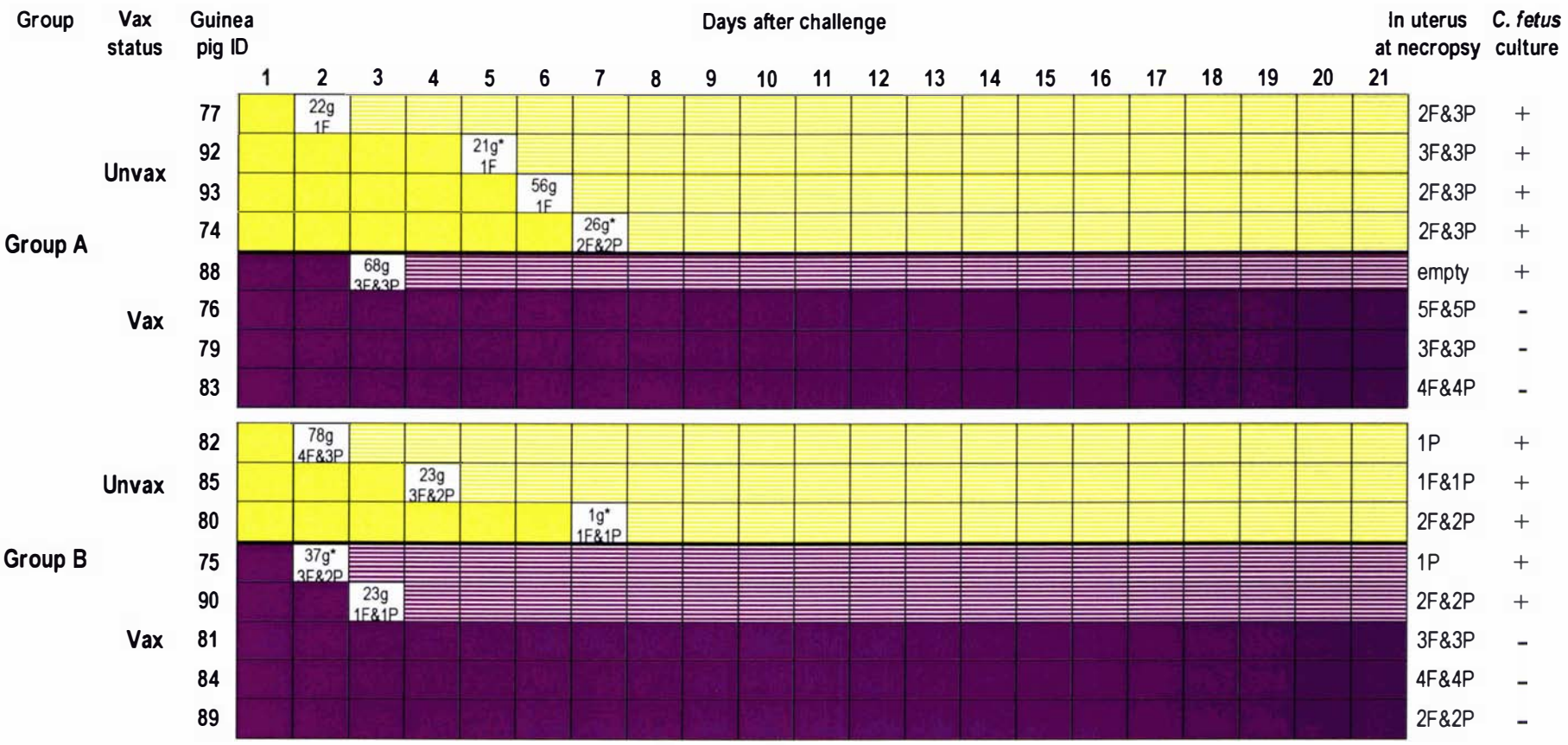


Figure 8.17 Summary of guinea pig abortion data in the vaccine challenge experiment with B1. The individually identified guinea pigs were housed in two groups, with unvaccinated (unvax) and vaccinated (vax) animals together. The data for the unvaccinated guinea pigs are in yellow and for the vaccinated guinea pigs, violet. The guinea pigs were weighed each day after challenge for 21 days. Solid colour indicates the daily change in weight was within the normal range (as determined in Section 8.2.4). The presence of foetal material in the cage indicated abortion and the aborting guinea pig was identified by vaginal bleeding and weight loss (g), and euthanased. The number of foetuses (F) and placentae (P) in the cage and in the uterus at necropsy was noted. All abortions were detected in the morning, except those indicated with *, which were detected in the afternoon. Guinea pigs that did not abort by 21 days after challenge were euthanased and necropsied and the number of foetuses and placentae in the uterus is noted. + or – indicates whether *C. fetus* subsp. *fetus* was cultured from the uterus of each guinea pig.

8.4.5 Protein profile and serological analysis of challenge inocula

The protein profile of the isolates used as the challenge strains were compared with the protein profiles of each challenge inoculum preparation (Figure 8.18, Panel A). No major change in expression of the protein bands between the preparations was detected, for either strain. Western blotting using pooled serum from 15 ewes vaccinated with Campylovexin® showed equivalent antibody binding between the preparations for each strain (Figures 8.18, Panel B). There was no significant antibody binding when pre-immune sheep serum was used in Western blotting (Figures 8.18, Panel C).

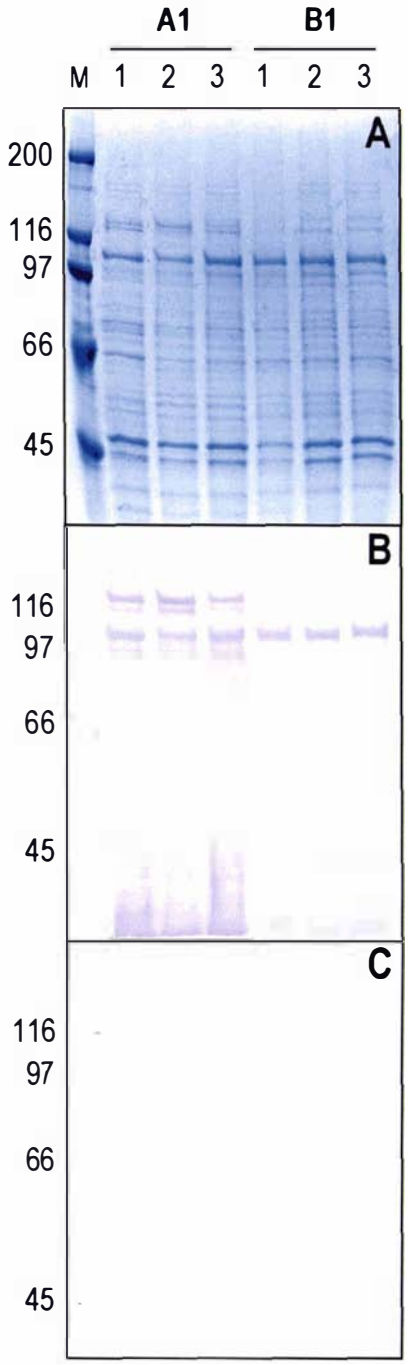


Figure 8.18 Protein profile and Western blotting of the challenge inocula in the MAD_{50} determination and vaccine challenge experiments, for both PFGE types A1 (isolate 5915) and B1 (isolate NS41). Lane 1: control protein preparation of the isolate. Lane 2: challenge inoculum used in the MAD_{50} determination experiment. Lane 3: challenge inoculum used in the vaccine challenge experiment. **A** Coomassie-stained SDS-PAGE gel. **B** Western blot using pooled immune sheep sera. **C** Western blot using pooled pre-immune sheep sera. M = molecular weight standards, protein standard bands were labelled with sizes (kDa).

8.4.6 Discussion

This study evaluated the efficacy of the vaccine Campylovexin®, using pregnant guinea pigs as a model, against homologous challenge with the vaccine strain (5915, PFGE type A1) and heterologous challenge with an isolate of the prevalent field strain PFGE type B1. Significantly fewer vaccinated guinea pigs aborted than unvaccinated guinea pigs, irrespective of challenge strain.

Campylovexin® was found to be protective against homologous challenge as no vaccinated guinea pigs aborted, compared with six abortions out of eight unvaccinated guinea pigs (75%). The level of challenge was demonstrated by the number of abortions in the control (unvaccinated) group, with manifestation of disease in 75% of the unprotected animals. The United States Department of Agriculture (USDA) requires that at least 80% of unvaccinated animals challenged with $100\times LD_{50}$ succumb to disease to demonstrate a valid test when assessing *Clostridium chauvoei* vaccine testing in guinea pigs (Supplemental assay method for potency testing products containing *Clostridium chauvoei* antigen, BBSAM0200.01, Center for Veterinary Biologics and National Veterinary Services Laboratories Testing Protocol, USDA Animal and Plant Health Inspection Service). This is close to the level attained with the homologous Campylovexin® challenge in the present study.

In contrast, the heterologous challenge appeared to be more abortifacient as all seven of the control guinea pigs aborted. Three of the nine vaccinated guinea pigs also aborted after this challenge, while six vaccinated guinea pigs did not abort (67%). It had been attempted to equate the challenge doses of the two vaccine challenge experiments, by the elucidation of the MAD_{50} of each strain. For reasons discussed in Section 8.3.5, it was decided to administer an equal dose. Dilution plating results indicated that the inoculum of B1 in the vaccine challenge experiment contained fewer viable *C. fetus* subsp. *fetus* cells than that of A1 (35,033 cfu and 39,600 cfu, respectively). However, the abortion rates of the control animals in the two vaccine challenge experiments (75% and 100%), suggest that the B1 challenge appeared to be more abortifacient than that of A1. Therefore, a direct comparison of the proportion of vaccinated animals that did not abort when challenged with homologous (100%) and heterologous (67%) strains should be interpreted with caution. However, within each experiment, there was a significant

difference between the numbers of vaccinated and unvaccinated guinea pigs that aborted.

For each strain, the protein profiles of the challenge inocula used in the MAD_{50} determination experiments were very similar to that used in the vaccine challenge experiments. The vaccine strain (isolate 5915) has been shown to alter the size of the Surface Layer Proteins (SLPs) expressed between different protein preparations (Chapter 6), however, there was no detected change in expression of the SLPs in the inocula used for the different experiments. This suggests that the guinea pigs were challenged with equivalent preparations of the same strain between the MAD_{50} determination experiment and the vaccine challenge experiment.

The average weight loss of the aborting guinea pigs in the vaccine challenge experiment using homologous challenge was greater than 50g (63g), as has been described previously (Bryner et al. 1978 and 1979; SultanDosa et al. 1983; Diker and Turutoglu 1995). Three out of the six guinea pigs that aborted in this experiment lost <50g upon abortion and one guinea pig lost <40g upon abortion (within the range of weight fluctuation for healthy guinea pigs determined in Section 8.2.4). These results were similar to those found in the MAD_{50} determination experiments. As already discussed, weight loss is not the only indicator of abortion and vaginal bleeding specified which particular guinea pig had aborted. The range of weight loss displayed by aborting guinea pigs may be due to the number and size of foetuses carried and the number of foetuses that had been expelled at the time of discovery of abortion, as was discussed for the MAD_{50} determination experiments. The total number of foetuses carried by guinea pigs that aborted in the vaccine challenge experiment using homologous challenge ranged from three to five.

In contrast, in the vaccine challenge experiment using heterologous challenge the average weight loss of aborting guinea pigs was 42g, as seven out of the 10 guinea pigs that aborted in this experiment lost <40g upon abortion. This may be due to the unusually high number of guinea pigs that had not expelled all foetuses and placentae before the abortion was discovered and the animal was euthanased. There was only one out of 10 (10%) aborting guinea pigs in this experiment that had expelled all foetuses and placentae before euthanasia and had an empty uterus upon necropsy (Figure 8.17).

This compared to four out of six (67%) aborting guinea pigs that had an empty uterus upon necropsy in the vaccine challenge experiment using homologous challenge (Figure 8.16). The difference in the proportion of aborting guinea pigs with an empty uterus upon necropsy may be due in part to the time of abortion. An unusually high number of aborting guinea pigs in the heterologous challenge experiment began aborting after the morning examination: four out of 10 guinea pigs (40%), compared with one out of six (17%) guinea pigs in the homologous challenge. For guinea pigs that began aborting after the morning check, abortion was discovered in the afternoon and euthanasia was performed promptly upon discovery of abortion to prevent unnecessary suffering. Therefore, there was less time for all the foetuses to be expelled (seven hours maximum) than if abortion had started after the afternoon check and had been discovered the following morning (up to 17 hours). As abortions in both the homologous and heterologous challenge trials occurred over a similar period of time (2-6 days and 2-7 days after challenge, respectively), the difference in the time of the day that abortions began is not considered to be significant.

However, it appears that challenge with the PFGE type A1 isolate prompted a greater percentage of the guinea pigs to abort all foetuses and placentae before discovery of abortion than challenge with PFGE type B1. In the MAD_{50} determination experiments, eight out of 12 (67%) guinea pigs that aborted after challenge with PFGE type A1 had empty uteri upon necropsy, compared with four out of 11 (36%) guinea pigs that aborted after challenge with PFGE type B1. These figures are similar to those obtained in the vaccine challenge trials with these strains (67% and 10%, respectively). Fisher's exact test was used to assess the association between *C. fetus* subsp. *fetus* strain and proportion of aborting guinea pigs with empty uteri with the resulting P-value ($P = 0.0105$) being significant. However, in these experiments a guinea pig abortion was considered to occur if one or more foetuses had been expelled and vaginal bleeding was evident. The proportion of foetuses that had been expelled upon discovery was not considered in the final abortion figures.

There are limitations to using guinea pigs as a model for *Campylobacter* abortion in sheep. There have been no studies showing the direct correlation of dose or disease between guinea pigs and sheep. In addition, the challenge dose of $100\times MAD_{50}$ is arbitrarily defined, instituted to provide a level of consistency between experiments.

Nevertheless, guinea pigs are useful as a model for the investigation of *C. fetus* subsp. *fetus* vaccine efficacy.

8.5 Conclusion

- In the pregnant guinea pig challenge model Campylovexin® vaccination provided a statistically significant level of protection against a homologous *C. fetus* subsp. *fetus* challenge of 100×MAD_{50} vaccine strain, and against a heterologous *C. fetus* subsp. *fetus* challenge using a PFGE type B1 isolate.

Chapter 9

General discussion

9.1 Introduction

Campylobacter spp. are an important cause of abortion in sheep, and the majority of *Campylobacter* sheep abortion in New Zealand is associated with *C. fetus* subsp. *fetus*. Campylovexin® is a single-strain, killed vaccine available in New Zealand for protection in sheep against abortion due to *C. fetus* subsp. *fetus*. Concern arose regarding the cross-protection offered by Campylovexin® against other strains of *C. fetus* subsp. *fetus* when *C. fetus* subsp. *fetus* abortions in apparently vaccinated ewes was reported (Fenwick et al. 2000). The overall aim of this project was to evaluate the efficacy of Campylovexin®, particularly relating to protection against other *C. fetus* subsp. *fetus* sheep abortion strains.

9.2 Strain typing of *Campylobacter fetus* subsp. *fetus*

Since the development of Campylovexin® in the 1980s, it has been known that there were at least two "strains" of *C. fetus* subsp. *fetus* causing abortion in sheep in New Zealand, as Animal Health Diagnostic Laboratories routinely serotyped *C. fetus* subsp. *fetus* sheep abortion isolates as "vaccine strain" or "non-vaccine strain" (Pauling 1988). The first genotypic sub-typing of a group of *C. fetus* subsp. *fetus* sheep abortion isolates was performed with 43 isolates from 10 New Zealand farms (Collins and Ross 1984). This study used the technique restriction endonuclease analysis (REA) and four different REA types were identified (Collins and Ross 1984). Soon after, seven REA types were found when this technique was applied to larger collections of *C. fetus* subsp. *fetus* isolates from 51 and 67 New Zealand farms, respectively (Collins and de Lisle 1985; de Lisle et al. 1987). In addition, the strain used in production of Campylovexin® was found to be different from the most commonly found type, REA type b (Collins and de Lisle 1985).

Pulsed-field gel electrophoresis is a highly discriminatory molecular typing technique (Owen et al. 1989; Gibson et al. 1995; On and Vandamme 1997; On 1998; Wassenaar et al. 1998; Newell et al. 2000), which overcomes the difficulty inherent with REA of comparing the complex profiles of poorly separated DNA bands between different isolates (Arbeit 1995). This typing method was applied in the present study to a large set of *C. fetus* subsp. *fetus* sheep abortion isolates collected from 374 farms from the Hawke's Bay in 1999 and throughout New Zealand in 2000 (Chapters 3 and 4). This is

the first reported PFGE typing study of a large collection of *C. fetus* subsp. *fetus* sheep abortion isolates.

Twenty-six distinct PFGE types were identified amongst the *C. fetus* subsp. *fetus* sheep abortion isolates from the Hawke's Bay and national studies, and these were arranged by cluster similarity into 11 PFGE groups (Chapters 3 and 4). The number of isolates in this study and the discriminatory power of PFGE have extended the findings of de Lisle et al. (1987) of seven REA types.

The large collection of *C. fetus* subsp. *fetus* isolates cultured from sheep abortion in New Zealand and the genotypic organisation of these isolates has enabled comparisons with strains saved from the previous study of de Lisle et al. (1987) and with further strains isolated during the course of this project. When isolates representative of the REA types identified in 1987 were typed using PFGE, they were found to be similar or indistinguishable to PFGE types found in 1999 and 2000 (Chapter 4). In addition, PFGE type B1 was predominant amongst the *C. fetus* subsp. *fetus* isolates cultured from sheep abortion in the Hawke's Bay in 1999 and in each region of New Zealand in 2000, and the isolate representative of REA type b was also PFGE type B1. The relative prevalence of the PFGE groups found in 1987 and 2000 suggests that there has been no major genotypic shift in the population of *C. fetus* subsp. *fetus* implicated in sheep abortion in New Zealand during this time. Further, On and Harrington (2001) found that the PFGE profile of the *C. fetus* subsp. *fetus* Type strain, isolated from an aborted sheep foetus in France in 1952 (Veron and Chatelain 1973), was found to be indistinguishable from that of an isolate from Canada. Similarly, in the present study, the *C. fetus* subsp. *fetus* Type strain was found to differ by the position of three bands with an isolate found in Canterbury in 2000 (Chapter 4), and this provides some further evidence for either the relative homogeneity of the *C. fetus* subsp. *fetus* population globally or the existence of clonal lines proposed by On and Harrington (2001). In order to explore these ideas, genotyping of many more isolates from different locations around the world is required with the use of standardised molecular typing conditions and analysis. The European Union financed a project (named Campynet) with the aim of standardisation of molecular typing methods to facilitate epidemiological studies of the foodborne pathogens, *C. jejuni* and *C. coli* (http://campynet.vetinst.dk). A standard PFGE protocol for these organisms has been devised to account for differences in the equipment used in

the European Union (http://campynet.vetinst.dk/PFGE.html). However, a standardised method for analysis of PFGE profiles to facilitate the comparison of data from one study to the next has not been resolved for the *Campylobacter* spp. This lack of standardisation of analysis may lead to confusion when interpreting and comparing genotypic results between studies, and limits the comparisons that may be made.

The present study has demonstrated for the first time that concurrent abortions in flocks may be associated with more than one *C. fetus* subsp. *fetus* strain. The route of introduction of *C. fetus* subsp. *fetus* infection to sheep is still poorly understood. However, two *C. fetus* subsp. *fetus* strains associated with abortions in a single flock raises the possibility of introduction of these different strains into a flock from potentially two different sources.

The PFGE type of the strain used in the manufacture of Campylovexin® (type A1) was the third most frequently found type in the national study in 2000, identified from 8% of the farms (Chapter 4). This type was isolated from regions in the South Island, although the finding that PFGE type A1 (or any other type) was not identified in a particular region does not preclude the existence of that type in that region. There was anecdotal evidence to suggest that there was cross-protection of the vaccine strain against other *C. fetus* subsp. *fetus* strains as there have been few reported cases of *C. fetus* subsp. *fetus* abortions in Campylovexin® vaccinated ewes (Pauling 1988; Marchant 1999). Aspects of this apparent cross-protection were examined in the present study with *in vitro* protein and serological studies, the Campylovexin® breakdown investigation, and the *in vivo* vaccine challenge study (Chapters 6-8).

9.3 *Campylobacter jejuni*

There is evidence that *C. fetus* subsp. *fetus* sheep abortion vaccines do not cross-protect against sheep abortion due to *C. jejuni* (Miller et al. 1964; Diker and Turutoglu 1995). Campylovexin® makes no claim of protection against *C. jejuni* abortion in sheep. However, little is known about *C. jejuni* in sheep abortion in New Zealand, and for this reason the *C. jejuni* isolates collected during the Hawke's Bay and national studies were typed using PFGE.

Indistinguishable or similar *C. jejuni* PFGE types were identified from different aborted foetuses from the same flock (Chapter 5), consistent with the role of *C. jejuni* as an infectious cause of abortion in sheep. Twelve distinct PFGE profiles were found amongst the 30 *C. jejuni* sheep abortion isolates from 25 farms from the national study in 2000 and the Hawke's Bay study in 1999 (Chapter 5). It appears that there was not a single *C. jejuni* PFGE type most frequently associated with abortion in sheep. There is one other report of molecular typing of a collection of *C. jejuni* sheep abortion isolates. Delong et al. (1996) also found that there was not a single type identified most frequently when REA was used to strain type 14 *C. jejuni* sheep abortion isolates from 12 farms in the USA. It appears from the present study that *C. jejuni* sheep abortion isolates are generally more genetically diverse from each other than the *C. fetus* subsp. *fetus* isolates from sheep abortion. This may be a reflection of the large species and environmental range of *C. jejuni* and possibly the propensity of this organism for the intestine (Penner 1988; On 1996)

A case investigation in the present study demonstrated that careful interpretation of laboratory results is important in respect to *C. jejuni* sheep abortion diagnoses (Chapter 5). Contamination of the foetal sample may result in the isolation of *C. jejuni*, even when this may not have been the cause of foetal death.

Investigation of the role of *C. jejuni* in sheep abortion in New Zealand falls outside the objectives of the current study. However, this is an area that requires further investigation, as this organism is implicated in between 10% (Chapter 5) and 43% (Anon 2000) of the *Campylobacter* sheep abortion cases in New Zealand annually. It is hoped that the findings reported here using PFGE typing may be of some value in future studies.

9.4 *In vitro* protein and serological studies

The potential cross-protection of Campylovexin® against other *C. fetus* subsp. *fetus* strains was first investigated with protein and serological studies (Chapter 6).

Campylobacter fetus subsp. *fetus* cells have an outer capsule made up of a layer of proteins called surface layer proteins (SLPs). Sheep that were exposed to *C. fetus* subsp.

fetus by vaccination or artificial challenge, or that aborted due to *C. fetus* subsp. *fetus* produced antibodies to SLPs (Myers et al. 1970; Grogono-Thomas et al. 2000 and 2003). Myers et al. (1970) and Grogono-Thomas et al. (2003) have evidence to suggest that antibodies to SLPs are important in protection against abortion due to *C. fetus* subsp. *fetus*.

In the present study, Western blotting showed that sheep vaccinated with Campylovexin® produced antibodies that recognised each of the expressed SLPs produced by the *C. fetus* subsp. *fetus* PFGE types found in New Zealand (Chapter 6). The comparison with Western blotting using pre-immune sera demonstrated that this antibody production was specific to Campylovexin® vaccination. It has previously been shown that SLPs are immunodominant protein antigens (Dubreuil et al. 1988), and that sheep vaccinated with *C. fetus* subsp. *fetus* produced antibodies to SLPs (Myers et al. 1970; Grogono-Thomas et al. 2003). In addition, Western Blotting and ELISA experiments have previously shown that antiserum raised to a purified SLP binds to SLPs of different sizes and from both serotype A and B strains (Pei et al. 1988; Fujimoto et al. 1991; Wang et al. 1993). Therefore, in the present study, antibody recognition of each of the expressed SLPs produced by the *C. fetus* subsp. *fetus* PFGE types suggests that vaccination of sheep with Campylovexin® elicits the production of antibodies against all the *C. fetus* subsp. *fetus* strains found in New Zealand.

There is some evidence that suggests that antibodies to SLPs are important in protection against sheep abortion due to *C. fetus* subsp. *fetus* (Myers et al. 1970; Grogono-Thomas et al. 2003). The next part of the present study involved openly seeking cases in which it was suspected that Campylovexin® failed to protect against *C. fetus* subsp. *fetus* abortion.

9.5 Campylovexin® breakdown investigation

There have been few reported cases of *C. fetus* subsp. *fetus* abortions in Campylovexin® vaccinated ewes (Pauling 1988; Marchant 1999). However, in 1996 an apparent vaccine breakdown occurred, when fifteen ewes aborted out of a flock of 300 vaccinated two-tooth ewes (Fenwick et al. 2000). *Campylobacter fetus* subsp. *fetus* was isolated from six foetuses, and the isolates were shown to have a different PFGE profile to the vaccine

strain. However, it was suggested that the ewes may have been exposed to high *C. fetus* subsp. *fetus* challenge when they were densely stocked each night in a small, "sacrifice" paddock (Fenwick et al. 2000).

Despite efforts to solicit reports of Campylovexin® failure in the present study, only one case was reported in which ewes that had been apparently vaccinated in the 2001 season subsequently aborted due to *C. fetus* subsp. *fetus* (Chapter 7). The management of this flock meant that there was likely to have been a high challenge to vaccinated ewes from unvaccinated aborting ewes.

There were two other reported cases of *C. fetus* subsp. *fetus* abortions in vaccinated ewes. Both cases involved abortion in ewes that had not been vaccinated in the current year and the PFGE type of the *C. fetus* subsp. *fetus* isolates from these cases was the same as the vaccine strain, PFGE type A1. This suggests that in these cases apparent vaccine breakdown was not due to lack of cross-protection against other strains.

It appears that there was not a single *C. fetus* subsp. *fetus* PFGE type associated with abortions in ewes vaccinated with Campylovexin®. As mentioned, in two of the three cases from 2001 the *C. fetus* subsp. *fetus* isolates were PFGE type A1. The *C. fetus* subsp. *fetus* isolate from the other case was PFGE type C1, and the isolates from the 1996 Manawatu breakdown case were identified in the present study as PFGE type B1. Just as there was not a particular PFGE type associated with *C. fetus* subsp. *fetus* abortion in vaccinated ewes, there was not a particular SLP type expressed by *C. fetus* subsp. *fetus* isolates from abortions in ewes vaccinated with Campylovexin®. The SLPs of the breakdown isolates were recognised by antibodies in pooled immune sera, indicating that the SLPs of these breakdown isolates were able to be recognised by immune animals. This suggests that despite the potential selective pressure of a vaccine on the *C. fetus* subsp. *fetus* population, there has not been the emergence of a strain of *C. fetus* subsp. *fetus* that expresses an SLP able to evade the immunity of vaccinated ewes.

High stocking density was a feature in the three apparent Campylovexin® breakdown cases (Chapter 7), as well as the 1996 Manawatu case (Fenwick et al. 2000). High stocking density management systems have been found to be associated with a higher

Campylobacter abortion rate than low stock density set-stocking management practices (Quinlivan and Jopp 1982). It appears that high stocking density may overwhelm immunity to *Campylobacter* disease and some flocks may require annual boosting of immunity, as recommended by the manufacturer.

It appears that despite the widespread occurrence of distinct strains of *C. fetus* subsp. *fetus* (Chapter 4), abortion due to *C. fetus* subsp. *fetus* is a rare event in ewes vaccinated with Campylovexin®. The observed paucity of *C. fetus* subsp. *fetus* abortion in sheep vaccinated with Campylovexin®, together with the recognition of proteins of the various *C. fetus* subsp. *fetus* PFGE types by antibodies from ewes vaccinated with Campylovexin®, indicates that Campylovexin® may provide protection against other *C. fetus* subsp. *fetus* strains. The protection of Campylovexin® against the most commonly occurring *C. fetus* subsp. *fetus* strain, PFGE type B1, was tested directly using an animal challenge model.

9.6 Investigation of Campylovexin® protection using an animal challenge model

Campylovexin® efficacy was tested using pregnant guinea pigs as a model, as has been described for the testing of other *C. fetus* subsp. *fetus* vaccines (Bryner et al. 1978, 1979 and 1988; Diker and Turutoglu 1995). This model was successfully implemented in the present study (Chapter 8). Initially, the strain used in the manufacture of Campylovexin® (strain 5915, PFGE type A1) was tested using the pregnant guinea pig model. The 5915 challenge strain was used in order to examine whether Campylovexin® provided protection against homologous challenge using this model. The results of this study indicated that Campylovexin® provides a statistically significant level of protection against homologous challenge using the pregnant guinea pig model.

Subsequently, an isolate of PFGE type B1 was used as the challenge strain in this model to examine the cross-protection offered by Campylovexin® against a heterologous *C. fetus* subsp. *fetus* strain (Chapter 8). An isolate of PFGE type B1 was chosen as the challenge strain because this type was the most commonly isolated in the Hawke's Bay regional study and the national study (Chapters 3 and 4), as well as being implicated in the 1996 Manawatu breakdown case (Fenwick et al. 2000). The actual isolate used as

the challenge strain was cultured from an aborted foetus from a flock in which 90 out of 330 ewes aborted (27%). In the pregnant guinea pig model, Campylovexin® provided a statistically significant level of protection against challenge with this strain.

9.7 Summary

Despite the range of *C. fetus* subsp. *fetus* PFGE types identified in this study, there was no evidence to suggest that the different strain types compromised Campylovexin® efficacy in protection against *C. fetus* subsp. *fetus* sheep abortion. The protein recognition by serum from ewes vaccinated with Campylovexin®, the lack of a robust vaccine breakdown case, and the demonstration of cross-protection in the guinea pig model, provide evidence that Campylovexin® appears to offer cross-protection against strains of *C. fetus* subsp. *fetus* causing abortion in sheep in New Zealand.

Appendix 1: Similarity matrix of *Campylobacter fetus* subsp. *fetus* PFGE types from Hawke's Bay in 1999
Calculation Method: Dice Coefficient

		1 B1	2 B2	3 F1	4 D1	5 F2	6 E1	7 E2	8 D2	9 A2	10 A1	11 C1
1	B1	100.0	92.9	78.6	75.9	74.1	71.4	71.4	69.0	66.7	64.3	60.0
2	B2	92.9	100.0	71.4	69.0	74.1	71.4	71.4	69.0	66.7	64.3	66.7
3	F1	78.6	71.4	100.0	75.9	88.9	64.3	64.3	69.0	81.5	78.6	66.7
4	D1	75.9	69.0	75.9	100.0	71.4	82.8	82.8	93.3	78.6	82.8	71.0
5	F2	74.1	74.1	88.9	71.4	100.0	74.1	66.7	71.4	84.6	81.5	62.1
6	E1	71.4	71.4	64.3	82.8	74.1	100.0	92.9	82.8	74.1	71.4	60.0
7	E2	71.4	71.4	64.3	82.8	66.7	92.9	100.0	82.8	74.1	71.4	60.0
8	D2	69.0	69.0	69.0	93.3	71.4	82.8	82.8	100.0	85.7	82.8	77.4
9	A2	66.7	66.7	81.5	78.6	84.6	74.1	74.1	85.7	100.0	88.9	75.9
10	A1	64.3	64.3	78.6	82.8	81.5	71.4	71.4	82.8	88.9	100.0	73.3
11	C1	60.0	66.7	66.7	71.0	62.1	60.0	60.0	77.4	75.9	73.3	100.0

Appendix 2: Pulsed-field gel electrophoresis data for the isolates from the Hawke's Bay 1999 study (page 1 of 2).

Isolate name	No. of farms	No. of isolates	PFGE type	Species[a]
CV1	1	1	E1	
CV2		1	E2	
CV3		1	E2	
CV4	1	1	B1	
CV5		1	B1	
CV6	1	1	B1	
CV7		1	B1	
CV8		1	B1	
CV9		1	B1	
CV10		1	B1	
CV11	1	1	B1	
CV12		1	B1	
CV13		1	B1	
CV14	1	1	B1	
CV15		1	B1	
CV16		1	B1	
CV17		1	B1	
CV18	1	1	B1	
CV19		1	B1	
CV20		1	B1	
CV21		1	B1	
CV22		1	B1	
CV23	1	1	B1	
CV24		1	B1	
CV25		1	B1	
CV26		1	B1	
CV27	1	1	B2	
CV28		1	B2	
CV29		1	B2	
CV30	1	1	A2	
CV31	1	1	B1	
CV32		1	B1	
CV33	1	1	B1	
CV35	1	1	C1	
CV36	1	1	B1	
CV37		1	B1	
CV38	1	1	B1	
CV39		1	D1	
CV40		1	B1	
CV41		1	B1	
CV42		1	B1	
CV45	1	1	F2	
CV46		1	F2	
CV47		1	F2	
CV48		1	F2	
CV49		1	F2	

Appendix 2: Pulsed-field gel electrophoresis data for the isolates from the Hawke's Bay 1999 study (page 2 of 2).

Isolate name	No. of farms	No. of isolates	PFGE type	Species[a]
CV50	1	1	B1	
CV51		1	B1	
CV52		1	B1	
CV53		1	B1	
CV54	1	1	B1	
CV71		1	B1	
CV72		1	B1	
CV73		1	B1	
CV55	1	1	B1	
CV56		1	B1	
CV57		1	F1	
CV58		1	F1	
CV59	1	1	B1	
CV60		1	B1	
CV61		1	B1	
CV62	1	1	B1	
CV68		1	B1	
CV69		1	B1	
CV70		1	B1	
CV63	1	1	B1	
CV64		1	B1	
CV65		1	B1	
CV66		1	B1	
CV67		1	B1	
CV77	1	1	B1	
CV78		1	B1	
CV79		1	B1	
CV80		1	B1	
CV75	1	1	B1	
CV81		1	B2	
CV82		1	B1	
CV83	1	1	D1	
CV84		1	D2	
CV85	1	1	B1	
CV86		1	B1	
CV34	1	1	CJ-1	*C. jejuni*
CV43	1	1	CJ-1	*C. jejuni*
CV44		1	CJ-1	*C. jejuni*
CV74	1	1	CJ-2	*C. jejuni*
	28	**85**		

[a] Species is *C. fetus* subsp. *fetus* unless stated otherwise.

Appendix 3: Similarity matrix of all *Campylobacter fetus* subsp. *fetus* PFGE types identified to date (page 1 of 2)
Calculation Method: Dice Coefficient

		1 B1	2 B3	3 B5	4 B2	5 B6	6 B4	7 F4	8 F1	9 D1	10 D3	11 J1	12 F2	13 F5	14 F3	15 E1	16 E2
1	B1	100.0	**96.6**	**92.9**	**92.9**	**92.9**	**88.9**	81.5	78.6	75.9	75.9	75.9	74.1	74.1	74.1	71.4	71.4
2	B3	**96.6**	100.0	**89.7**	**89.7**	**89.7**	**85.7**	78.6	75.9	73.3	73.3	73.3	71.4	71.4	71.4	69.0	69.0
3	B5	**92.9**	**89.7**	100.0	**92.9**	**92.9**	**88.9**	74.1	71.4	69.0	69.0	69.0	74.1	74.1	74.1	71.4	71.4
4	B2	**92.9**	**89.7**	**92.9**	100.0	**92.9**	**88.9**	74.1	71.4	69.0	69.0	69.0	74.1	74.1	74.1	71.4	71.4
5	B6	**92.9**	**89.7**	**92.9**	**92.9**	100.0	**88.9**	74.1	71.4	69.0	69.0	69.0	74.1	74.1	74.1	71.4	71.4
6	B4	**88.9**	**85.7**	**88.9**	**88.9**	**88.9**	100.0	69.2	66.7	64.3	64.3	78.6	69.2	69.2	69.2	66.7	66.7
7	F4	81.5	78.6	74.1	74.1	74.1	69.2	100.0	**96.3**	78.6	71.4	64.3	**92.3**	**92.3**	**92.3**	66.7	66.7
8	F1	78.6	75.9	71.4	71.4	71.4	66.7	**96.3**	100.0	75.9	69.0	62.1	**88.9**	**88.9**	**88.9**	64.3	64.3
9	D1	75.9	73.3	69.0	69.0	69.0	64.3	78.6	75.9	100.0	**86.7**	73.3	71.4	71.4	71.4	82.8	82.8
10	D3	75.9	73.3	69.0	69.0	69.0	64.3	71.4	69.0	**86.7**	100.0	66.7	64.3	71.4	64.3	75.9	75.9
11	J1	75.9	73.3	69.0	69.0	69.0	78.6	64.3	62.1	73.3	66.7	100.0	57.1	57.1	57.1	62.1	62.1
12	F2	74.1	71.4	74.1	74.1	74.1	69.2	**92.3**	**88.9**	71.4	64.3	57.1	100.0	**92.3**	**92.3**	74.1	66.7
13	F5	74.1	71.4	74.1	74.1	74.1	69.2	**92.3**	**88.9**	71.4	71.4	57.1	**92.3**	100.0	**92.3**	66.7	66.7
14	F3	74.1	71.4	74.1	74.1	74.1	69.2	**92.3**	**88.9**	71.4	64.3	57.1	**92.3**	**92.3**	100.0	66.7	66.7
15	E1	71.4	69.0	71.4	71.4	71.4	66.7	66.7	64.3	82.8	75.9	62.1	74.1	66.7	66.7	100.0	**92.9**
16	E2	71.4	69.0	71.4	71.4	71.4	66.7	66.7	64.3	82.8	75.9	62.1	66.7	66.7	66.7	**92.9**	100.0
17	D2	69.0	66.7	69.0	69.0	69.0	64.3	71.4	69.0	**93.3**	**86.7**	66.7	71.4	78.6	71.4	82.8	82.8
18	K2	66.7	64.3	59.3	59.3	59.3	61.5	84.6	81.5	85.7	78.6	71.4	76.9	76.9	76.9	66.7	66.7
19	I1	66.7	64.3	66.7	66.7	66.7	61.5	76.9	74.1	71.4	71.4	64.3	76.9	84.6	76.9	66.7	66.7
20	A2	66.7	64.3	66.7	66.7	66.7	61.5	84.6	81.5	78.6	78.6	57.1	84.6	92.3	84.6	74.1	74.1
21	A7	66.7	64.3	66.7	66.7	66.7	61.5	84.6	81.5	78.6	71.4	57.1	84.6	84.6	92.3	74.1	74.1
22	A4	64.3	62.1	64.3	64.3	64.3	59.3	81.5	78.6	75.9	75.9	55.2	81.5	88.9	88.9	71.4	71.4
23	A6	64.3	62.1	64.3	64.3	64.3	59.3	81.5	78.6	82.8	75.9	62.1	88.9	81.5	81.5	78.6	71.4
24	A3	64.3	62.1	64.3	64.3	71.4	59.3	81.5	78.6	75.9	69.0	62.1	81.5	81.5	81.5	64.3	64.3
25	A1	64.3	62.1	64.3	64.3	64.3	59.3	81.5	78.6	82.8	75.9	62.1	81.5	81.5	81.5	71.4	71.4
26	A5	64.3	62.1	64.3	64.3	71.4	59.3	81.5	78.6	82.8	75.9	62.1	81.5	81.5	81.5	71.4	71.4
27	H1	64.3	62.1	64.3	64.3	64.3	74.1	66.7	64.3	69.0	62.1	69.0	66.7	66.7	66.7	64.3	71.4
28	G1	64.3	62.1	57.1	57.1	57.1	51.9	66.7	71.4	69.0	69.0	62.1	59.3	59.3	59.3	64.3	64.3
29	C2	60.0	58.1	60.0	60.0	60.0	55.2	62.1	66.7	71.0	71.0	58.1	62.1	69.0	69.0	60.0	60.0
30	C1	60.0	58.1	60.0	66.7	60.0	55.2	62.1	66.7	71.0	71.0	58.1	62.1	69.0	62.1	60.0	60.0
31	K1	57.1	55.2	50.0	50.0	50.0	59.3	74.1	71.4	75.9	69.0	69.0	66.7	66.7	66.7	57.1	57.1

Appendix 3: Similarity matrix of all *Campylobacter fetus* subsp. *fetus* PFGE types identified to date (page 2 of 2)
Calculation Method: Dice Coefficient

		17 D2	18 K2	19 I1	20 A2	21 A7	22 A4	23 A6	24 A3	25 A1	26 A5	27 H1	28 G1	29 C2	30 C1	31 K1
1	B1	69.0	66.7	66.7	66.7	66.7	64.3	64.3	64.3	64.3	64.3	64.3	64.3	60.0	60.0	57.1
2	B3	66.7	64.3	64.3	64.3	64.3	62.1	62.1	62.1	62.1	62.1	62.1	62.1	58.1	58.1	55.2
3	B5	69.0	59.3	66.7	66.7	66.7	64.3	64.3	64.3	64.3	64.3	64.3	57.1	60.0	60.0	50.0
4	B2	69.0	59.3	66.7	66.7	66.7	64.3	64.3	64.3	64.3	64.3	64.3	57.1	60.0	66.7	50.0
5	B6	69.0	59.3	66.7	66.7	66.7	64.3	64.3	71.4	64.3	71.4	64.3	57.1	60.0	60.0	50.0
6	B4	64.3	61.5	61.5	61.5	61.5	59.3	59.3	59.3	59.3	59.3	74.1	51.9	55.2	55.2	59.3
7	F4	71.4	84.6	76.9	84.6	84.6	81.5	81.5	81.5	81.5	81.5	66.7	66.7	62.1	62.1	74.1
8	F1	69.0	81.5	74.1	81.5	81.5	78.6	78.6	78.6	78.6	78.6	64.3	71.4	66.7	66.7	71.4
9	D1	**93.3**	85.7	71.4	78.6	78.6	75.9	82.8	75.9	82.8	82.8	69.0	69.0	71.0	71.0	75.9
10	D3	**86.7**	78.6	71.4	78.6	71.4	75.9	75.9	69.0	75.9	75.9	62.1	69.0	71.0	71.0	69.0
11	J1	66.7	71.4	64.3	57.1	57.1	55.2	62.1	62.1	62.1	62.1	69.0	62.1	58.1	58.1	69.0
12	F2	71.4	76.9	76.9	84.6	84.6	81.5	88.9	81.5	81.5	81.5	66.7	59.3	62.1	62.1	66.7
13	F5	78.6	76.9	84.6	92.3	84.6	88.9	81.5	81.5	81.5	81.5	66.7	59.3	69.0	69.0	66.7
14	F3	71.4	76.9	76.9	84.6	92.3	88.9	81.5	81.5	81.5	81.5	66.7	59.3	69.0	62.1	66.7
15	E1	82.8	66.7	66.7	74.1	74.1	71.4	78.6	64.3	71.4	71.4	64.3	64.3	60.0	60.0	57.1
16	E2	82.8	66.7	66.7	74.1	74.1	71.4	71.4	64.3	71.4	71.4	71.4	64.3	60.0	60.0	57.1
17	D2	100.0	78.6	78.6	85.7	78.6	82.8	82.8	75.9	82.8	82.8	69.0	62.1	77.4	77.4	69.0
18	K2	78.6	100.0	69.2	84.6	84.6	81.5	88.9	81.5	88.9	88.9	74.1	59.3	69.0	69.0	**88.9**
19	I1	78.6	69.2	100.0	84.6	76.9	81.5	74.1	74.1	74.1	74.1	66.7	59.3	69.0	69.0	59.3
20	A2	85.7	84.6	84.6	100.0	**92.3**	**96.3**	**88.9**	**81.5**	**88.9**	**88.9**	74.1	59.3	75.9	75.9	74.1
21	A7	78.6	84.6	76.9	**92.3**	100.0	**96.3**	**88.9**	**81.5**	**88.9**	**88.9**	74.1	59.3	75.9	69.0	74.1
22	A4	82.8	81.5	81.5	**96.3**	**96.3**	100.0	**85.7**	**78.6**	**85.7**	**85.7**	71.4	57.1	80.0	73.3	71.4
23	A6	82.8	88.9	74.1	**88.9**	**88.9**	**85.7**	100.0	**85.7**	**92.9**	**92.9**	71.4	57.1	73.3	73.3	78.6
24	A3	75.9	81.5	74.1	**81.5**	**81.5**	**78.6**	**85.7**	100.0	**85.7**	**92.9**	64.3	57.1	66.7	66.7	71.4
25	A1	82.8	88.9	74.1	**88.9**	**88.9**	**85.7**	**92.9**	**85.7**	100.0	**92.9**	71.4	57.1	73.3	73.3	78.6
26	A5	82.8	88.9	74.1	**88.9**	**88.9**	**85.7**	**92.9**	**92.9**	**92.9**	100.0	71.4	57.1	73.3	73.3	78.6
27	H1	69.0	74.1	66.7	74.1	74.1	71.4	71.4	64.3	71.4	71.4	100.0	57.1	66.7	66.7	71.4
28	G1	62.1	59.3	59.3	59.3	59.3	57.1	57.1	57.1	57.1	57.1	57.1	100.0	60.0	60.0	50.0
29	C2	77.4	69.0	69.0	75.9	75.9	80.0	73.3	66.7	73.3	73.3	66.7	60.0	100.0	**93.8**	66.7
30	C1	77.4	69.0	69.0	75.9	69.0	73.3	73.3	66.7	73.3	73.3	66.7	60.0	**93.8**	100.0	66.7
31	K1	69.0	**88.9**	59.3	74.1	74.1	71.4	78.6	71.4	78.6	78.6	71.4	50.0	66.7	66.7	100.0

Appendix 4: Pulsed-field gel electrophoresis data for the isolates from the 2000 national study.

	Isolate name	Diagnostic laboratory	No. of farms	No. of isolates	PFGE type	Species[a]
Gisborne	OC8	Alpha-Sci	1	1	B5	
	OC9	Alpha-Sci		1	B1	
	OC204	Alpha-Sci	1	1	B1	
Waikato	OC93	Alpha-Sci	1	1	B1	
	OC94	Alpha-Sci	1	1	B1	
	OC95	Alpha-Sci		1	B1	
	OC98	Alpha-Sci	1	1	B1	
	OC99	Alpha-Sci	1	1	B1	
	OC100	Alpha-Sci	1	1	B1	
	OC149	Alpha-Sci	1	1	B1	
	OC150	Alpha-Sci		1	B1	
	OC151	Alpha-Sci		1	B1	
	OC260	Alpha-Sci	1	1	B1	
	OC261	Alpha-Sci		1	B1	
	OC262	Alpha-Sci	1	1	B1	
	OC264	Alpha-Sci	1	1	B1	
	OC265	Alpha-Sci		1	B1	
			11	**17**		
Hawke's Bay	NS1	AgriQuality	1	1	B1	
	NS2	AgriQuality		1	B1	
	NS3	AgriQuality	1	1	B1	
	NS4	AgriQuality		1	B1	
	NS5	AgriQuality		1	B1	
	NS21	AgriQuality		1	B1	
	NS22	AgriQuality		1	B1	
	NS6	AgriQuality	1	1	B1	
	NS7	AgriQuality		1	B1	
	NS10	AgriQuality		1	CJ-1	*C. jejuni*
	NS11	AgriQuality		1	CJ-1	*C. jejuni*
	NS9	AgriQuality	1	1	CJ-1	*C. jejuni*
	NS8	AgriQuality	1	1	F4	
	NS47	AgriQuality		1	F4	
	NS48	AgriQuality		1	F4	
	NS17	AgriQuality	1	1	D1	
	NS12	AgriQuality	1	1	B1	
	NS13	AgriQuality		1	B1	
	OC28	AgriQuality	1	1	B1	
	OC29	AgriQuality		1	B1	
	NS14	AgriQuality	1	1	B1	
	NS15	AgriQuality		1	B1	
	NS18	AgriQuality		1	B1	
	NS19	AgriQuality		1	B1	
	NS20	AgriQuality		1	B1	

	Isolate name	Diagnostic laboratory	No. of farms	No. of isolates	PFGE type	Species
Hawke's Bay (continued)	NS25	AgriQuality	1	1	B1	
	NS26	AgriQuality		1	B1	
	OC41	AgriQuality	1	1	J1	
	NS23	AgriQuality	1	1	B2	
	NS24	AgriQuality		1	B2	
	NS27	AgriQuality	1	1	B1	
	NS28	AgriQuality		1	B1	
	NS29	AgriQuality		1	B1	
	NS30	AgriQuality	1	1	B1	
	NS31	AgriQuality		1	B1	
	NS32	AgriQuality		1	B1	
	NS33	AgriQuality		1	B1	
	OC64	AgriQuality	1	1	F2	
	NS38	AgriQuality	1	1	B1	
	NS39	AgriQuality		1	B1	
	NS34	AgriQuality	1	1	B1	
	NS35	AgriQuality		1	B1	
	NS40	AgriQuality		1	B1	
	NS49	AgriQuality	1	1	B1	
	NS50	AgriQuality		1	B1	
	NS51	AgriQuality		1	B1	
	NS44	AgriQuality	1	1	B1	
	NS45	AgriQuality		1	B1	
	NS46	AgriQuality		1	B1	
	OC79	AgriQuality	1	1	B1	
	OC81	AgriQuality	1	1	B1	
	OC109	AgriQuality	1	1	B1	
	OC110	AgriQuality		1	B2	
	NS52	AgriQuality	1	1	B1	
	NS53	AgriQuality		1	B1	
	NS54	AgriQuality		1	B1	
	NS55	AgriQuality		1	B1	
	NS56	AgriQuality		1	B1	
	OC152	AgriQuality	1	1	B1	
	OC153	AgriQuality		1	B1	
	OC154	AgriQuality		1	B1	
	OC155	AgriQuality		1	B1	
	OC162	AgriQuality	1	1	D1	
	NS66	AgriQuality	1	1	B1	
	NS67	AgriQuality	1	1	C1	
	NS68	AgriQuality		1	C1	
	OC212	AgriQuality	1	1	B1	
	OC213	AgriQuality		1	B1	
	OC220	AgriQuality	1	1	F2	
	OC222	AgriQuality	1	1	B2	
	OC223	AgriQuality		1	B2	
	OC224	AgriQuality		1	B2	
	OC225	AgriQuality		1	B2	
	OC30	AgriQuality	1	1	B1	

	Isolate name	Diagnostic laboratory	No. of farms	No. of isolates	PFGE type	Species
Hawke's Bay (continued)	OC115	AgriQuality	1	1	B4	
	OC116	AgriQuality		1	B4	
	OC191	AgriQuality	1	1	B2	
			33	**77**		
Manawatu	OC3	AgriQuality	1	1	B1	
	OC1	AgriQuality	1	1	B3	
	OC2	AgriQuality		1	B3	
	OC24	AgriQuality	1	1	B1	
	OC25	AgriQuality		1	B1	
	OC26	AgriQuality	1	1	B1	
	OC33	AgriQuality	1	1	B1	
	OC34	AgriQuality		1	B2	
	OC35	AgriQuality	1	1	B1	
	OC67	AgriQuality	1	1	B1	
	OC66	AgriQuality	1	1	B1	
	OC68	AgriQuality	1	1	B1	
	OC69	AgriQuality		1	B1	
	OC70	AgriQuality		1	B1	
	NS41	AgriQuality	1	1	B1	
	NS42	AgriQuality		1	B1	
	NS43	AgriQuality		1	B1	
	OC75	AgriQuality	1	1	B1	
	OC76	AgriQuality	1	1	D1	
	OC77	AgriQuality	1	1	B1	
	OC78	AgriQuality		1	B1	
	OC80	AgriQuality	1	1	B1	
	OC118	AgriQuality	1	1	B1	
	OC117	AgriQuality	1	1	B1	
	OC119	AgriQuality	1	1	B1	
	NS59	AgriQuality	1	1	B1	
	OC163	AgriQuality	1	1	B1	
	OC194	AgriQuality	1	1	B1	
	OC210	AgriQuality	1	1	B1	
	OC211	AgriQuality	1	1	B1	
	OC221	AgriQuality	1	1	B1	
	OC236	AgriQuality	1	1	B1	
	OC90	MML	1	1	B1	
	OC111	MML	1	1	B1	
			26	**34**		
Wairarapa	OC4	AgriQuality	1	1	B1	
	OC5	AgriQuality		1	B1	
	OC6	AgriQuality	1	1	F4	
	OC7	AgriQuality	1	1	D1	
	OC65	AgriQuality	1	1	A2	
	OC91	AgriQuality	1	1	B1	
	OC112	AgriQuality	1	1	F4	
	OC113	AgriQuality		1	F4	
	OC114	AgriQuality		1	F4	

	Isolate name	Diagnostic laboratory	No. of farms	No. of isolates	PFGE type	Species
Wairarapa (continued)	OC156	AgriQuality	1	1	B6	
	OC192	AgriQuality	1	1	B1	
	OC193	AgriQuality		1	B1	
	OC208	AgriQuality	1	1	B1	
	OC209	AgriQuality		1	B1	
	OC266	me	1	1	B2	
	OC259	AgriQuality		1	CJ-3	*C. jejuni*
	OC31	AgriQuality	1	1	CJ-1	*C. jejuni*
	OC32	AgriQuality		1	CJ-1	*C. jejuni*
			10	**16**		
Marlborough	OC96	Alpha-Sci	1	1	B1	
	OC97	Alpha-Sci		1	B1	
	OC207	Alpha-Sci	1	1	B1	
	OC263	Alpha-Sci	1	1	B1	
	OC13	LabWorks	1	1	A2	
	OC43	LabWorks	1	1	B1	
	OC47	LabWorks	1	1	A2	
	OC49	LabWorks	1	1	B1	
	OC50	LabWorks		1	B1	
	OC142	LabWorks	1	1	B1	
	OC143	LabWorks		1	B1	
	OC175	LabWorks	1	1	B1	
	OC188	LabWorks	1	1	B2	
	OC135	LabWorks	1	1	D1	
	OC136	LabWorks		1	D1	
	OC137	LabWorks		1	D1	
	OC138	LabWorks		1	CJ-1	*C. jejuni*
	OC139	LabWorks		1	CJ-1a	*C. jejuni*
			11	**18**		
Canterbury	OC92	AgriQuality	1	1	B1	
	OC164	AgriQuality	1	1	B1	
	OC147	Alpha-Sci	1	1	B1	
	OC148	Alpha-Sci		1	B1	
	OC202	Alpha-Sci	1	1	B1	
	OC203	Alpha-Sci		1	B1	
	OC205	Alpha-Sci	1	1	B1	
	OC206	Alpha-Sci		1	B1	
	OC11	LabWorks	1	1	B1	
	OC12	LabWorks	1	1	K1	
	OC14	LabWorks	1	1	A2	
	OC15	LabWorks		1	A2	
	OC17	LabWorks	1	1	B2	
	OC18	LabWorks	1	1	B1	
	OC19	LabWorks		1	B1	
	OC42	LabWorks	1	1	B1	
	OC44	LabWorks	1	1	B1	
	OC45	LabWorks	1	1	B1	
	OC46	LabWorks		1	B2	

	Isolate name	Diagnostic laboratory	No. of farms	No. of isolates	PFGE type	Species
Canterbury (continued)	OC48	LabWorks	1	1	F2	
	OC56	LabWorks	1	1	B1	
	OC59	LabWorks	1	1	D1	
	OC60	LabWorks	1	1	B1	
	OC61	LabWorks		1	B1	
	OC62	LabWorks	1	1	F4	
	OC63	LabWorks		1	F4	
	OC102	LabWorks	1	1	D1	
	OC103	LabWorks		1	D1	
	OC104	LabWorks	1	1	A2	
	NS60	LabWorks	1	1	B1	
	NS61	LabWorks		1	B1	
	NS62	LabWorks		1	B1	
	NS63	LabWorks		1	B1	
	NS64	LabWorks		1	B1	
	NS65	LabWorks		1	B1	
	OC130	LabWorks	1	1	B1	
	OC134	LabWorks	1	1	A1	
	OC140	LabWorks	1	1	F3	
	OC141	LabWorks		1	B1	
	OC145	LabWorks	1	1	D1	
	OC146	LabWorks	1	1	B6	
	OC144	LabWorks	1	1	B1	
	OC174	LabWorks	1	1	B1	
	OC178	LabWorks	1	1	B1	
	OC179	LabWorks	1	1	I1	
	OC180	LabWorks	1	1	B1	
	OC182	LabWorks	1	1	B1	
	OC183	LabWorks	1	1	B1	
	OC184	LabWorks		1	B1	
	OC185	LabWorks		1	B1	
	OC186	LabWorks		1	F4	
	OC187	LabWorks		1	B1	
	OC189	LabWorks	1	1	A1	
	OC190	LabWorks	1	1	B2	
	OC71	LabNet	1	1	A2	
	OC88	LabNet	1	1	B2	
	OC51	LabWorks	1	1	CC-1	*C. coli*
	OC132	LabWorks	1	1	CJ-7	*C. jejuni*
	NS77	LabWorks	1	1	CJ-2	*C. jejuni*
			40	**59**		
Otago	OC20	LabNet	1	1	B1	
	OC37	LabNet	1	1	B1	
	OC38	LabNet		1	B1	
	NS36	LabNet	1	1	B1	
	NS37	LabNet		1	B1	
	NS70	LabNet		1	B1	
	NS57	LabNet	1	1	B1	

	Isolate name	Diagnostic laboratory	No. of farms	No. of isolates	PFGE type	Species
Otago (continued)	NS58	LabNet	1	1	B1	
	NS69	LabNet		1	B1	
	NS72	LabNet		1	B1	
	NS73	LabNet		1	B1	
	NS74	LabNet	1	1	A1	
	NS78	LabNet		1	A1	
	NS71	LabNet	1	1	A1	
	NS76	LabNet		1	A5	
	NS75	LabNet	1	1	B1	
	OC216	LabNet	1	1	B1	
	OC58	LabWorks	1	1	A2	
	OC170	LabNet	1	1	B1	
	OC172	LabNet	1	1	B1	
	OC199	LabNet	1	1	F5	
	OC201	LabNet	1	1	B1	
	OC234	LabNet	1	1	B1	
	OC239	LabNet	1	1	D1	
	OC251	LabNet	1	1	B1	
	OC254	LabNet	1	1	B1	
	OC255	LabNet	1	1	B1	
	OC124	LabNet	1	1	B1	
	OC160	LabNet	1	1	A1	
	OC219	LabNet	1	1	A1	
	OC217	LabNet	1	1	A1	
	OC226	LabNet	1	1	D1	
	OC231	LabNet	1	1	B1	
	OC238	LabNet	1	1	A1	
	OC252	LabNet	1	1	A1	
	OC253	LabNet	1	1	B1	
	OC74	LabNet	1	1	CJ-4	*jejuni*
	OC158	LabNet	1	1	CJ-4	*jejuni*
	OC161	LabNet	1	1	CJ-2	*jejuni*
	OC196	LabNet	1	1	CJ-2	*jejuni*
	OC250	LabNet	1	1	CJ-4	*jejuni*
			33	**41**		
Southland	OC82	AgriQuality	1	1	B1	
	OC21	LabNet	1	1	B1	
	OC22	LabNet	1	1	B1	
	OC23	LabNet	1	1	B1	
	OC36	LabNet	1	1	B1	
	OC39	LabNet	1	1	A1	
	OC40	LabNet	1	1	A1	
	OC72	LabNet	1	1	B1	
	OC73	LabNet	1	1	B2	
	OC84	LabNet	1	1	B1	
	OC86	LabNet	1	1	B1	
	OC87	LabNet	1	1	B1	
	OC121	LabNet	1	1	B2	

	Isolate name	Diagnostic laboratory	No. of farms	No. of isolates	PFGE type	Species
Southland (continued)	OC122	LabNet	1	1	B2	
	OC123	LabNet	1	1	B1	
	OC125	LabNet	1	1	B1	
	OC126	LabNet	1	1	B1	
	OC127	LabNet	1	1	A3	
	OC128	LabNet	1	1	B1	
	OC129	LabNet	1	1	A1	
	OC159	LabNet	1	1	B1	
	OC167	LabNet	1	1	G1	
	OC168	LabNet	1	1	B1	
	OC195	LabNet	1	1	B2	
	OC197	LabNet	1	1	A4	
	OC200	LabNet	1	1	A1	
	OC214	LabNet	1	1	B1	
	OC215	LabNet	1	1	B1	
	OC228	LabNet	1	1	B2	
	OC229	LabNet	1	1	B1	
	OC230	LabNet	1	1	D1	
	OC233	LabNet	1	1	B1	
	OC235	LabNet	1	1	B1	
	OC237	LabNet	1	1	A1	
	OC240	LabNet	1	1	H1	
	OC241	LabNet	1	1	B1	
	OC242	LabNet	1	1	B1	
	OC243	LabNet	1	1	A1	
	OC244	LabNet	1	1	B1	
	OC245	LabNet	1	1	A1	
	OC248	LabNet	1	1	B1	
	OC249	LabNet	1	1	B1	
	OC256	LabNet	1	1	B2	
	OC257	LabNet	1	1	B1	
	OC258	LabNet	1	1	B1	
	OC85	LabNet	1	1	CJ-5	*jejuni*
	OC89	LabNet	1	1	CJ-6	*jejuni*
	OC157	LabNet	1	1	CJ-2	*jejuni*
	OC165	LabNet	1	1	CJ-2	*jejuni*
	OC169	LabNet	1	1	CJ-4	*jejuni*
	OC173	LabNet	1	1	CJ-8	*jejuni*
	OC218	LabNet	1	1	CJ-5	*jejuni*
	OC227	LabNet	1	1	CJ-2	*jejuni*
	OC232	LabNet	1	1	CJ-4	*jejuni*
	OC246	LabNet	1	1	CJ-2	*jejuni*
	OC247	LabNet	1	1	CJ-1a	*jejuni*
			56	**56**		

[a] Species is *C. fetus* subsp. *fetus* unless stated otherwise.

Appendix 5: Similarity matrix of *Campylobacter jejuni* PFGE types - ***KpnI***
Calculation Method: Dice Coefficient

		1 CJ-1	2 CJ-1b	3 CJ-1a	4 CJ-7	5 CJ-2a	6 CJ-2	7 CJ-2b	8 CJ-4	9 CJ-2c	10 *coli*T	11 CJ-5	12 CJ-3	13 CJ-6	14 CC-1
1	CJ-1	100.0	**92.9**	**92.9**	32.0	29.6	29.6	28.6	25.0	23.1	21.4	15.4	14.8	14.8	13.3
2	CJ-1b	**92.9**	100.0	**85.7**	40.0	37.0	37.0	35.7	33.3	30.8	28.6	15.4	14.8	7.4	13.3
3	CJ-1a	**92.9**	**85.7**	100.0	32.0	22.2	29.6	35.7	25.0	23.1	14.3	23.1	14.8	14.8	20.0
4	CJ-7	32.0	40.0	32.0	100.0	58.3	58.3	56.0	47.6	52.2	8.0	8.7	41.7	0.0	0.0
5	CJ-2a	29.6	37.0	22.2	58.3	100.0	**92.3**	**88.9**	43.5	**88.0**	14.8	16.0	15.4	23.1	20.7
6	CJ-2	29.6	37.0	29.6	58.3	**92.3**	100.0	**88.9**	43.5	**88.0**	14.8	16.0	15.4	23.1	27.6
7	CJ-2b	28.6	35.7	35.7	56.0	**88.9**	**88.9**	100.0	41.7	**84.6**	7.1	30.8	14.8	22.2	26.7
8	CJ-4	25.0	33.3	25.0	47.6	43.5	43.5	41.7	100.0	45.5	8.3	9.1	26.1	8.7	23.1
9	CJ-2c	23.1	30.8	23.1	52.2	**88.0**	**88.0**	**84.6**	45.5	100.0	7.7	16.7	16.0	24.0	21.4
10	*coli*T	21.4	28.6	14.3	8.0	14.8	14.8	7.1	8.3	7.7	100.0	23.1	14.8	22.2	20.0
11	CJ-5	15.4	15.4	23.1	8.7	16.0	16.0	30.8	9.1	16.7	23.1	100.0	16.0	32.0	42.9
12	CJ-3	14.8	14.8	14.8	41.7	15.4	15.4	14.8	26.1	16.0	14.8	16.0	100.0	23.1	20.7
13	CJ-6	14.8	7.4	14.8	0.0	23.1	23.1	22.2	8.7	24.0	22.2	32.0	23.1	100.0	34.5
14	CC-1	13.3	13.3	20.0	0.0	20.7	27.6	26.7	23.1	21.4	20.0	42.9	20.7	34.5	100.0

*coli*T indicates the *C. coli* Type strain (NCTC 11366)

Appendix 6: Similarity matrix of *Campylobacter jejuni* PFGE types - ***Sma*I**
Calculation Method: Dice Coefficient

		1 CJ-1	2 CJ-1a	3 CJ-1b	4 CJ-2*	5 CJ-5	6 CJ-3	7 CC-1	8 CJ-7	9 *jejT*	10 CJ-6	11 *coliT*	12 *CffT*	13 CJ-4
1	CJ-1	100.0	**87.5**	**87.5**	46.2	44.4	35.3	22.2	14.3	12.5	11.8	10.5	0.0	0.0
2	CJ-1a	**87.5**	100.0	**75.0**	46.2	33.3	35.3	22.2	14.3	12.5	11.8	10.5	0.0	0.0
3	CJ-1b	**87.5**	**75.0**	100.0	30.8	44.4	23.5	22.2	0.0	12.5	11.8	0.0	0.0	0.0
4	CJ-2*	46.2	46.2	30.8	100.0	13.3	42.9	0.0	36.4	15.4	14.3	25.0	0.0	16.7
5	CJ-5	44.4	33.3	44.4	13.3	100.0	21.1	20.0	0.0	11.1	0.0	9.5	0.0	23.5
6	CJ-3	35.3	35.3	23.5	42.9	21.1	100.0	10.5	13.3	23.5	22.2	10.0	0.0	12.5
7	CC-1	22.2	22.2	22.2	0.0	20.0	10.5	100.0	0.0	11.1	10.5	9.5	8.7	11.8
8	CJ-7	14.3	14.3	0.0	36.4	0.0	13.3	0.0	100.0	14.3	13.3	23.5	10.5	15.4
9	*jejT*	12.5	12.5	12.5	15.4	11.1	23.5	11.1	14.3	100.0	0.0	21.1	0.0	0.0
10	CJ-6	11.8	11.8	11.8	14.3	0.0	22.2	10.5	13.3	0.0	100.0	20.0	9.1	0.0
11	*coliT*	10.5	10.5	0.0	25.0	9.5	10.0	9.5	23.5	21.1	20.0	100.0	16.7	11.1
12	*CffT*	0.0	0.0	0.0	0.0	0.0	0.0	8.7	10.5	0.0	9.1	16.7	100.0	10.0
13	CJ-4	0.0	0.0	0.0	16.7	23.5	12.5	11.8	15.4	0.0	0.0	11.1	10.0	100.0

CJ-2* indicates types CJ-2, CJ-2a, CJ-2b and CJ-2c (indistinguishable with restriction enzyme *Sma*I)
*jej*T indicates the *Campylobacter jejuni* subsp. *jejuni* Type strain (NCTC 11351)
*coli*T indicates the *C. coli* Type strain (NCTC 11366)
*Cff*T indicates the *C. fetus subsp. fetus* Type strain (NCTC 10842)

Appendix 7: National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) protein-protein search results of sequence: M I S K S E in positions 1-6.

http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

BLASTP 2.2.5 [Nov-16-2002]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

RID: 1046915394-07120-24505

Query= (6 letters)

Database: All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF
1,348,542 sequences; 431,791,111 total letters

>gi|94639|pir||B36717 S-layer protein - Campylobacter fetus (strain VC119)
(fragments)
Length = 42

Score = 22.7 bits (46), Expect = 572
Identities = 6/6 (100%), Positives = 6/6 (100%)

Query: 1 MISKSE 6
MISKSE
Sbjct: 1 MISKSE 6

>gi|2920661|gb|AAC04580.1| surface layer protein [Campylobacter fetus]
Length = 1112

Score = 22.7 bits (46), Expect = 572
Identities = 6/6 (100%), Positives = 6/6 (100%)

Query: 1 MISKSE 6
MISKSE
Sbjct: 1 MISKSE 6

>gi|2120535|pir||I40711 sapB protein - Campylobacter fetus
gi|802000|gb|AAA79683.1| SapB
Length = 936

Score = 22.7 bits (46), Expect = 572
Identities = 6/6 (100%), Positives = 6/6 (100%)

Query: 1 MISKSE 6
MISKSE
Sbjct: 1 MISKSE 6

```
Database: All non-redundant GenBank CDS
  translations+PDB+SwissProt+PIR+PRF
    Posted date:  Feb 26, 2003 10:23 PM
  Number of letters in database: 431,791,111
  Number of sequences in database:  1,348,542

Lambda     K      H
   0.329    0.255     1.57

Gapped
Lambda     K      H
   0.294    0.110    0.610


Matrix: PAM30
Gap Penalties: Existence: 9, Extension: 1
Number of Hits to DB: 7,822,130
Number of Sequences: 1348542
Number of extensions: 32067
Number of successful extensions: 1094
Number of sequences better than 20000.0: 1089
Number of HSP's better than 20000.0 without gapping: 1089
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1094
length of query: 6
length of database: 431,791,111
effective HSP length: 0
effective length of query: 9
effective length of database: 431,791,111
effective search space: 3886119999
effective search space used: 3886119999
T: 11
A: 40
X1: 15 ( 7.1 bits)
X2: 35 (14.8 bits)
X3: 58 (24.6 bits)
S1: 34 (18.1 bits)
S2: 34 (17.6 bits)
```

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SUPERVISOR'S DECLARATION

This is to certify that the research carried out for the Doctoral thesis entitled "*Campylobacter* abortion in sheep: a study of strain types and vaccine protection" was done by Sally Mannering in the Institute of Veterinary, Animal, and Biomedical Sciences, Massey University, Palmerston North, New Zealand. The thesis material has not been used in part or in whole for any other qualification, and I confirm that the candidate has pursued the course of study in accordance with the requirements of the Massey University regulations.

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CANDIDATE'S DECLARATION

This is to certify that the research carried out for my Doctoral thesis entitled "*Campylobacter* abortion in sheep: a study of strain types and vaccine protection" in the Institute of Veterinary, Animal, and Biomedical Sciences, Massey University, Palmerston North, New Zealand is my own work and that the thesis material has not been used in part or in whole for any other qualification.

Candidate's Name Sally Mannering

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Date 09 May 03