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Diagnosis of bovine venereal campylobacteriosis in New Zealand

A thesis presented in partial fulfilment of the requirements for the
Master of Applied Science
at Massey University
Palmerston North, New Zealand

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2005

Acknowledgements

I would like to thank Prof. Tim Parkinson for his help through this learning process. Your support, constructive criticism, good advice and good humour were always uplifting and helped me through this experience. I'll miss helping out in the large animal handling practice, your life stories, your spicy humour and your always positive feedback. Thank you for letting me free to work, organize and decide.

My special thanks to Cord Heuer for his good advice and constant support while doing the statistical analysis of these data.

This work would not have been achieved without the help of Liz Gillespie, Robin Whitson, Sheila Ramsay, Wendy, Bo, Puppy, Tiger and Horace who were by my side every step of the way taking care not only of me but also my animals. I thank you for your friendship, your support, the nice BBQs shared and for keeping your door open for me....I'll never forget you.

Wendy Gill and Debbie Delport, thanks for bearing with me these two years, thanks for knowing where the boss was and thanks for answering that phone so nicely even if it was the third time in an hour that I called!!!!!

My deep thanks also to the 8th floor technical staff past and present at IVABS, namely Lynn Rogers, Rebecca Pattison, Hamish Mack, Anne Midwinter, Dianne Knight and Laryssa Howe. My work would have never been possible without each one of you. I thank you for your technical support, for having been always open to discuss my results with me, for teaching me the techniques I used during this work, for bearing with me through my ups and downs, for giving me a place in your rooms, benches, machines and hearts. I am sure you won't miss my screaming, singing, loud voice and constant chitchat!!!!!

Alex Grinberg, thanks heaps for your points of view, they always came in good times.

Peter Wildbore my thanks for your support, for being always there, for putting up with my last minute orders and for making my life easier.

Kim Dowson, thanks heaps for your help in the last stage of my field work. You're great to work with.

To my witches: Fernanda, Maria, Alejandra and Klelia, and my morelock apprentices Victor and Nacho. What can I say to you....you are coming with me wherever life takes me and a piece of me stays with each one of you. I love you all. Thanks for all the good moments, for the big laughs and the support through tough times, the amazing food, your amazing culture but most of all thank you for allowing me to have true friends so far away from home.

To my partner Alexis for embarking with me in this adventure to far away lands, for making me a better person. I love you heaps.

To LASA for being always cheerful, for sharing great BBQs and for being our repesentation in these far lands. Keep up the good work guys!!!!!

To my parents, for giving me life, for always allowing me to fly as far as my wings would take me even if that caused you pain, for teaching me good values and for helping me being the person that I am today. And thanks Juan for my lovely niece. I'll be home soon.

To my friends back home, thanks for your support, for your good advice, for keeping in touch and for the gossip updates. I'll be back soon girls!!!!

To the life of my two Hereford boys, bulls 29 and 92... Thank you for being such good animals to work with.

To IVABS and Meat and Wool Innovation for funding this work, and to Livestock Improvement Corporation for providing me so many times with working material.

To NZODA for allowing me to come to New Zealand and fulfil this life-long dream. Sue Flynn and Sylvia Hooker at International Student Support Office, Massey University, thank for your support, your great people!!!!

I dedicate this work to my two grand mothers

Carmen Uriarte and Tilia Flematti,

two amazing women

I'll always love you.

Abstract

Bovine venereal campylobacteriosis (BVC) is a venereal disease causing infertility in cows due to early embryonic loss. Caused by *Campylobacter fetus* subsp *venerealis* (*Cfv*), which lives in the preputial crypts of older asymptomatic carrier bulls and the vagina of carrier cows. Diagnosis of the disease is difficult as it is a fastidious organism to culture and the serological methods which have been developed to diagnose BVC, exhibit substantial cross-reactivity with *Campylobacter fetus* subsp *fetus* (*Cff*), due to the close genomic and phenotypic relationship of the two subspecies. Definitive differentiation between *Cfv* and *Cff* requires molecular biology techniques.

In New Zealand, *Cfv* was last isolated in 1993 and suspected again in 2001/2002 after the use of a recently-developed IgA ELISA test that claimed to be 98.5% specific for *Cfv*. A nation wide study showed that there was no relationship between the test's results and the reproductive performance of the herds. Most of the positive results obtained were false probably due to cross-reactions with *Cff*. The lack of positive isolations of *Cfv* raised questions about the sensitivity of the isolation methods used.

Five experiments were undertaken. Experiments 1–3 examined the microbiological methods that are used to isolated *Cfv*. Results indicated that the optimal samples and culture conditions were preputial washes or scrapes from bulls, or vaginal washes (20 ml PBS) from cows, enriched in Lander's medium for three days before subculture onto blood agar. All cultures need to be undertaken under microaerophilic conditions. These experiments also addressed the reliability of the IgA ELISA for animals managed according to the husbandry conditions that prevail in New Zealand. Results showed that there was a great deal of within- and between- animal variation in ELISA values and that there was substantial cross reactivity with *Cff*. (ICC ranged from 0.29 to 0.03 depending on the Group and all heifers challenged with *Cff* became positive to IgA ELISA). In consequence, the test appears to be of limited diagnostic value in situations where cattle may be cross contaminated with *Cff*.

Experiment 4 evaluated a PCR method for identification of *Cfv* in preputial washings that had been inoculated with small numbers of the organism,

whilst Experiment 5 used the same method to attempt to identify *Cfv* in preputial washings or scrapings that had been collected from experimentally-infected bulls. Positive results were obtained with as little as 880 *Cfv* cells/ml of preputial wash after samples had been concentrated by centrifugation and filtration (5 µm and 0.8µm pore size filters). The PCR also detected the presence of *Cfv* in two bulls, 48 h after infection.

It was concluded that the IgA ELISA test is unlikely to be suitable for use in New Zealand, due to the risk of cows being contaminated with *Cff* from the sheep with which they are co-managed. Microbiological identification, whether through PCR or culture and isolate, although difficult, remains the definitive diagnostic method. If the presence of the disease in New Zealand is to be confirmed or refuted, careful and methodical collection of samples from animals, whose history suggests the possibility of the condition, will be required.

Abbreviations

AFLP	Amplified Fragment Length Polymerase
BHI	Brain heart infusion
BVC	Bovine venereal campylobacteriosis
Cff	*Campylobacter fetus* subsp. *fetus*
CFU	Colonies forming units
Cfv	*Campylobacter fetus* subsp. *venerealis*
C-G	Cytocine-guanine
CVM	Cervicovaginal mucous
DNA	Deoxyribose Nucleic Acid
EVs	Elisa Values
FA	Fluorescent antibody
gly	glycine
H_2S	Sulphur hydroxide
ICC	Itraclass correlation coeficient
IG	Immunoglobulin
IgA, IgG, IgM	Immunoglobulins A, G and M respectively
$MgCl_2$	Magnesium chloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed Filed Gel Electrophoresis
SLPs	S. layer protein, Surfase layer proteins
µl	Microlitre
µm or um	micron
VMAT	Vaginal Mucus Agglutination test

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Chapter 1: Bovine venereal campylobacteriosis

1.1 Introduction

Bovine Venereal Campylobacteriosis (BVC) or Vibriosis is a venereal disease caused by *Campylobacter fetus* subsp. *venerealis (Cfv)*. Described in United Kingdom by McFadyean et al. in 1913 (referred by Veron et al. (1973) for the first time as a "vibrio like" organism it was first classified into the *Vibrio* family. It causes infertility, reduced calf crop and significant losses to the farmer once introduced in the herd (pregnancy rates can fall to 10% in susceptible herds) (Lawson and Mc Kinnon, 1952).

Adult animals brought in from outside the farm is the normal way by which disease is introduced to in a clean herd. After acquiring the disease females generate immunity that last 2 to 4 years but bulls can stay as asymptomatic carriers of *Campylobacter* for life.

The main problem with BVC is the difficulty with its diagnosis. *Cfv* is a very slow growing fastidious Gram-negative (Gram -ve) bacterium, microaerophilic, and susceptible to be overgrown by contaminants present in the samples. Isolation being very difficult, several indirect diagnostic methods have been developed. Until today none is sensitive and/or specific enough to be used on its own, and the only definitive result arises from microbiologic isolation of the organism from clinical samples.

In New Zealand the disease was positively diagnosed for the last time in 1993 (Loveridge and Gardner, 1993) although it was suspected again in 1996 (Black and Orr, 1996). In 2001 large animal practitioners from the Taihape area also suspected the presence of *Cfv* in five beef herds (Hughes, 2001), which showed chronically poor reproductive performance. However, isolation of *Cfv* was not achieved from bull samples, so a recently-developed IgA ELISA test (1994; Hum et al., 1991) was used to elucidate the situation. Interpretation of the results suggested endemic campylobacteriosis in those herds. Later studies, done on the behaviour of this IgA ELISA test under New Zealand conditions (McFadden et al., 2004a) showed disagreement between the test results and the reproductive performance of the herds tested. At this point, *Cfv* had not been isolated from 54 preputial washes from bulls from affected herds. The questions

arose then: Is the IgA ELISA reacting with some other bacterium? Are the isolation methods used in New Zealand for *Cfv* accurate enough to deliver trustworthy diagnoses?

It is the aim of this section to review the disease; its characteristics, taxonomy, pathogenesis, immunity, treatment and control. Special attention will be put to the diagnostic methods available.

1.2 The Genus Campylobacter

This genus was proposed by Sebalt and Veron in 1963 for some bacteria hitherto classified under the genus *Vibrio* (Taxonomy, 2001). Nowadays, this genus includes 16 species and 8 subspecies (On, 2001; Vargas et al., 2003) of *Campylobacter* (Table 1.1). It belongs to the *Campylobacteraceae* family along with *Arcobacters* (Vargas et al., 2003). They are Gram negative, spirally curved, S-shaped or "seagull" shaped (cocoid in older cultures) motile (one single polar flagellum) rods that measure 0.2-0.8 μm in diameter and 0.5-0.8 μm in length. They have mainly microaerophilic requirements (5% O_2, 10% CO_2, 85% N_2) although some are anaerobic (Taxonomy, 2001). Their metabolism is respiratory, oxidase positive and nitrates reducers. The guanine/cytosine content of their DNA is: 29-39 mole% (Lander and Gill, 1985).

They are ubiquitous in nature (soils, water, animals, plants, etc) and some are pathogenic for humans and/or animals, producing severe economic losses in animal production (intestinal and reproductive diseases) and serious health problem in humans (e.g. food born diseases). They are target of heavy interest from the research community and increasingly sophisticated tools are applied for their isolation and study (On, 2001).

Table 1.1: List of *Campylobacter* species, subspecies and varieties belonging to the genus *Campylobacter* (adapted from On (2001)

Species	Subspecies	Variety
C. mucosalis		
C. hyointestinalis	*hyointestinalis*	
	lawsonii	
C. fetus	*fetus*	
	venerealis	
C. lanienae		
C. concisus		
C. curvus		
C. showae		
C. rectus		
C. sputorum	*sputorum*	
	paranreolyticus	
C. hominis		
C. gracilis		
C. upsaliensis		
C. helveticus		
C. lari		
C. jejuni	*jejuni*	
	doylei	
C. coli		
C coli		*hyolei*

1.3 Bovine Venereal Campylobacteriosis (BVC)

Caused by *Cfv*, BVC produces infertility in cattle. Disease is mainly characterized by irregular oestrous cycle lengths due to early embryo losses, an increase in the number of services per conception, a decrease in the pregnancy rates, a reduced calf crop, an increase in the length of the mating period in non-seasonal mating systems and abortion in approximately 10% of females. Females develop immunity and typically are self-cured 6 months after infection. Convalescent immunity lasts for 2 to 4 years. Some cows may however carry the *Cfv* in their vagina during normal pregnancy and remain carriers after calving and until the following mating season.

The bull can act as an asymptomatic carrier and is responsible for perpetuating the disease in a herd. Insemination using infected semen constitutes also a potential means disease spread.

1.4 Taxonomy

Campylobacter fetus subsp. *venerealis* was isolated for the first time in 1913 by McFadyean (referred by Veron et al. (1973) from an aborted bovine foetus. The author described it as a spirillum. Smith in 1918 (referred by Veron et al. (1973) cultured some "vibrios" from aborted calves and called them *Vibrio fetus* based on their morphology. Two different *Vibrios* were found; one was called *Vibrio fetus* var. *venerealis* (only found in reproductive tracts of females and males and in aborted foetuses of cattle) and the other was called *Vibrio fetus* var. *intestinalis* (found in reproductive tracts of male and female cattle, in the intestines of bovine and ovine stock and in aborted foetuses of sheep and cattle).

Davis et al. (1962) considered that the genus *Vibrio* included many heterogeneous bacteria, so they broke it down into: *Vibrios, Aeromonas* and *Pseudomonas* and *Comonas* on the basis of morphology and biochemistry tests. Berg et al. (1971) revised the taxonomy of certain *Vibrio fetus* strains on the basis of biochemical studies (catalase test, glycine tolerance and H_2S production and antigen agglutination tests (for heat-labile and heat-stable antigens). They reclassified *Vibrio fetus* into 5 groups: A1 (gly -, H_2S -), A sub 1 (gly -, H_2S +), A2 (gly +, H_2S+), B (gly +, H_2S +) and C (gly variable, H_2S +) and suggested that *Vibrio fetus* var. *venerealis* (groups A1 and A sub1) and *Vibrio fetus* var.

intestinalis (groups A2, B and C) should not be two different species since they shared many antigens, survival characteristics and biochemistry. Veron and Chaterlain (1973) revised this issue again because they also considered that several very dissimilar bacteria were classified under the genus *Vibrio*. They proposed then a new genus, to be called *Campylobacter*, which would include Gram negative, curved, motile (single polar flagellum), microaerophilic, non sugar-fermenting bacteria with a guanine-cytocine (G-C) content of between 29-36%; they left those bacteria that fermented sugars and had a G-C content of between 40-53% under the genus *Vibrio*.

The classification criteria for this new genus *Campylobacter* were: production of catalase, production of H_2S and growth in a medium containing 1% glycine. They included in this new genus the following bacteria: *Campylobacter fetus* subsp. *fetus* (causing abortion in sheep and sporadic abortion in cattle), *Campylobacter fetus* subsp. *venerealis* (causing infertility and sporadic abortion in cattle), *Campylobacter fetus* subsp. *venerealis* biovar *intermedius*, *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter sputorum* subsp. *sputorum* and *Campylobacter sputorum* subsp. *bubulus*. The Approved list of Bacterial Names, in which this new genus *Campylobacter* was included, was published in 1980 (Skerman et al., 1980).

Between 1974 and 1988, 12 new species of *Campylobacter* were discovered, from a wide range of hosts and habitats (On, 2001) and the genus includes today the bacteria listed in Table 1.1.

Even though the problem of taxonomy has been addressed, there remains much misunderstanding with nomenclature of *C. fetus* subspecies. This problem arose when, Smibert (1974) published a list of *Campylobacter* names in the 8th edition of Bergey's Manual of Determinative Bacteriology. He named *Campylobacter fetus* subspecies differently to Veron and Chatlerain (1973), which made it more difficult for the research community to fully understand the already changing nomenclature of this genus.

Subsequent authors have tried to clarify this problem (Hemmisen, 1981) and others have addressed the validity of the names used by Veron and Chaterlain in 1973 (Yabuuchi, 1983). Until today several authors only name the bacteria to the species level (not subspecies) and others directly confuse them. Table 1.2 shows

a list of published papers where the species or subspecies of *Campylobacter* has been changed with respect to Veron and Chaterlain's classification of 1973.

Table 1.2: Literature in which the species or subspecies of *Campylobacter* have been changed with respect to Veron and Chaterlain (1973).

Publication	Species quoted	Veron and Chaterlain 1973
Schurig et al. (1978)	*Campylobacter fetus* subsp. *intestinalis*	*Campylobacter fetus* subsp. *fetus*
Roberst (1979)	*Campylobacter fetus* subsp. *fetus*	*Campylobacter fetus* subsp. *venerealis*
	Campylobacter fetus subsp. *intestinalis*	*Campylobacter fetus* subsp. *fetus*
Agumbah et al. (1979)	*Campylobacter fetus subsp. intestinalis*	*Campylobacter fetus* subsp. *fetus*
Border et al. (1980)	*Campylobacter fetus* subsp. *fetus*	*Campylobacter fetus* subsp. *venerealis*
Von Bispin et al. (1981)	*Campylobacter fetus* subsp. *fetus*	*Campylobacter fetus* subsp. *venerealis*
	Campylobacter fetus subsp. *intestinalis*	*Campylobacter fetus* subsp. *fetus*
Corbeil et al. (1981)	*Campylobacter fetus* subsp. *fetus*	*Campylobacter fetus* subsp. venerealis

Salama et al. (1992), concluded that *Campylobacter* species classified by Veron and Chaterlain where differentiated by genomic sizing. They found that *Cff*, *Cfv* and *Cfv* biovar *intermedius* had genomic sizes of 1.1 Mb, 1.3 Mb and 1.5 Mb respectively, supporting Veron and Chaterlain's (1973) classification.

In the case of the BVC, it is caused by *Cfv* and not by *Cff*, which actually affects sheep causing abortion storms and sporadic abortion in cattle.

1.5 Pathogenesis

The initial research on the diseases caused by *Cfv* dates back to the early 20th century. In reading this literature, attention has to be given to correctly determine which *Campylobacter* the paper refers to since when such research was being carried out, the causal organism was still called *Vibrio fetus*.

1.5.1 Transmission

Bovine venereal campylobacteriosis is a true venereal disease in cattle (Lander and Gill, 1985). Its only transmission path is through mating of an infected individual with a susceptible receptor. There is no horizontal or vertical transmission between the female and its offspring or between two female or male herd mates (Newsam and Peterson, 1964). There are also reports of transmission through fomites (insemination pipettes, dirty artificial vaginas) and through infected semen from AI centres. Intravenous and subcutaneous transmission routes have also been experimentally evaluated but are not efficient means of transmission (McEntee et al., 1954a).

1.5.2 Pathogenesis in cows

McEntee et al. (1954a) used artificial insemination with semen from infected bulls and semen spiked with *Cfv* to reproduce the disease in dairy heifers. The animals showed long oestrous cycles (range 27 to 52 days) at least once after infection and an average of 5 services per pregnancy in both infected groups, compared to one service per pregnancy in the control (uninfected) group. The mucus during oestrus was cloudy in infected animals and became clearer before pregnancy took place. Newsam (1960) experimentally produced the disease using infected bulls. Only 57% of the females became infected showing a conception rate to first service of 21%. Even if many animals did not become infected to first exposure they still were susceptible to become infected if re-exposure took place. Four year later, Newsam et al. (1964) deposited a recently isolated strain of *Cfv* inside the vagina of cows. The organism rapidly invaded the uterus and survived there for 13 weeks (Newsam and Peterson, 1964). No heifer became infected when the inoculum was deposited on the vulval lips (McEntee et al., 1959; Newsam and Peterson, 1964).

However, Clark (1971) reported that in naturally infected animals, the

organism reached the uterus by Day 12-14 post-infection. The uterus would gradually clear the infection (6 to 12 weeks) but the organism could persist for longer periods (6-18 months) inside the vagina (vaginal carrier status), making the cow infective to susceptible bulls. If *Cfv* was inoculated directly into the uterus, clearance of this organ would occur much faster than in naturally infected animals (Schurig et al., 1974). Infection would be finally cleared from the whole reproductive tract at different times in different animals but, the cow may become vaginal carrier again if mated to an infected bull, even when convalescent (Clark, 1971; Corbeil et al., 1981; Schurig et al., 1974; Schurig et al., 1978). In this case vaginal invasion will occur but not uterine invasion, so the animal will be able to carry a normal pregnancy (Corbeil et al., 1981).

Several explanations have been proposed to explain why the decrease in fertility happens in cows infected with *Cfv*. Some have said that the resulting mild inflammatory response of the uterine wall (mild endometritis defined by focal lymphocytic and plasmatic lesions of the endometrium) after invasion of *Cfv* makes it inhospitable to the embryo (Clark, 1971; Peterson and Newsam, 1964; Schurig et al., 1974). Taking into account that *Cfv* invasion of the uterus takes place at the same time that an embryo would reach the uterus this could be a good explanation. This endometritis may last 3 to 4 months, which would explain the loss of the pregnancy, the abnormally long cycles and the length of time needed for the cow to become pregnant (Clark, 1971; Schurig et al., 1974). Ware (1980) proposed that *Cfv* might decrease the dissolved oxygen tension resulting in a sub-optimal environment for the embryo to attach, mainly, since the embryo is highly dependant on the oxygen tension of the uterus before implantation and after. Lastly, Stalhem et al. (1975) studied the effect of *Cfv* on bovine uterine tube organ cultures and found that *Cfv* caused loss of cilia hence, ciliary movement. *Mycoplasma* spp, *Trichomona fetus,* on the other hand, did not. Taking into account that the embryo depends highly on ciliary movement to reach the uterus and implant, this could also contribute to the early embryo losses in newly infected cows or heifers.

Be that as it may, it is clear that after infection, the organism invades the uterus transforming it into an aggressive environment for the embryo. Infection is eventually cleared from the uterus but may survive for a long time in the vagina.

Once fully cleared, the cow is immune to re-infection for 2 to 4 years, at which time most animals would become susceptible again (Corbeil et al., 1981). The immune response of the cow and the evasion mechanism of *Cfv* to that immune response is covered in section 6.2.

At herd level, if infected bulls are used at a rate of one bull to 40 cows, around 60% of the females would become infected in a single cycle (Newsam, 1960). Any additional bulls become infected when mating with cows that return to oestrus. Such newly-infected bulls will run with other cows, infect them, and so on. By the end of the mating season, probably 90% of the herd of cows will be infected and the symptoms at herd level will be marked (Carrol and Hoerlein, 1972).

1.5.3 Pathogenesis in the bull

The process of infection in the male is completely different. A male can be infected when mating with an infective female or when a semen sample is taken using contaminated collection equipment (Clark, 1971). The prepuce of the bull has crypts in the region where the preputial mucosa joins the penile mucosa (fornix). Here *Cfv* finds the microareophilic conditions needed for growth and multiplication, along with low levels of contamination of other organisms that could compete with it. The deeper these crypts the better the conditions are for *Cfv*, and as they deepen with aging of the bulls, the older the bull, the more prone to maintain *Cfv* in its prepuce. There are no symptoms in these animals, no systemic invasion and no transport into the gonads or reproductive glands (Samuelson and Winter, 1966; Wagner et al., 1965).

Early research was done on the differences of the incidence of *Cfv* in old or young bulls, the length of their infective period and their capacity of becoming carrier bulls. The definitions of “old” and “young” in different publications varies; Wagner et al. (1965)considered young bulls those 6 years old or less, Philpott (1968) considered bulls less than 4 years old as young, Ladds et al. (1973) set the limit at 3 years old or less. Given any of these limits all the authors concluded that older bulls would show higher incidences of carrier stages than younger bulls. Table 1.3 shows those results.

Table 1.3: Results of differences in the incidence of *Cfv* of young and old bulls in early research.

Author	Age ranges	Incidence	Length of infectivity
Wagner et al. (1965)	Less than 6 years old	1.7%	
	More than 6 years old	46.7%	
Philpott (1968)	Less than 4 years old		Short
	More than 4 years old		Long
Carrol et al. (1972)	Less than 5 years old		Short
	More than 5 years old		Long
Ladds et al. (1973)	Young: 9 months to 3 years of age	1.4%	
	Mature: 3.5 to 7 years of age	9.7%	
	Old: more than 7 years of age	11.3%	

Young bulls (1-3 years old bulls) could clear *Cfv* infection in a couple of weeks, while older bulls (4 and older) will maintain the infection for very long periods (years).

A few reports have shown that the incidence of *Cfv* infection in bulls does not change with age. This question was specifically addressed by Dufty et al. (1975) and Bier et al. (1977) since the use of young bulls was being postulated as means of controlling the disease in herds. Their main conclusion was that the use of such animals to decrease spread of campylobacteriosis in a herd was of questionable value.

It is generally accepted nowadays that the older the bull the longer it retains infection in the deep crypts of the prepuce, although the use of young bulls to decrease the spread of the disease is not without risk.

The number of viable cells encountered inside the prepuce needs to be taken into account. This represents the inoculum that bulls can deposit in the females´ vaginas during mating. This number ranges from less than 1×10^2 cells to more than 2×10^5, a variability that is found not only between bulls but also in

the same bull at serial samplings. The number of organisms recovered decreases when the bull mates many females during the same day (Clark, 1971).

1.6 Immunity

One of the most puzzling characteristics of this disease is the carrier stage that occurs in males and females. Much research has been done to find out how the organism stays viable for such long periods in the same animal.

1.6.1 The Surface Layer Proteins (SLPs/S-layer proteins) of *Cfv*

Campylobacter fetus are Gram negative bacteria. Such type of bacteria are covered by single molecule arrays of SLPs, which are critical for their persistence in their natural environments in the host (Dworkin and Blaser, 1997). These SLPs are the proteins responsible for generating the immune response by the host, as they are the surface antigens presented to the host (sheep or cattle). Once this happens and the host secretes the specific immunoglobulin (IG) to attack the SLPs expressed by the organism, a shift in their expression results in a different antigen being presented. The secreted IG cannot recognise the new protein and a new immune response is generated. This cycle repeats itself continually in time, allowing the agent to survive in a hostile environment without being completely destroyed (Garcia et al., 1995).

References to this antigenic variation occur in the literature as early as 1975/1976 (Corbeil et al., 1975b; McCoy et al., 1976) when it was observed that antibodies from heifers that were vaginal carriers would agglutinate the isolate achieved on one day but would not agglutinate the isolate achieved one week later. *Campylobacter fetus* subsp. *venerealis* isolates from the same animal were shown to be antigenically different from those of the infective strain two weeks post-infection (Schurig et al., 1978). Changes in the heat-labile surface factors occurred in isolates obtained from the same animals over time. The DNA fingerprints of the isolates were highly conserved, but the expression of these heat-labile surface antigens changed during the carrier stage (Wesley and Bryner, 1989). The S-layer proteins of the subsequent isolates obtained after infecting heifers with *Cfv* were tested against monoclonal antibodies. Those antibodies showed different reactivity to the different isolates, showing that these proteins were responsible for the antigenic variation (Wang et al., 1993). This was again

shown in a study in Brazil (de Vargas et al., 2002) where naturally infected animals showed that persistence of *Cfv* in their reproductive tract was dependant on a shift in the expression of its S-layer proteins.

Another important characteristic of these SLPs is that laboratory adapted strains will lose this protein layer after repeated passages (Blaser and Pei, 1993; Sara and Sleytr, 2000). This is important to take into account since persistence of the agent in the host does depend on the presence of this layer.

1.6.2 Immunity in the female

Plastridge et al. (1964) found that when heifers are infected for the first time, *Cfv* was recoverable for at least 10 months post-infection. Wilkie et al. (1972) investigated the origin, class and specificity of the immunoglobulins produced after parenteral or local infection of heifers with *Cfv* was investigated. It was found that the local immunity was mediated by IgA immunoglobulins whereas parenteral infection evoked a systemic immune response with production of IgG and IgM, which then diffused into the uterine and vaginal lumen. Winter (1973) published a review on current knowledge about immunogenicity of *Cfv*, in which she commented on the hypothesis that uterine and vaginal immunity involved different mechanisms. The inoculum of *Cfv* is deposited into the cranial vagina by an infected bull. Invasion of the uterus occurs few days later (by Week 3 post-infection) and the organism will then be gradually cleared from the uterus into the vagina, where it can survive for long periods, before being totally eliminated (even if the cow got pregnant and carried a pregnancy to term). At uterine level, the immune response is seen as an accumulation of plasma cells and lymphocytes within the mucosa (Clark, 1971).

These immune responses take time, but once immunity is acquired, animals will eventually regain fertility. Although this immunity does protect the cows against uterine re-invasion, it does not protect them against vaginal colonisation with *Cfv*. Actually, 30 to 70% of cows could become re-infected (only at vaginal level), although their fertility would not be affected (Clark, 1971). Should they be mated to infected bulls and become pregnant, the organism could persist in the vagina for long times, even after the cow calved.

The antibodies secreted at vaginal level are of the IgA class only and they do not posses opsonic or bactericidal actions but they work immobilizing the

invading organisms not allowing them to bind to the epithelial surface. Since IgA does not promote phagocytosis or presentation of the antigen to antibody-forming immune cells (lymphocytes B) no memory immune response (booster response) will be elicited by a second invasion by *Cfv* (Tizard, 1995). Other immunoglobulins (IgG and IgM) have been demonstrated by immunofluorescent assays.

Intravaginally exposed animals responded mainly with IgA that is secreted into cervico-vaginal mucus (CVM). If exposure is parenteral, IgG will be present in the CVM. Both IGs have strong immobilisation characteristics. IgA immobilises the organism at a rate of one molecule of IgA per *Cfv* cell, although it does not promote opsonisation and phagocytosis. IgG on the other hand, also clumps the organism and is effective in promoting opsonisation and phagocytosis (Corbeil et al., 1974b). Corbeil et al. (1974a) reported the order of appearance of the immunoglobulins in CVM and in the uterus of locally and systematically immunized animals. In CVM of locally infected animals, IgM appeared first, then IgA, and finally some IgG. IgA was the only one still present 10 months after infection. In the uterus, the main immunoglobulin present was IgG. In parenterally immunised animals, IgG was the main immunoglobulin both in uterus and CVM. The presence of IgG in uterus accounts for the rapid clearance of *Cfv* from the uterus. The presence of IgA alone in the vagina is the cause of the persistence of *Cfv* in this organ. Further research showed that local infection had no influence on the immunoglobulin concentrations found in serum, thus secretion of IgA is entirely local (Van Aert et al., 1977).

Two reviews about immunity of *Cfv* infection (Corbeil and BonDurant, 2001; Corbeil et al., 1981) also concluded that the first immunoglobulin to appear in CVM is IgA; later on IgG1 and IgG2 could appear. *Campylobacter fetus* subsp. *venerealis* could evade the immune response of IgA by shifts in the expression of the surface layer proteins, allowing persistent infection of cows and development of the vaginal carrier stages.

However, since IgG immunoglobulins are more efficient in opsonisation and phagocytosis; their presence at high concentrations in the uterus, explains why this organ clears infection faster than the rest of the tract, and the reason that uterine invasion after first exposure is rare even when *Cfv* persists in the vagina.

1.6.3 Immunity in the male

Infection in the male occurs locally in the crypts of the mucosa of the prepuce (Samuelson and Winter, 1966). Immunoglobulins are concentrated in preputial fluids of infected bulls (Corbel, 1974). Even though the *Cfv* infection of the prepuce is superficial, there is a certain degree of immunity against the organism in young bulls. Hence, such animals clear the infection in a short period of time, exhibiting a subsequent period of refractoriness to re-infection (Winter, 1973).

Bier et al. (1977) experimentally infected bulls of different ages to study how the immunoglobulins were secreted into preputial fluids. They found that the most concentrated immunoglobulin was IgG. IgA was the second highest but IgM was present in very low concentrations. The IgG2 subgroup was higher in old bulls, while in young bulls the predominant IgG subclass varied. The IgG/IgA ratio for young infected bulls was: 2.78 ±2.10 and 2.87 ± 1.51 for controls. In old bulls, this ratio was 3.93 ± 3.12 and 2.43 ± 0.96 for infected and control animals respectively. Some degree of agglutination by both IgA and IgG occurred, but it was generally incomplete. They also concluded that superficial antigen variability also happens in males, and this alteration occurred in the heat labile surface antigen (S-layer). As in females, histological findings the observed included diffuse infiltration of mononuclear cells (mainly lymphocytes and plasma cells) within the lamina propia of the preputial mucosa. Clusters of plasma cells were found at the top of the dermal papillae. These were more numerous in older bulls. No other histological changes were found. Even with all these observations, no development of antibodies could be demonstrated in the preputial cavities of carrier bulls. The titres of immunoglobulins encountered in this experiment were found before and/or after infection, thus no pattern of immunoglobulin secretion could be concluded and the refractory period previously described by Winter (1973) could not be confirmed. The lymphoid and plasma cells accumulated in the preputial mucosa are responsible for the secretion of the IG found, although there was no significant relationship between infection status, mucosal infiltration of lymphocytes and plasma cells and concentrations of immunoglobulins in the preputial fluids (Corbeil et al., 1981; Flower et al., 1982).

1.7 Clinical signs

Most infected animals show little or no symptoms. Cows may have a mild purulent vaginal discharge that is normally only observed at the moment of sampling. The most spectacular signs are seen at herd level, which will depend on the level of previous exposure that the particular herd has.

1.7.1 Naïve herd

When an infected bull is brought into a naïve (never previously exposed) herd at a rate of 1 bull per 40 cows, 57% will become infected to a single service. Although these animals suffer a mild endometritis and salpingitis, this is normally not seen by the farmer due to the absence of external signs (Cipolla et al., 1994; Hartley, 1952). The one sign that the farmer does see is abnormal returns to oestrus of cows that have been mated. The returns to oestrus will be repeated and with inter-service intervals of irregular length (tending to be longer than normal). At the end of the mating season the pregnancy rates can be as low as 10 to 30% in a mating season that is restricted to 60 days (Carrol and Hoerlein, 1972; Newsam, 1960; Te Punga and Boyes, 1958). If mating is of no strict duration, over time, it can be seen that the mating and the calving seasons will start to spread from 99% calving in a 60 days calving season before infection to a 7 months calving period 3 years later (Carrol and Hoerlein, 1972).

After between 2 and 4 returns to oestrus, cows become pregnant and maintain pregnancy to term. Between 1 and 10% of cows abort their fetus between the 4th and the 7th month of pregnancy. Abortuses present non-specific lesions such as lymphoid proliferation and focal accumulation of mononuclear cells in most organs (Jeffrey and Hogg, 1988).

Calving cows will be naturally immunized for 2 to 4 years. They can become vaginal carriers and maintain infection from the one mating season into the following one, ensuring the presence of *Cfv* in the following year.

Uninfected bulls become infected from mating infected cows and may remain lifelong carriers depending on their age. They show no external symptoms and the semen is normal (Hartley, 1952).

1.7.2 Chronically infected herd

If the herd remains infected, there is likely to be a mixed population of

infected and uninfected animals. In this case since older cows will have developed a degree of immunity, herd pregnancy rates are higher than in naïve females mated to herd bulls (either maiden heifers or first calvers) but still not up to normal standards. Heifers will probably have a pregnancy rate of approximately 20% (Hartley, 1952). The overall pregnancy rate may reach 85% (Carrol and Hoerlein, 1972), which is low for the top 75% percentile beef herds in New Zealand (McFadden et al., 2004b).

Over time, the pregnancy rates stabilize at between 75% and 85%, depending on the proportion of naïve heifers, naturally immunized cows and cows that lose immunity over time (Carrol and Hoerlein, 1972). Calving will continue to spread if not kept between boundaries, abortions will be sporadic (Hartley, 1952) and bulls will all be infected.

1.7.3 Immunized herd

Vaccination of all animal (males, females and incoming heifers) results in elimination of the disease from carriers and prevention of infection in naïve heifers (albeit not at 100% efficacy). If males are not vaccinated, they will remain as carriers. The response to the vaccine will depend on the categories of stock that the farmer chooses to vaccinate. This will be further explained in section 9.2.2.

1.7.4 Summary

The pattern of infertility and/or abortion in an infected herd will depend on the proportion of susceptible heifers, carrier and clean bulls, and carrier and convalescent cows that the particular herd presents. The chronic low fertility will be a sure sign of the disease, and the degree of decrease in fertility will depend on the combination of all these categories of animals along with any other measure taken by the farmer to control the problem (Carrol and Hoerlein, 1972).

1.8 Diagnosis

The diagnosis of BVC has always been troublesome. Only a positive microbiologic isolation is conclusive proof that the disease is present, but a negative result does not mean it is absent. The fastidious cultural needs of *Cfv* have presented researchers and clinicians with many difficulties to try and

develop a reliable method to diagnose the disease.

The presence of BVC can be suspected from the herd's reproductive performance history (see Section 1.7). When this is the case, appropriate samples have to be taken from the animals, perhaps using specific media for transport and isolation. Management of the diagnosis of *Cfv* can be best considered in terms of the sampling, transport, isolation and diagnostic methods that are approved or recommended by OIE (OIE, 2004). Other methods that are not recognized by OIE are also available.

1.8.1 Collection of samples for microbiology

The sampling method differs in females, males and aborted foetuses, and also according to the diagnostic method that is to be used.

1.8.1.1 Sampling from cows

Vaginal or cervicovaginal mucus is used for most diagnostic tests and microbiologic isolation. Before any sampling is done, the vulva area has to be properly cleaned. Faeces and dry mucus must be cleared from the vulva, followed by a thorough washing, drying and aseptic cleaning of the area. The use of alcohol or Virkon is recommended after washing. A sterile speculum can also be used, which some say is essential to obtain a good sample (OIE, 2004). There are three ways of obtaining a sample of vaginal mucus:

1. Vaginal swabbing.
2. Vaginal mucus suction, using Bartlett's pipette or insemination pipettes.
3. Washing of the vaginal cavity.

Other methods have been described but their impracticality has made them obsolete.

1.8.1.2 Sampling from bulls

Three types of sample are useful in the male: preputial mucus or smegma, preputial washes and semen. Good restraining facilities have to be available and more than one person has to be present during sampling for safety purposes (OIE, 2004). The collection of preputial smegma can be done using a Bartlett's pipette, a scrapper or by washing an artificial vaginal after semen collection (Tedesco et al., 1977). All have to be collected under the most aseptic conditions

possible. This is very important due to the potential for contamination growth that exists within the preputial cavity.

Tedesco et al. (1977) have compared the scraper, the prepucial aspiration with Bartlett's pipette and the preputial wash. They reported that there was significantly less contamination and more successful isolations of *Cfv* using the scraper than the other two methods. Dufty et al. (1969) have found that the use of Bartlett's pipette is uncomfortable for bulls. In many preputial washes urine was present, although this did not greatly influence the percentage of positive results.

The washing/dilution medium has also been subject to a lot of research. Currently, the use of Phosphate-Bovine Serum (PBS) or saline is recommended (Hum, 1987; Lander, 1990a; OIE, 2004) but in the past many other media have been used (e.g. sodium thioglycolate (Modolo et al., 2000), peptone broth (Dufty and McEntee, 1969) and peptone water (Tedesco et al., 1977)

1.8.1.3 Sampling from aborted foetuses

Preferred samples from an abortus are: maternal placenta, foetal stomach contents, lungs and liver. All have to be collected under the most aseptic conditions possible, and sent to the laboratory cooled and in a sealed container (OIE, 2004). If possible, transport media should be inoculated at the time of collection with stomach contents, which is the most fruitful sample from foetuses (Campero et al., 2003; Varga et al., 1986)

1.8.2 Direct diagnosis: microbiologic isolation

The main problems with isolation of *Cfv* are its slow growth, susceptibility of overgrowth of contaminants (Lander, 1990b), and specificity of the atmospheric conditions for its growth. All samples come from very contaminated environments: vagina, prepuce and aborted foetuses (Tait et al., 1983). To ensure survival of the *Cfv* and inhibition of other contaminants samples from foetuses should be sent to the laboratory for isolation as soon as possible, and samples from females, males and foetuses are normally inoculated into transport media at the time of collection.

1.8.2.1 Transport enrichment media

The use of media that are enriched and selective is designed to help

survival of *Cfv* during transport into the laboratory for further isolation and to inhibit growth of contaminants. Much research has been done towards finding the best or most suitable transport media.

Since *Cfv* is a gram-negative bacterium, antibiotics against growth of Gram-positive bacterium along with anti-fungal drugs are included in most of these media. Some authors have developed solid transport enrichment media (Clark and Dufty, 1978; Clark et al., 1969; Clark et al., 1974; Dufty, 1967; Dufty and McEntee, 1969; Garcia et al., 1983; Garcia et al., 1984; Plastridge et al., 1964; Shepler et al., 1963; Winter and Caveney, 1978) and others have developed liquid transport enrichment media (Bolton et al., 1983; Lander, 1990a, b). The most widespread alternatives are:

1. Clark's medium: solid medium containing 5-fluoro-uracil, Polymixin B sulphate, brilliant green (anti Gram positive bacteria), nalidixic acid and cycloheximide (anti-fungal) on a bovine serum base. This medium serves well but its preparation is difficult and time consuming. It is used mainly in Australia and Uruguay for preputial scrapings (Clark and Dufty, 1978; Repiso et al., 2002).
2. Lander's Medium: liquid broth containing bacteriological charcoal (for selection of *Campylobacter* over other contaminants), "campylobacter growth supplement" (Oxoid), haemolysed horse blood, vancomycin, polymixin B sulphate cycloheximide, trimethoprim and 5-fluorouracil, on a Mueller-Hinton broth base. It is mainly used for preputial washes and vaginal washes in New Zealand and the UK (Lander, 1990b; McFadden et al., 2004a)
3. Modified SBL medium: modified by Clark (1985), this is a semisolid medium containing agar, sodium thioglycolate, sodium glycerophosphate, aqueous calcium chloride, cysteine hydrochlorhydrate, aqueous methylene blue, polimyxin, novobiocin, bacitracin and cycloheximide on a SBL base. This medium is used in France.

Many authors have compared transport media but results are very different and not repetitive. Little agreement exists as to which one is the best.

1.8.2.2 Isolation media for Campylobacter fetus subsp. venerealis

Again there has been a lot of work in this area over the years to develop a medium for *Cfv* isolation. These media are normally of use after the use of transport enrichment media.

Many media are available for isolation. Some examples of solid media to be used include (OIE, 2004):

1. Skirrow medium: solid medium containing polymixin B sulphate, trimethoprim and vancomycin cycloheximide, on a defibrinated horse blood and sheep blood agar base.
2. Clark's selective medium: solid medium containing peptone, sodium chloride beef extract, defibrinated sheep blood, bacitracin, polymixin B sulphate, novobiocin and cycloheximide on an agar base.

Non-selective media can also be used after the use of transport enrichment. Any blood-based medium like Columbia agar, or blood agar enriched with sheep or horse blood could be use.

1.8.2.3 Atmospheric conditions

Campylobacter fetus subsp. *venerealis* is a microaerophylic bacterium. It needs a gas mixture of no more than 5 to 10% oxygen, 5 to 10% carbon dioxide and 5 to 9 % hydrogen for optimal growth (Kiggins and Plastridge, 1956).

Media have to be incubated at 37°C ± 1°C under these atmospheric conditions. It takes between 4 to 7 days for *Cfv* colonies to be visible. A negative result after 4 days cannot be declared as negative. The use of a control plates is advisable at each stage of culture to ensure that conditions are appropriate for *Cfv* growth. *Campylobacter fetus* subsp. *venerealis* colonies are 1-3 mm wide, slightly pink, round, convex, smooth shiny and with regular edges (OIE, 2004).

1.8.2.4 Sample handling

It is important that the sample and/or inoculated media arrive in the laboratory as soon as possible, as this will improve chances of isolation of the organism.

Samples from females

A lot of work has been done on the handling of samples to ensure that *Cfv*

will be recovered. For example in females, vaginal mucus may be too viscous and liquefaction may be necessary. This is achieved by addition of an equal volume of a cysteine solution to the sample and waiting for 15 to 20 min before inoculation of the diluted sample into any other medium (OIE, 2004). It can also be filtered before using for isolation or other indirect diagnostic techniques (Shires and Kramer, 1974).

A lot of work has been done to find the best combination of sampling methods with transport media with little agreement between reports. The only common agreement in the literature is that the use of the such media is of crucial importance to ensure isolation of the organism (Andrews and Frank, 1974; Clark et al., 1969; Dufty and McEntee, 1969; Hum et al., 1994; Newsam, 1960).

Samples from males

Much research has been done on how to deal with samples from males. One problem is the large volumes of those samples (at least 10 ml). Some authors concentrated the samples by centrifugation, inoculating transport media with pellets (Bier et al., 1977; Clark et al., 1969; Clark et al., 1974; Lander, 1990a; MacLaren and Agumbah, 1988), some let the sample stand for some time before inoculating transport medium with the supernatant (Repiso et al., 2001), others filtered an aliquot of the sample through filters with a pore size of between 0.65 and 0.60 μm (Lander, 1990b; Plumer et al., 1962) and many combined two or the three methods (Clark and Dufty, 1978; Dufty, 1967; Modolo et al., 2000; von Bispin et al., 1981; Winter et al., 1967). All was done with the aim of capturing any *Cfv* cells present in the sample. Isolation of *Cfv* can also be done from semen (Dunn et al., 1965; Winter et al., 1965).

Many combinations of transport and enrichment media, sample treatment and isolation media were evaluated (see references above). None have delivered repeatable results. The main conclusion that can be drawn from all these papers is that there is not one exclusive way to ensure isolation of *Cfv*.

1.8.3 Identification of *Cfv*

Identification of *Campylobacter* to the subspecies level is very difficult. One of the problems is that many of them can be present at the same time in the samples. Some of the most common ones are: *Cfv*, *Cff* and *C. jejuni, C. bubulus*

(McFadden et al., 2004a; Samuelson and Winter, 1966). *Campylobacter fetus* subsp. *venerealis* shares a close genomic relationship with *Cff* (Harvey and Greenwood, 1983; Roop et al., 1984) and, consequently, these two subspecies have many characteristics in common both at genomic and phenotypic level.

Be that as it may, the first step into identifying the growing colonies is to perform a Gram stain. *Campylobacter* appears as a small (0.5-0.8 µm long and 0.3-0.4 µm wide) thin and curved Gram negative (reddish) bacillus, which can be seen as a short form (comma shaped), medium length (S-shaped) or long form (helical or spiral shape) invariably separated from each other (On, 2001). In older cultures they may present a round form.

1.8.3.1 Biochemical characterisation

Several biochemical or phenotypic tests were designed to identify *Campylobacter* spp. according to the response to different culture conditions such as salt concentration, temperatures, reagents in the media, etc. Different publications do not completely agree on the results of these biochemical tests for the whole *Campylobacter* genus. Tests are not standardized, the results vary with the conditions and they are very susceptible to human misreading (On, 1996). Tests currently recommended by OIE (2004) are shown in Table 1.4.

However results of these tests and others available vary from source to source (Table 1.5, (Bergey and Holt, 1993; MacFaddin, 2000; Nachamkin, 1995; Quinn et al., 1994; Quinn et al., 2002)

When looking at Tables 1.4 and 1.5 it can be seen that biochemical characterisation between *Cff* and *Cfv* lacks repeatability. There are only a few tests to differentiate them, which do not always produce the same results. Atypical characteristics may lead to wrong classification of the isolate. For example strains of *Cfv* that are tolerant to Glycine have been reported, which have been classified as *Campylobacter fetus venerealis* biovar *intermedius* (Vargas et al., 2003).

Table 1. 4: Differential characteristics of the genus *Campylobacter* (OIE, 2004)

	Oxidase	Catalase	H_2S[(a)]	25°C	42°C	Glycine 1%	NaCl 3.5%	Cephalothin	Nalidixic acid
C. fetus* subsp. *venerealis	+	+	–	+	V[(b)]	–	–	S	V
C. fetus* subsp. *fetus	+	+	–	+	V[(b)]	+	–	S	R
C. jejuni	+	+	–	–	+	+	–	R	V
C. sputorum* biovar *sputorum	+	–	+	–	+	+	V	S	V
C. sputorum* biovar *faecalis	+	+	+	–	+	+	V	S	V
C. sputorum* biovar *paraureolyticus	+	–	+	–	+	+	V	S	V

(a) = On triple sugar iron agar medium; (b) = Although *C. fetus* does not belong to the thermophilic *Campylobacter* spp., growth of this species at 42°C has been reported (12, 41); (+) = positive reaction or growth and (–) = negative reaction or absence of growth of the strain on an appropriate medium under specified conditions; V = variable results; S = sensitive; R = resistant.

1.8.3.2 Other identification tests

The difficulties observed in identifying *Campylobacter* species through biochemical characteristics, has prompted the advent of several other techniques. Many of them involve molecular biology. Such tests are to be applied to pure cultures isolated from clinical samples.

On (1996) reviewed the identification methods for the genus *Campylobacter*. Many methods are available to assist with the evaluation of biochemical characterisation (Computer schemes, probability matrices, computer-assisted evaluation of probabilistic matrices), together with serological tests, lecitin agglutination tests, cellular fatty acids profiling, pyrolisis mass spectrophotometry, protein profiling, nucleic acid probes, polymerase chain reaction (PCR) along with other genomic methods. The main conclusion from the review, however, is that even if progress has been made towards the development of methods to accurately identify *Campylobacter* species and subspecies, there is still a need for a cost-effective method for accurate and rapid identification of *Campylobacter* species (On, 1996).

Table 1.5: Differences among published sources on the diagnostic biochemistry of *Cfv* and *Cff*. Grey areas show distinctive test in each source.

Book	Quinn et al. (1994)		Quinn et al. (2002)		Bergey (1993)		Nachamkin (1995)		MacFaddin (2000)		Cowan et al. (1999)		Lander et al. (1985)		Prescott (1990)		Roberts (1971; 1986)	
Test	Cff	Cfv	Cff	Cfv	Cff	Cfv	Cff	Cfv	Cff	Cfv	Cff	Cfv	Cff	Cfv	Cff	Cfv	Cff	Cfv
Growth at 25 °C	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+
Growth at 42 °C	-	-	-	-	+	-	-	-	-	-			-	-	V	-	-	-
Catalase reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-			+	+	-	-					+	+	+	-	+	+
H_2S production (lead acetate)	+	-	+	-	n/a	n/a	+	-	+	-	+	-	n/a	n/a	-	-	-	-
Nalidixic Acid sensitivity.	R	R	V	R	R	R	R	R	R	R	n/a	n/a	V	R	R	R	R	R
Cephalothin sensitivity	S	S	S	S	S	S	S	S	S	n/a	n/a	n/a	S	S	S	S	S	S
Growth on 1% glycine	n/a	n/a	+	-	+	-	+	-	+	-	+	-	+	-	n/a	n/a	+	-
Growth on 3.5% NaCl	n/a	n/a	-	-	-	-	-	-	-	-	-	-	-	-	n/a	n/a	n/a	n/a
Hippurate hydrolysis	n/a	n/a	n/a	n/a	-	-	-	-	-	-	n/a	n/a	-	-	-	-	-	-
Oxydase reaction	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	+	+	n/a	n/a

Polymerase Chain Reaction (PCR)

Based on previous work from Wesley et al. (1991), Blom et al. (1995) published a PCR-DNA probe technique used for identification of *C. fetus* species to the species level. It targets a fragment of the 16S rDNA gene, which encodes for the synthesis of ribosomal RNA (16S ribosomal DNA [rDNA]). This gene is ubiquitous, its function is conserved, it is easy to sequence, and large databases are available for sequence alignment and identification (Drancourt et al., 2000). It has highly conserved regions along with hypervariable regions. By targeting the hypervariable regions specific species of bacteria can be identified. This technique in particular, allowed a fast identification of food borne *C. coli* that infect humans. Oyarzabal et al. (1997) selected short sequences of the hypervariable regions of the 16S-rDNA gene to produce specific primers for the *C. fetus* species. This method identified *C. fetus* from other *Campylobacter* species.

Based on previous work from Pannachio et al. (1993) Hum et al. (1997) published another PCR method in Australia. This technique used two pairs of oligonucleotide primers (MG3F, MG4R and VenSf, VenSR). The first pair of primers targets the *C. fetus* species delivering a band of 960 base pairs (bp) and the second pair of primers targets *Cfv* specifically delivering a band of 142 bp. A total of 99 strains were analysed and an agreement in the final identification of the strains between the PCR and biochemical characterisation of 80.8% was reported. Those strains in which disagreement occurred were re-identified by using the PCR, and the authors attributed the discrepancies to a lack of proper standardisation of the biochemical tests. This was the first PCR technique that was apparently able to differentiate between *Cfv* and *Cff* and represented a huge step forward, since PCR is a quick and very accurate technique for identification of the presence of species-specific DNA.

Many authors have evaluated this PCR method subsequently. Newell et al. (2000) and Vargas et al. (2003) agreed with the band lengths reported by Hum et al. (1997), although Wagenaar et al. (2001) and Muller et al. (2003) reported a 750 bp long subspecies band. However, there is a general agreement among all that this method seems to be of use for identification of *C. fetus* to the subspecies

level.

Pulsed Field Gel Electrophoresis (PFGE)

This technique has also been evaluated for differentiation of *C. fetus* subspecies. It basically works by digesting the DNA with a specific restriction enzyme and then subjecting the product to electrical pulses from 3 different directions to separate DNA fragments. Fujita et al. (1995) used this technique with the restriction enzymes called Sma I and Sal 1 to test 21 strains *C. fetus* (20 *Cff* and 1 *Cfv*). The pattern of DNA fragments obtained for *Cfv* was completely different from the patterns obtained for *Cff*. They concluded that this method was reliable when working with fresh cultures, and that it was a good and fast way of distinguishing *Cfv* from *Cff*.

Twenty-eight of 31 *C. fetus* isolates analysed by PFGE followed by PCR, biochemical characterisations and 16S rDNA sequencing achieved the same final diagnosis (On and Harrington, 2001). For the three strains where discrepancies were observed, full and reduced probabilistic identification matrixes were used to clarify the diagnosis. In one of the three cases, the diagnosis obtained by PFGE and PCR was confirmed by the use of these matrixes. It was concluded that both PCR anf PFGE were good methods for differentiation between *Cfv* and *Cff*, but that diagnosing *C. fetus* subspecies should involve more than one diagnostic method (On and Harrington, 2001).

Vargas et al. (2003) evaluated used biochemical characterisation, PFGE and PCR to differentiate between *C. fetus* subspecies. They found an agreement of 90.9% between the three methods and the discrepancies were explained with reference to a lack of standardization and objectivity of the biochemical characterization. Interestingly, Fujita et al. (1995) and Vargas et al. (2003) found similar DNA profiles obtained by PFGE between strains of *Cfv* coming from the same herd or geographic region and suggested the existence of different clones in different geographic locations.

Amplified Fragment Length Polymorphism (AFLP)

This technique works by digestion of chromosomal DNA with specific restriction enzymes and ligation of the products to specific adapters (i.e. known

DNA sequences). The product is then submitted to preselective PCR with primers that will recognize the adapters, and the amplified product is finally submitted to electrophoresis and sequenced. Newell et al. (2000), Duim et al. (2001) and Wagenaar et al. (2001) have concluded that it is a good method for differentiation amongst *C. fetus* subspecies.

1.8.4 Serology

Due to the fact that *Cfv* is very difficult to isolate from field samples, many other indirect approaches to diagnosis have been developed since early stages of the discovery of this disease.

1.8.4.1 Immunofluorescence

Mellick et al (1965) attempted the use of fluorescent antibody (FA) techniques on preputial washes for the first time. Results were encouraging since complete agreement between FA results and microbiological isolation was found. However, the nuisance was that cross-reactivity occurred with *Cff* (*Vibrio fetus*). Some time later, Winter et al. (1967) evaluated the use of FA and isolation on the field. A total of 269 samples from 145 bulls (preputial smegma using Barttlet's technique) were processed using both techniques. Agreement was found on 122 negative samples and 44 positive samples. Twelve bulls were positive only to FA and 8 only to isolation. They reported that even though both techniques were useful, serial samples should be taken, none of both diagnostic methods should be used by itself and a single negative result from either of them should not be taken as definitive. In the same year, (Dufty, 1967) found that 94 of the 100 samples (preputial smegma collected using Barttlet's technique) evaluated by FA delivered positive results, although there was again cross-reaction with *Cff*. This sensitivity was by far the highest of the four methods used in this trial (isolation with selective media, isolation after filtration and isolation after deep freezing with glycerol) but even so, it was still advised that there should be at least four serial samplings before diagnosing an animal as negative. Lein et al. (1968) and Ruckerbauer et al. (1974) supported these results.

In females, the FA test is not widely used because it does not differentiate between *C. fetus* subspecies (found in the vagina of cows as a result of faecal

contamination). The test was proposed to be used in females only for screening purposes at herd level (Carrol and Hoerlein, 1972). Shires et al. (1974) criticized this strategy considering that isolation of *Cff* from cattle was rare in all but post-abortion samples. In their case, filtration of the cervico-vaginal mucous improved the results from FA by eliminating the interference of blood or purulent material in the sample and non-specific background fluorescence. Andrews et al. (1974) pointed out that females become clear of the organism 6 months after infection, thus *Cfv* might no longer be present when the sample is collected.

In 2002 the FA technique was formally assessed for sensitivity, specificity, detection limit and observer effects (Ferreira Figueredo et al., 2002). Subspecies of *C. fetus* could not be differentiated, but the sensitivity and specificity for differentiating *C. fetus* from other *Campylobacter* species were 92.59% and 88.89% respectively, and the detection limits were $1x10^4$ CFU/ml for PBS and non-centrifuged preputial samples and $1x10^2$ CFU/ml for centrifuged material. Observers did not have any effect on the final results.

The use of FA has largely been discredited now, although it is still occasionally used as a confirmation of microbiological tests (OIE, 2004).

1.8.4.2 Vaginal Mucus Agglutination Test (VMAT)

As early as 1952, VMAT was being investigated as a diagnostic field test with generally encouraging results (Lawson and Mc Kinnon, 1952). The test was adapted from an existing VMAT for trichomoniasis, which depends on the presence of antibodies in vaginal mucous. Using VMAT, it was found that IgA against *Cfv* appears in CVM between 3 to 5 weeks after infection and lasts at high levels up to 40 weeks (Corbeil et al., 1975b; Corbeil et al., 1975c; Wilkie et al., 1972). Carrier cows may not develop agglutinating titres, although some animals become positive and remain so for several years. Many authors agree that this test can be very useful at herd level (50% of infected animals will be detected by VMAT), although results at individual animal level can be very misleading (Hartley, 1952; Lawson and Mc Kinnon, 1952; MacLaren and Agumbah, 1988; Newsam, 1960). The test can also produce false positives if blood is present in the sample, or due to the presence of agglutinins against *Cff* (Clark, 1971).

Newsam et al. (1967) standardized a method for preparation of the antigen because this was found to be essential for comparison of results that were being reported from all over the world. Hewson et al. (1985) also found that the results of the VMAT were highly dependant on the quality of the antigen produced and that for no apparent reason all antigens autoagglutinated after some time.

Moreover, Clark et al. (1970) observed an increase in false positive results for VMAT after the repetitive sampling of cows. The probable reason for this is the occurrence of mild trauma caused during repetitive sampling, which can cause transfer of agglutinins from plasma.

Even though VMAT has limitations, it was and is, still widely used at herd level for the diagnosis of the presence of the organism, delivering satisfactory results (Healey et al., 1960; Leaver and Hart, 1960; Murnane et al., 1959; Newsam, 1960; Newsam and Peterson, 1964; OIE, 2004).

1.8.4.3 Agglutination tests developed for sera

Hartley (1952) developed an agglutination tests for sera, but it is no longer used because *Cfv* elicits mainly a local immune response with secretion of IgA into the vaginal secretions, and only a short term IgG response. There is no long-term specific IgG response in the serum of naturally infected animals.

1.8.4.4 ELISA tests for vaginal mucous samples

Due to the problems observed with the use of the VMAT, an ELISA test for the diagnosis of infection with *Cfv* based upon identification of the presence of IgG in samples of vaginal mucous was developed (Hewson et al., 1985). A strain of *Cfv* was used for the production of a sonicated antigen which was expected to increase the amount of binding sites, avoid the problem of autoagglutination seen with the VMAT and overcome the problem of the quality of standard solution of antigens seen with VMAT. The conclusion was that the ELISA test offered a number of advantages from the VMAT: i) it detected positive animals earlier than the VMAT (confirmed by culture), ii) the ELISA could be used during oestrus and iii) only a small amount of vaginal mucous is needed. It was observed that running this test on the purulent samples of the early

infection could cause false positive results. Hence, since this test also produced occasional false negative results, it might be of better use at herd level. The authors remarked that and that microbiological isolation of the organism remained the only method to deliver unequivocal positive results.

Based on these results, an indirect ELISA test for the identification of immunoglobulin A (IgA ELISA) in vaginal mucous samples was developed in Australia (Hum et al., 1991). It was postulated that it would be a better method of diagnosing the disease since IgA is locally secreted into the vagina after *Cfv* infection. The IgA ELISA was evaluated in the field and a specificity of 98.5% for the identification of *Cfv* infected herds was reported (Hum et al., 1994). Although several observations were discussed about this test by the authors (undulating pattern of titres appearance, the occurrence of a small proportion of false positive results, a possible cross-reactivity with *Cff*) and no reports on sensitivity accompanied the results, its use was advised at herd level. This IgA ELISA test is now used at national level in Australia to evaluate infection with *Cfv* status of herds and is recognized by OIE as diagnostic test for BVC. This test only identifies IgA and thus possible vaccination immunity will not interfere with the results.

In other words, there are a number of weaknesses of the IgA ELISA test that could affect its diagnostic value under field conditions. For example in New Zealand, most of the beef herds are reared alongside sheep. Since sheep may carry *Cff*, contamination of farm soil, grasses and waters with *Cff* is likely where both species graze together. This could mean that vaginal invasion of *Cff* in cows in New Zealand (via faecal contamination) might be more frequent than in Australia here the species are farmed separately. Taking this into account, together with the previous acceptance from Hum et al., (1994; 1991) that an earlier sensitisation with *Cff* could be the reason for the false positive results they observed, and results obtained from the use of this IgA ELISA test on New Zealand beef herds which showed a high proportion of false positive results (McFadden et al., 2004a), it may be considered that the test under New Zealand conditions could not achieve the 98.5% specificity achieved under Australian conditions.

These and other aspects that might influence the results obtained by this

test: i) the advice given for sampling only animals more prone to be affected and ii) the use of an adapted laboratory strain of *Cfv* to coat to the ELISA plates will be discussed in Chapter 3.

1.8.4.5 ELISA test for serum

An ELISA test for the identification of IgG immunoglobulins against *Cfv* in serum after vaccination has also been developed. This test provides a good measure of the immune response after vaccination and a means of evaluating the need for booster vaccinations. It is not suitable to be used as means of diagnosis in the field (Repiso et al., 2002).

1.8.5 Other diagnostic methods used

The use of PCR followed by restriction endonuclease analysis on samples of bull semen experimentally infected in the laboratory with *Cfv* was evaluated. As few as 3 *Cfv* cells per sample could be detected and no cross-reactions of the primers were seen with any other *Campylobacter* species or any of the normal preputial contaminants present in semen (Eaglesome et al., 1995).

1.8.6 Summary

Despite the development of many diagnostic techniques, none is sensitive and/or specific enough to be used on its own. Thus, at least two methods should be used to diagnose this disease. One should be microbiological isolation, since it is the only means of obtaining an unequivocal positive diagnosis. To improve isolation rates, inoculation onto transport media as soon as the sample is taken has been advised. Abortions are the most fruitful samples, since *Cfv* is present at high concentrations in foetal stomach content of foetuses aborted due to this infection. Results of any of the serological tests should be used at herd level due to their lack of sensitivity and/or specificity. Moreover, a negative result from any of the diagnostic methods does not guarantee that a herd is disease-free.

1.9 Treatment, control and prevention

The susceptibility of *Cfv* to a wide range of antibiotics and antiseptics means that it is potentially possible to treat affected animals.

1.9.1 Treatment

Many different treatments have been studied in bulls all with high rates of success. The early recipes include:

- Daily preputial infusions of 0.5 to 1 lt of a 3% solution of hydrogen peroxidase followed by 300 ml of an 0.5-0.7% aqueous solution of furaltadone hydrochloride (Winter et al., 1967);
- Preputial washes with a combination of penicillin and streptomycin (Clark, 1971);
- Preputial washes with a combination of streptomycin, neomycin, and erythromycin.
- Other combinations of tetracycline, chloramphenicol have also been suggested (von Bispin et al., 1981).

All are generally successful, whether given by the systemic or local route (preputial infusion). After treatment, four consecutive negative samplings should follow any of the treatments to diagnose the animals as disease-free. Moreover, successfully treated bulls are susceptible to re-infection as soon as re-exposure happens (Clark, 1971; Dufty, 1967).

Another approach available for infected bulls would be to cull them and substitute them by young, clean, virgin bulls. This approach is fine for bulls of no special genetic merit, but treatment should probably be advised in the case of high merit bulls (Hartley, 1952; von Bispin et al., 1981).

Infection of semen can also be eliminated with antibiotics such as streptomycin, penicillin and sulphanilamide (Hartley, 1952; McEntee et al., 1954b). Polymixin B has also been evaluated in combination with streptomycin and penicillin (Howard et al., 1982; Shin et al., 1985). Likewise, a combination of gentamycin, tylosin and lincospectin was good for treating semen against *Cfv* and also against *Mycoplasma* and *Ureoplasma* species (Shin et al., 1988). Eaglesome et al. (1994) evaluated the use of photosensitive additives to semen. Three photosensitive agents were added to the semen (hematoporphiryne, hematoporphyrin derivate and thiopyronine), which was treated it afterward by irradiation. This treatment was good against *Mycoplasma canadense, Ureoplasma diversum, Mycoplasma bovigenitalium* and *Leptospira pomona*, but was ineffective against *Leptospira hardjo, Mycoplasma bovis* and *Cfv*.

Vaccination can also be use as a means of treating infected bulls (Section 1.9.2.2).

The practicality of treating cows has been a long-standing matter of debate. Hartley (1952) reported successful cow treatment by intra-uterine injection of solutions of streptomycin or combinations of penicillin and propanadine. Te Punga et al. (1958) successfully prevented the disease by uterine irrigation of a solution of streptomycin four days after inseminating heifers with infected semen in New Zealand. However, when Healey et al. (1960) evaluated a range of antibiotic administration regimes in an attempt to cure, or prevent, BVC after mating by infected bulls, none were sufficiently reliable or practical for field use. After these early publications, the discovery that vaccination was an effective method of treatment for females meant that research on antibiotic use in females was abandoned.

1.9.2 Control

1.9.2.1 Reproductive management rules and artificial insemination

Effective control of this disease was achieved with the introduction of Artificial Insemination (AI). Once natural mating could be avoided and semen could be treated, spread of *Cfv* infection could be prevented. Before this, some reproductive management rules were introduced that would promote the self-limiting aspect of this disease in a herd. These included:

- The separation of a herd into infected and non-infected animals was proposed (Hartley, 1952).
- Clean bulls should run with clean cows and heifers and older possible infected males should be joined to convalescent cows (Dewes, 1961; Hartley, 1952; Leaver and Hart, 1960; Young, 1965).
- If possible, all newly bought heifers should only be mated to newly bought virgin bulls, leaving the rest of the cows to run with the older bulls (Hartley, 1952).
- The lending and borrowing of bulls should be avoided since the health status of these animals could not be ensured.
- If any older animals were to be introduced into a closed herd, 90 days of sexual rest should be given to cows. Bulls should be mated with 4 or 5 virgin

heifers to check their infective status (Ball et al., 1987; Hartley, 1952).

- Good fencing should be put in place and culling of known-infected bulls carried on (Dewes, 1961).

If all these rules were put in place in a farm, the disease should self eliminate from the herd in two or three years.

1.9.2.2 Vaccination

Another approach to control is vaccination. This tool started to be developed in the 1960s. Early reports showed that subcutaneous injection of a suspension of *Cfv* cells produced rapid recovery of fertility in heifers (Te Punga, 1962). Work done in Australia at the same time focused on intravaginal inoculation of live and dead *Cfv* cells suspensions in the hope of generating good immune responses. Immunity was only achieved by inoculation of live bacteria (Newsam, 1965b, c). Some time later, Clark et al. (1968a; 1968b) reported that after subcutaneous injection of phenol-killed whole *Cfv* cell suspensions, had curative effects and prevented re-infection of cured females and males. Thereafter, many researchers have focused on different methods of vaccine preparation (Clark et al., 1972a), age of vaccination (Clark et al., 1972b), dosages and the effects of single or double vaccination (Clark, 1975), the best timing for vaccination (Berg and Firehammer, 1978) and the use of annual or biannual boosters (Clark and Dufty, 1982; Hoerlein and Carrol, 1970). The use of *Cff* cells for the formulation of vaccines against *Cfv* was also evaluated since they share many antigens (Bouters et al., 1973; Clark et al., 1975). The results were encouraging, as no significant differences were found between vaccines made of different *Cfv* strains (Eaglesome et al., 1986), vaccines made from *Cfv* biovar *intermedius* (Johns et al., 1977), or even dual vaccines prepared from strains of *Cfv* and *Cfv* biovar *intermedius* (Clark et al., 1977). Cell-free vaccines have also been evaluated for their effectiveness to prevent or cure BVC with encouraging results (Schurig et al., 1978). Finally, passive immunisation of animals was evaluated. This also appeared to have a curative effect (Berg et al., 1979).

The pattern of immunoglobulin secretion promoted by these vaccines was also studied. In females, IgG and IgA increased in both serum and vaginal mucous after vaccination, although no consistent relationship between the two

was observed (Schurig et al., 1975). In males diffusion of IgG and IgM into the prepuce along with local secretion of IgA occurs after vaccination (Van Aert et al., 1976). Border et al. (1980) reported that it was the capsular antigens (K) of *Cfv* that elicited immunity. Further investigation showed that antibodies against K antigens were secreted 4 weeks after vaccination and antibodies against somatic antigen (O) were secreted within 6 week of vaccination (Vasquez et al., 1983).

Hjerpe (1990) published a comprehensive review on the subject of vaccination against *Cfv*. According to this material, vaccines develop opsonins of the IgG class against superficial heat-labile K antigens of *Cfv* cells. The IgG diffuses into the vagina and uterus. The presence of IgG in the vagina does not last for a long period and bacterial invasion may still occur although fertility is not lost (vaginal carrier stage). In males, immunization results in the presence of IgG, IgM and IgA in the preputial cavity. Hence, vaccination will prevent infection of bulls and transmission of the disease to susceptible cows. Vaccines are based on oil or aluminium hydroxide adjuvants. The oil adjuvant promotes longer lasting immunity although granulomatosis may develop at the injection site. If the vaccine is oil based, then injection should be subcutaneous with a dose given 1 to 7 months before mating plus an annual boost. Males require a double vaccination (1 and 8 weeks before mating) plus an annual booster. If the vaccine is aluminium hydroxide based it may be administered subcutaneously or intra-muscularly with the first dose 6 weeks before mating, a booster 10 days before mating and an annual booster thereafter at least 10 days before mating. For bulls a double vaccination is recommended (2 and 4 weeks before mating) plus an annual booster 4 weeks before mating.

Some authors have had problems curing BVC in bulls using only vaccination. The complementary use of an intra muscular injection of 6.25g of procaine penicillin plus dihydrostreptomycin sulphate was proposed as an support of vaccination (Hum et al., 1993) .

Some commercial laboratories have also developed polyvalent vaccines against as spectrum of common reproductive diseases. The effectiveness in preventing BVC of one vaccine containing *Leptospira interrogans* serovar *pomona, Haemophilus somnus, Cff, Cfv*, IBR and BVD antigens in aluminium

hydroxide adjuvant and another vaccine containing the same antigens except *Haemophilus somnus* was evaluated (Cobo et al., 2003). It was found that these vaccines produced no immunity against *Cfv*. This shows that it is better to have proper knowledge of the disease status of the herd than trying to prevent all of the possible diseases blindly.

1.9.3 Prevention

If the herd is free of BVC and no preventive vaccine is available, introduction of only virgin bulls should be done in case of need. If an older non-virgin bull is to be used, treatment with antibiotics (locally and systemically) should be implemented an at least 4 consecutive samplings should deliver negative results before allowing mating (Hartley, 1952). If new female stock is needed, the farmers must have certainty about their health status and the history of the provider in that matter. A sexual rest of at least 6 months should be allowed before mating.

If vaccination is available, the previously explained vaccination plans should be applied (Section 1.9.2.2).

In all cases, the application of the reproductive management rules explained in Section 1.9.2.1 are useful to prevent spread of the disease in case of introduction into a herd.

1.10 Bovine genital campylobacteriosis in New Zealand

The disease was well known in Australasia in the decades between 1950-1980 and many studies on campylobacteriosis have been published in New Zealand and Australian Journals (Clark, 1971; Hartley, 1952; Hemmisen, 1981; Newsam, 1960; Newsam and Peterson, 1964; Te Punga, 1958, 1962; Te Punga and Boyes, 1958; Te Punga et al., 1964; Te Punga and Moyle, 1961).

The implementation of AI together with reproductive management rules (Section 9.2.1.) decreased the prevalence of the disease in New Zealand, until it was thought that it no longer existed in the country. The organism was last isolated in 1993 in New Zealand (Loveridge and Gardner, 1993), although it was suspected again in 1996 (Black and Orr, 1996).

In the early 21st century, the presence of *Cfv* in some herds in the Taihape

area was suspected (Hughes, 2001). The reproductive history of these herds resembled that of endemic campylobacteriosis, whilst tests for other potential causes of low fertility had failed to identify any other source of the problem. The IgA ELISA was used in an attempt to demonstrate the presence of *Cfv* in those herds. According to the rules of interpretation of the test (Hum et al., 1994) it was concluded that the disease was present. However, the organism was never isolated from any of preputial washes taken from bulls on farm and at an abattoir.

One year later, the IgA ELISA test was used to evaluate the presence of *Cfv* in a dairy herd in the Gisborne area (Tattersfield, 2002). It was found that, according to the tests results the infection with *Cfv* was in the herd, although, again, the organism was not isolated from any of the preputial washes obtained from the bulls.

Results from research by McFadden et al. (2004a) at Massey University showed that there was no relationship between the fertility of beef herds and the proportion of positive results to the IgA ELISA test in those herds. Moreover, when the standard of interpretation given with the test was used, positive animals were widespread throughout the country. Again the agent was not isolated from the 54 samples collected from bulls as part of the survey, although both *Cff* and *C. jejuni* were commonly present.

The reproductive histories of the researched herds in 2001 and 2002 do agree with bovine genital campylobacteriosis. Natural mating is still used in both beef and dairy productions (Anonymous, 2005; McFadden et al., 2004b). The country counts with the right geography and management to promote this disease to be maintained in some animals (hill country where no control over the animals is exercised other than joining the bulls to the cow herd and taking them out once the mating season ends; cows grazing off farm or in hill country where no control over possible abortions is done). Only a few carrier animals (females or males) in a herd that undergoes natural mating for some time during the year are enough to maintain a certain proportion of the bovine population as constant reservoirs of the organism. These animals will change from on year to the next but if the disease is present in New Zealand, it will surely be quartered in a few animals per herd.

1.11 Conclusions

In the light of all the above, it may be said that BVC presents many challenges to the clinician, the microbiologist and the epidemiologist in New Zealand.

It is a disease that if endemically present in a herd, the symptoms will be subtle enough for the farmer to need time to recognize that a problem is present and call the veterinarian (Hughes, 2001). Once the clinician is involved, the fact that the disease has not been diagnosed in New Zealand for so long means that it will probably be one of the last causes of infertility to be considered. When *Cfv* is finally suspected, the challenge is now to try and diagnose it. Microbiological isolation should always be sought but the extremely fastidious characteristics of this organism renders isolation a very erratic means of undoubtful diagnosis.

If other means of diagnosis are used, such as the IgA ELISA test, there is no certainty about the results delivered by this test under New Zealand conditions. In the light of the evidence shown in previous sections it appears that there are major problems in the use or interpretation of the ELISA test in New Zealand herds, which need to be observed before it can be widely used in the field.

None of the diagnostic method developed until now are sensitive and/or specific enough to be used alone, while culture should always be the goal. However, isolation of the organism had not proved possible in any samples taken from bulls and cows in New Zealand during 2001/2002. This raises the question if the isolation methods used in New Zealand are adequate for the agent to be isolated. Perhaps, since none of the diagnostic methods has yet proven effective, evaluation of the newly developed techniques is needed. For example, preliminary evaluation of PCR as a diagnostic technique to be applied to clinical samples from bulls has been done and encouraging results were obtained (Pannachio et al., 1993).

Every effort should be made for the diagnosis of this disease if present in New Zealand. Once confirmed, its characteristics are such that in two or three years of proper reproductive management, application of artificial insemination and maybe vaccination is enough to control it and possibly eradicate it. But until some proper attempt of diagnosis is made nation wide, no sure statement about

its presence or absence can be made.

1.12 Hypothesis

In the light of the above evidence it appears that there are some difficulties in using the IgA ELISA test in New Zealand herds, which need to be understood before it can be widely used on the field. Moreover, it may also be that the cultural methods used in New Zealand are not adequate for the agent to be isolated either at sampling level or at media level. Furthermore, exploration of other means of diagnosis needs to be researched.

It is the aim of this work to:

1) Evaluate possible cross-reactions of the IgA ELISA test with other *Campylobacter* subspecies
2) Evaluate the sampling methods used in New Zealand
3) Evaluate the use of the PCR technique (Hum et al., 1997; Pannachio et al., 1993) on clinical samples from bulls.

Chapter 2: General Materials and Methods

2.1 Animals

Heifers used in these trials (n=18) were acquired from the Sheep and Beef Research Unit Hauorongo (Massey University, Palmerston North). They were all virgin animals and were kept together at the Large Animal Teaching Unit (LATU, Massey University, Palmerston North) under grazing conditions until assigned into treatment groups. They were supplemented, if required, with high quality hay. Once formed, groups were strictly isolated from each other so that not even the water troughs were shared.

A total of 4 virgin bulls were acquired at 18 months of age from a neighbouring bull fattening farm. They were also kept at LATU in 2 separate pens at a rate of 2 bulls per pen. They were fed high quality hay and were kept apart from females at all times except when they were used for mating

2.2 Incubation conditions for inoculated media.

Unless otherwise specified, incubation of inoculated plates or bottles of media was always done at 37°C under microaerophylic conditions generated with the use of a single use disposable microaerophylic gas generating system (Gas sachet; AnaeroPack System™, Pack-MicroAero 2.5L, Ngaio Diagnostics, Nelson, New Zealand) within hermetic anaerobiosis jars and/or boxes for 5 days.

2.3 Preparation of inoculi

Campylobacter fetus subsp. *fetus* strain NS41 (strain NS41 isolated from an aborted sheep foetus by S. Mannering, Massey University, 2003) was kept frozen at –70°C in glycerol. One week before challenging the animals, ten blood agar plates (BA) were inoculated with the frozen strain and incubated (as in section 2.2). Twenty-four hours before challenge, the inoculum was prepared by harvesting the growth of 5 plates, suspending it into 10 ml of BHI broth (Appendix 1) and incubating it under microaerophylic conditions. Subsequently, 6 ml of the inoculum were used to challenge heifers while the rest was taken to the laboratory for CFU/ml counts (see section 2.6).

Campylobacter fetus subsp. *venerealis* strain NCTC 10354 (strain NCTC

10354 (ATCC 19438, CCUG 538, CIP 6829) type strain, New Zealand Reference Culture Collection Medical Section, ESR, Wellington) was received from ESR in a freeze-dried pellet. The pellet was reconstituted according to supplier's instructions and was then inoculated onto 10 blood agar plates and incubated (as in section 2.2). Colonies were then harvested from the plates, mixed with glycerol, frozen at –70°C and used as *Cfv* cell stock. One week before animal challenge, 10 blood agar plates were inoculated with the stocked *Cfv* cells and incubated as explained in section 2.2. Twenty-four hours before animals were challenged, the inoculum was prepared by harvesting the growth of 5 plates, suspending it into 10 ml of BHI broth (Appendix 1) and incubating for 1 day under microaerophylic condition at 37°C. A total of 6 ml of the inoculum was used to challenge heifers and the reminder was used for CFU/ml counts (section 2.6).

For bull challenges, a total of 20 blood agar plates were inoculated with the stock *Cfv* cells and incubated under microaerophylic conditions at 37°C one week prior to the challenge. On the day of the bull challenge, the inoculum was prepared by harvesting the growth from 10 plates and diluting it into 22 ml of PBS (appendix 1). Each bull was challenged with 10 ml of this inoculum. The remainder was used for CFU/ml count (Section 2.6).

2.4 Post-infection sample handling

2.4.1 Inoculation of media used for microbiological isolation of *Cfv* and *Cff* from females

Blood agar and Skirrow's agar: inoculation of blood agar plates and Skirrow's agar plates (Appendix 1) was done within 1 min of sampling (isolation plates). Plates were placed in the incubator (Section 2.2) within 30 minutes of inoculation.

Blood Agar and Lander's medium: inoculation of blood agar plates (isolation plates) and Lander's medium broths (Appendix 1) was done within 1 min of sampling the heifers. Lander's medium cultures required special treatment (Lander, 1990a). They were first incubated for three days under microaerophylic conditions at 37°C after which an aliquot of 100 µl of the broth was subcultered

onto blood agar (isolation plate) and incubated (as in section 2.2).

Vaginal swab: When the sampling method used was the vaginal swab, inoculation of blood agar plates was done by spreading half of the harvested vaginal mucus from the swab directly onto the plate, without rotating the swab around. Then, the swab was rotated and the Skirrow's agar plates were inoculated with this second half of the mucus harvested in the swab. If Lander's medium was being used, the swab was first used for inoculation of the blood agar plates as described above. Then the top of the swab, which contained the rest of the harvested vaginal mucus, was cut, placed into the Lander's broth, and incubated as in section 2.2.

Vaginal washes: When the sampling method used was the vaginal wash, an aliquot of 100 μl of the harvested vaginal mucus was used to inoculate blood agar plates and an aliquot of 1 ml of the same sample was used to inoculate Lander's medium. All the media used were incubated within 30 minutes of inoculation.

2.4.2 Inoculation of media used for microbiological isolation of *Cfv* from males

Blood Agar: Inoculation of blood agar plates was done with an aliquot of 100μl of each sample (isolation plate). All plates were inoculated within an hour of sample collection.

Lander's medium: an aliquot of 1 ml from each of the samples was used to inoculate Lander's medium, which was then incubated for three days at 37°C under microaerophylic conditions. An aliquot of 100 μl of the broth was then subcultered onto blood agar (Lander, 1990a) and incubated (isolation plate). All media were inoculated within an hour of sample collection.

2.5 Laboratory procedures for phenotypic characterization

The isolation plates were checked for colony growth 5, 7 and 10 days after sowing. If no growth was observed by Day 10, the plates were discarded and the results were reported as negative for microbiology. Any colony found was subjected to the following biochemical characterization procedure:

The colonies obtained were Gram stained and tested for oxidase using

oxidase detection strips (Medvet Science PTY Ltd, The Barton, South Australia). A small proportion of the colony was put in contact with the oxidase strip and if the strip turned dark blue the colony was reported as positive for oxidase reaction. Colonies were also tested for catalase reactivity. A small proportion of the colony was harvested with a glass stick and placed into 3 ml of a 3% hydrogen peroxide solution. If bubbles were formed the colony was reported as positive for catalase reaction.

If the colony was Gram negative and positive to catalase and oxidase reactions it was inoculated onto three BA plates and incubated (Plates 1a, 1b and 1c). After 5 days, Plates 1a and 1b were checked for purity and the following tests were performed on them:

1) Cephalothin and Nalidixic acid sensitivity: Half the growth from each plate was harvested and suspended into 3ml of PBS (Appendix 1). An aliquot of 100μl of this suspension was spread over a blood agar plate, which was divided in two halves. One disk of Cephalothin (Cephalothin 30 μg disks, OXOID Limited, Basingstok, UK) was placed in one half and one disk of Nalidixic acid (Nalidixic acid 30 μg disks, OXOID Limited, Basingstok, UK) was placed in the other half. Plates were then incubated and growth was checked after 5 days. If there was no growth around the disk (inhibition zone), the colony was considered 'sensitive'; otherwise the result was 'resistant'.
2) Heat tolerance: One colony was subcultured onto two blood agar plates and incubated, one at 42°C and the other at 25°C for 5 days. Results were reported as positive (growth observed) or negative (no growth observed).
3) Growth in 1% glycine: one colony was harvested and inoculated onto two modified blood agar/glycine plates containing 1% glycine and incubated (Appendix 1). Results were reported as positive (growth observed) or negative (no growth observed).
4) Hippurate hydrolysis: half the growth from plates 1a and 1b was harvested and suspended in 0.4 ml of sterile distilled water. One Hippurate disk (BBL™ TAXO™ HIP, Becton Dickinson and Company, Sparks, MD, USA) was added to the suspension and incubated in water

bath at 37°C for 2 h. Then, 0.2 ml of ninhydrin reagent (Bio Merieux® SA. Global Science, New Zealand) was added to each tube and incubated again at 37°C for 10 min. A deep purple color was reported as a positive result.

5) H_2S production (lead acetate strip): one colony was harvested and inoculated into two BHI/Cysteine 200 ml broths (Appendix 1) and incubated with a lead acetated strip (PL Precision Laboratories, Cincinnati, OH, USA) suspended above the broth. Where the acetate strip turned black the result was positive (H_2S had been produced).

Control plates inoculated with *Cfv*, *Cff* and *C. jejuni* underwent the same biochemical characterization procedures in parallel.

Plate 1c was checked for purity and frozen at –70°C with glycerol. After phenotypic characterization, the frozen isolate was kept only if confirmed to be *C. fetus* subsp. *fetus,* or *C. fetus* subsp. *venerealis.*

Table 2.1 shows the interpretation of these phenotypic characterization procedures.

2.6 Colonies forming units (CFU/ml) count

The CFU/ml count was set up using the following methodology:

1) The residual inoculum was brought back into the microbiology laboratory.
2) It was centrifuged at 5000g for 10 min and the supernatant discarded.
3) The pellet was reconstituted into 1 ml of phosphate buffered saline (PBS, Appendix 1).
4) Ten consecutive tenfold dilutions were made into sterile PBS from the reconstituted pellet.
5) An aliquot of 100 µl of each dilution was inoculated onto a blood agar plate (Columbia sheep blood agar plates (BA); Fort Richard Laboratories Ltd, Auckland, New Zealand) and spread using a sterile glass spreader.
6) The ten plates were incubated for 4 days at 37°C under microaerophylic conditions.
7) Colonies were counted only on plates where between 200 and 20 colonies had grown. The final result for the plate was the number of colonies

counted multiplied by 10 elevated to the dilution exponent. For example, if dilution 7 delivered 20 colonies, then the final CFU/ml for that particular plate was: 20×10^7.

8) The final CFU/ml value for the inoculum was calculated as the average.

Table 2.1: Interpretation of the biochemical characterisation procedures for field isolates. Grey cells represent biochemical differences among these three *Campylobacters* (Cowan and Steel, 1999; Quinn et al., 2002)

Test	*C. fetus subsp. venerealis*	*C. fetus subsp. fetus*	*C. jejuni*
Gram	Negative	Negative	Negative
Oxidase	Positive	Positive	Positive
Catalase	Positive	Positive	Positive
Heat tolerance: 42°C	Negative	Negative	Positive
Heat tolerance: 25°C	Positive	Positive	Negative
Hippurate hydrolysis	Negative	Negative	Positive
Nalidixic	Resistant	Resistant	Sensitive
Cephalothin	Sensitive	Sensitive	Resistant
1% glycine	Negative	Positive	Positive
H_2S production (lead acetate strip)	Negative	Positive	Positive

2.7 Sampling

2.7.1 Preparation and Sampling methods used in heifers.

2.7.1.1 Preparation

Heifers were all sampled on the same day. If more than one group was sampled, the first group to be sampled was the Control, so that there was no

possibility of contamination of the facilities. Between groups, the crushes were sprayed with disinfectant (left for 15 min) and hosed with a pressure hose until no remaining faeces, hair or urine were present. Animals were put into crushes, and their heads were secured in head bales. Tails were tied to the side to avoid re-contamination of the vulva once it was clean.

The vulva was cleaned with warm water and cotton wool and dried with cotton wool. After this Virkon was sprayed and dried. Care was taken to never introduce water or Virkon inside the vagina. Gloves were worn at all times and changed between animals and during cleaning and sampling. The vulva labia of all animals were opened before introduction of the sampling instrument to ensure that no contamination was introduced during sampling.

2.7.1.2 Vaginal swab sampling for microbiological isolation

To allow sampling at an adequate depth, special swabs were prepared. A sterile swab was plugged to the end of an insemination pipette. The instrument was then fitted into a plastic clear sheath and the whole instrument was sterilized by autoclaving. The sheath protected the swab as it was inserted through the opened vulva into the vagina. Once inside the vagina, the sheath was left in place and the swab was pushed as far into the vagina as possible to achieve sampling closest to the cervix. The swab was moved around in the cranial vagina to achieve proper soaking with vaginal mucus. It was finally pulled back into the sheath and the whole instrument was removed from the tract.

2.7.1.3 Vaginal wash sampling for microbiological isolation

After preparation of the heifers, 10 ml of warm sterile PBS (Appendix 1) were infused by syringe into the vagina through a sterile insemination pipette. The liquid was then flushed up and down the pipette several times using the syringe to maximize the harvest of partially liquefied vaginal mucus. The sample was then placed inside a sterile glass container.

2.7.1.4 Vaginal swab for IgA ELISA test

A sterile cotton swab (20 cm long) was introduced through the opened vulva into the vagina and was allowed to soak with vaginal mucus by moving it around the vagina and against the vaginal wall. It was then placed into PBS

containing 0.05% Tween 20 (PBST, Appendix 1) according to the protocol of IgA ELISA test. Gribbles Veterinary Pathology Laboratories (142 Botanical Road, Palmerston North, New Zealand) for processed the samples. The same technician processed all samples without knowing their origin.

2.7.2 Preparation and sampling methods used for bulls

2.7.2.1 Preparation

Bulls were put into a crush and their heads were secured. Tails were tied to the side to avoid tail flicking during sampling and the hind right leg restrained with a rope to avoid kicking. The hairs around the preputial orifice were clipped to a length of 5-6 cm and the area was cleaned with cotton wool soaked in 96% ethanol. Urination was stimulated after this by the ethanol itself or by vigorous massage of the prepuce. Once the bull urinated, the area was cleaned again with cotton wool soaked in 96% ethanol, before sampling.

2.7.2.2 Preputial scraping

A sterile re-usable preputial scraper (Elastécnica, Tecnología en Caucho, Lomas de Zamora, Buenos Aires, Argentina) was introduced through the preputial orifice, taking special care not to touch the hairs around the preputial orifice. It was pushed towards the distal part of the preputial sheath and was then pushed and pulled along the preputial sheath at least 20 times. The scraper was then removed from the prepuce taking care not to touching the preputial hairs. The resulting sample was washed into 10 ml of sterile warm PBS (Appendix 1). The samples temporarily stored in an anaerobic jar/box under microaerophylic conditions (Gas sachet) until reaching the microbiology laboratory. All samples were processed for microbiology within one hour of collection.

2.7.2.3 Preputial wash

Between 30 and 50 ml of warm sterile PBS (Appendix 1) were flushed into the preputial sheath of the bulls via a 53 cm long uterine mare infusette (National Veterinary Supplies Ltd, Palmerston North, New Zealand). The preputial orifice was held closed by applying pressure with the hand. Once the fluid was inside, it was pushed up and down the prepuce at least 20 times by

preputial massage. Special care was taken to get the fluid to go as distally as possible inside the prepuce. The wash was then retrieved by aspiration by syringe, and transferred into a sterile 50 ml PP-Test tube (CellstarR, Greiner bio-one, Raylab Ltd, New Zealand). It was then placed into an anaerobic jar/box under microaerophylic conditions until it reached the microbiology laboratory. All samples were processed within one hour of collection.

2.8 Basic operating procedures for IgA ELISA test

The Elizabeth Macarthur Agricultural Institute (Melbourne, Australia) provided all reagents for the IgA ELISA test.

According to the standing operating procedures manual provided, the ELISA plates are coated with the provided *C. fetus* subsp *venerealis* antigen (4144 strain (CSL)) and stored frozen at -20°C. The three Controls provided were kept frozen: Low positive control 306/6 (MSW Agriculture, Armidle); Negative Control T244, T263, T260, T303 (NSW Agriculture, EMAI Dairy) and High positive control (NSW Agriculture, Armidale AN95/301, 306, 307, 309).
When the swab samples arrived at the laboratory they were mixed for one hour or placed on the plate rocker overnight. Plates coated with the antigen were thawed at room temperature for 24h and washed three times with reverse osmosis water.

A dilution 1/10 of the vaginal swab sample (test sample) was made by adding an aliquot of 50 µl of the sample into 450 µl of PBST. Controls were diluted in the same manner.

An aliquot of 100 µl of the test samples was placed in duplicate in the wells of the ELISA plate along with 5 high positive, 5 negative, 3 low positive and 3 PBST diluent controls. The plate was then incubated at 37°C on a plate shaker for 1 h. Thereafter, an aliquot of 100 µl of anti-bovine (rabbit) IgA conjugate (ICN code 641751) was added to the wells and the plate was again incubated at 37°C for 1 h shaking at speed 2 of 10. After the plate had been washed three times with reverse osmosis-generated water, 100 µl of anti-rabbit IgG conjugate (ICN code 674371) were added to the wells, and the plate was incubated again at 37°C on a plate shaker for 1 h. After the plate had been

washed, 100 µl of the TMB substrate provided was added to the wells, the plate was kept in the dark for 10 min. The optical densities (OD) of the wells in the plate were then read at 450 nm on a microplate reader.

The resulting ODs are registered. The final result for the IgA ELISA test measured in Elisa Values (EV) was calculated manually applying the following formula:

$$EV = \frac{\text{mean OD sample - mean OD negative control}}{\text{mean OD strong positive control – mean OD Negative control}} \times 100$$

EVs between 23 and 33 were categorized inconclusive (suspicious). Values greater then 33 were accepted as positives and values below 23 were accepted as negatives.

Quality control for this test:

- Strong positive, weak positive, negative and PBST controls should always be included in the ELISA plate.
- The mean OD of the strong positive and the mean OD for the weak positive should be within ±0.2 and ±0.1 from the targeted OD respectively.
- The difference in OD between PBST and the negative control should not be greater than 0.05.
- If the coefficient of variance of the OD of the duplicate in each test sample is greater than 15%, then the sample should be retested.
- The weak positive control is included to ensure that the reaction is identifying the sample within the inconclusive range.

2.9 PCR technique for identification of Cfv cells

The PCR was set and run according to the descriptions published by Hum et al. (1997) with little modification. A 2µl aliquot of the final sample suspension was used as template. The primers (Inivitrogen, New Zealand) used were MG3F (forward species primer) 5'GGTAGCCGCAGCTGCTAAGAT3', MG4R (reverse species primer) 5'TAGCTACAATAACGACAACT3', VenSF (forward subspecies primer) 5'CTTAGCAGTTTGCGATATTGCCATT3', and VenSR (reverse subspecies primer) 5'GCTTTTGAGATAACAATAAGAGCTT3' (Hum et al., 1997). The buffer (10x Reaction buffer without $MgCl_2$ (1.25 ml of 100

mM Tris-HCL (pH 9.0) and 500mM KCL) and the magnesium ($MgCl_2$ solution: 1 ml of a 25mM solution) used were provided with the Cloned *Taq* polymerase (SuperTaq™, 250 U, Ambion, GeneWorks, New Zealand). The appropriate volumes of each ingredient were used to achieve the following final concentrations: 1.125 U of *Taq*, 1.5 mM MgCl2, 0.1 mM of each dNTP (dNTP Mix, Finnzymes OY, Finland), 0.5 μM of each primer completed with sterile bi-distilled water to a total volume of 13 μl. The reagents and the template were mixed into a 0.5 μl thin-wall flat cup PCR tubes (Axygen Scientific, Global Science, New Zealand). The total reaction volume was 15 μl.

Reactions were performed in a GeneAmp PCR System 2400 (Perkin Elmer Corporation, USA) following 1 cycle at 95°C for 10 min to rupture whole cells, 30 cycles of 95°C for 20 sec, 50°C for 20 sec and 72°C for 2 min, followed by 1 cycle of 10 min at 72°C to ensure complete extension. The mixture was then kept at 4°C.

Finally, 10 μl of the PCR product were used with 6x SDS buffer, loaded into the wells of a 2% agarose gel (UltraPure™ Agarose, Invitrogen, New Zealand) and separated by electrophoresis in 0.5 TBE buffer at 100V/cm for 70 min. A 1kb molecular weight marker (1 kb Plus DNA ladder, Inivitrogen, New Zealand) was loaded to measure the bands obtained. The gels were stained with ethidium bromide for 10 min and visualized using an UV transiluminator (BioRad Gel Doc 2000) with a Quantity One 4.5.2 computer program. A positive control for *Cfv*, a positive control for *Cff*, a control for *C.jejuni* and a negative control (no template) were run within each reaction.

Chapter 3: Diagnosis of BVC in experimentally infected heifers.

3.1 Introduction

Between 2000 and 2005, three reports of investigations of beef and dairy herds in New Zealand with low pregnancy rates for the presence of bovine venereal campylobacteriosis (BVC) have been published (Hughes, 2001; McFadden et al., 2004a; Tattersfield, 2002). In each of these herds, there had previously been negative results for a range of diagnostic tests performed to detect the presence of other reproductive diseases (trichomoniasis, mineral deficiencies, toxicoses, BVD, leptospirosis), so, as the reproductive symptoms displayed by the herds were very reminiscent of BVC (Hartley, 1952), the presence of that disease was suspected. However, *Cfv* was not isolated from any of the herds, despite a concerted effort to collect samples from animals that were at risk of carrying the infection. Hughes (2001) was unable to isolate the organism from preputial washes from a number of bulls, whilst Tattersfield (2002) reported 100% negative results for isolation from vaginal swabs collected from 30 cows and from samples from culled bulls. As a result of the inability to culture *Cfv,* from the herds he investigated, Hughes (2001) sent a number of samples to Australia to be screened using a recently developed IgA ELISA test (Hum et al., 1994). This test looked promising, because it represented a cheap and fast way of diagnosing BVC in herds, and because only 10 of the cows most likely to be affected had to be sampled. The results came back positive and the test was imported to New Zealand for generalised use in potentially affected herds. According to the rules of interpretation of the IgA ELISA test (i.e. that 3 positive animals in 10 sampled is taken to indicate that the herd is affected by BVC; Carrus et al.(1997), Hughes (2001) concluded that the disease was apparently endemic in the beef herds he tested (3 positive and 3 suspicious results in 40 sampled cows from one herd and 6 positives out of 10 sampled cows from another herd). Tattersfield (2002) found similar results in a dairy herd in the Gisborne region (4 positive cows out of 10 sampled). The latter author noted, however, that effort needed to be put into attempting the isolation of the organism and that validation of the IgA ELISA test for use in New Zealand was

needed. In 2002, McFadden and co-workers initiated such a nationwide evaluation of the performance of this IgA ELISA test under New Zealand conditions. They sampled 10 cows/herd from 125 beef herds that were classified as low, medium and high fertility herds (herd type) and obtained samples from dairy herds that used AI only. They then compared the observed IgA ELISA results between herd types with the presence of possible risk factors for the spread of a venereal disease. Microbiological isolation of *Cfv* was attempted from 54 bulls, from 9 low fertility herds that had had more than 3 cows which were positive to the IgA ELISA, using preputial washes and Lander's transport enrichment medium. They reported that there was no relationship between the IgA ELISA test results and the herds' fertility, or the presence of risk factors for venereal disease (McFadden et al., 2004a). Moreover, culture of the organism was not successful. It was therefore considered that all the positive results should be considered as false positives, due to the lack of signs of BVC in the beef herds and the lack of sexual contact with bulls in the AI dairy herds. From this assumption, they calculated that the IgA ELISA test presented a 28% false positive rate from an analysis of the results generated by 206 sampled beef cows from high fertility herds and a 10% false positive rate from 52 sampled dairy cows that had been bred solely by AI. They speculated about the possibility that the IgA ELISA test might be cross-reacting with other *Campylobacter* species, in particular *C. fetus* subsp. *fetus* (*Cff*). The endemic infection of sheep flocks with *Cff* and the substantial similarities between the two *Campylobacter* subspecies supported this notion (McFadden et al., 2004a).

The key observations made in these three reports were:

1) Attempts were made to isolate *Cfv* in the three different studies, from samples collected from males (preputial washes) and females (vaginal swabs), all without positive results (Hughes, 2001; McFadden et al., 2004a; Tattersfield, 2002). This created uncertainty about the sensitivity of culture from preputial washes. However, McFadden et al. (2004a) isolated other *Campylobacter* species (*C. jejuni, C. fetus* subsp. *fetus*) and related organisms (*Arcobacter cryaerophilus* and *Helicobacter cinaedi)* from 8 samples from 3 farms.
2) Two evaluations of herds with fertility problems showed positive IgA

ELISA test results in all cases (Hughes, 2001; Tattersfield, 2002). The one nationwide study using IgA ELISA test (McFadden et al., 2004a) suggested that there may be false positive results to this test. It was therefore postulated that *Cfv* was probably not present in New Zealand and that the ELISA results might be explained in terms of cross-reactivity with *Cff*.

In the light of this evidence, two areas of study were found to need further research:

1) Sensitivity of microbiologic isolation methods (including sampling methods and isolation media) should be evaluated.
2) Performance of the IgA ELISA test should be studied under controlled conditions and its potential cross-reactivity with *Cff* evaluated.

3.2 Experiment 1: ELISA and microbiological studies of heifers challenged with Campylobacter fetus

3.2.1 Materials and methods

3.2.1.1 Animals, Group assignment criteria and inoculation

A description of the animals, their management and husbandry is given in Chapter 2.

Groups were defined as follows: Group *Cff* (n=6), challenged with *Campylobacter fetus* subsp. *fetus;* Group *Cfv* (n=6), challenged with *Campylobacter fetus* subsp. *venerealis* and Control Group (n=6), challenged with sterile phosphate buffered saline (PBS). Details of the preparation of materials for these challenges are given in Chapter 2.

Animals were challenged twice (24h apart), during oestrus. It was decided that the first 6 heifers in oestrus would become Group *Cfv*, the second 6 heifers would become Group *Cff* and the last 6 heifers would become the Control group. Oestrus was synchronized in all heifers with a single injection of $PGF_{2\alpha}$ (Lutalyse 5 mg, Pharmacia Company Limited, Auckland, New Zealand). Oestrus was observed 48 h later. Only 8 heifers showed heat; 6 of which were assigned to Group *Cfv* and were challenged as explained above. The rest of the heifers were re-synchronized with a second injection of 5 ml of Lutalyse 11 days after the first

injection. Heat was observed in these 12 animals 48 h after second injection and the first 6 to show oestrus were assigned to Group *Cff*. The rest of the heifers were assigned to Control Group. All heifers except one (Control group) had been observed in heat within 24 h of the time of challenge. Table 3.1 shows concentrations of viable cells (measured in colonies forming units per millilitre; CFU/ml) in the inocula used to challenge the heifers.

3.2.1.2 Sampling

Each animal was sampled 24 h after the second challenge (Day 0) and at weekly intervals thereafter. The sampling method used in this trial for both microbiology and IgA ELISA was the vaginal swab (see Chapter 2). The media were blood agar and Skirrow's agar (see Appendix 1). At each sampling, samples for IgA ELISA test were taken after the sample for microbiology.

Table 3.1: Inoculum used in each animal at each challenge measured in colonies forming unit per millilitre (CFU/ml)

Group	Challenge	CFU	Volume used/heifer	Strain
Cff	1	$4.00x10^8$	1 ml	NS41
	2	$3.00x10^8$	1 ml	NS41
Cfv	1	8.35×10^8	1 ml	NCTC 10354
	2	$1.00x10^9$	1 ml	NCTC 10354
Control	1		1 ml	PBS
	2		1 ml	PBS

Linear regression was used to compare Elisa Values (EV) of the IgA ELISA test within groups. Results obtained for the IgA ELISA test were transformed by natural logarithms to achieve normality and to apply generalized mixed model regression assuming a normal distribution. Variances were adjusted for the correlation between subsequent measurements on the same animal. The null-hypothesis of equal means between sampling days within group was evaluated with 95% confidence (P≤0.05). Pearson's residuals were pltted to check for patterns of distribution. An intraclass correlation coefficient (ICC; a measure of

correlation between duplicates) was calculated per cow within each Group. In addition, 46 samples were tested by the IgA ELISA before and after freezing to assess the influence of freezing on the results. Results were evaluated by ANOVA and evaluated by the intraclass correlation coefficient (ICC). All analyses were carried out using SAS (2003) and Microsoft® Excel 2000

3.2.2 Results and discussion

None of the heifers showed any clinical symptoms or the presence of vaginal discharges after challenge.

Mean (geometric, ± SEM) EVs for each Group are shown in Figure 3.1. Pearson's residuals showed no pattern for any of the Groups.

Suspicious EVs (i.e. between 23 and 33 VE; Hum et al.(1994) were present prior to challenge in Group *Cff* and Control animals. Despite the fact that all these heifers were known to be virgin individual values above the negative limit (EV=23) were obtained in 8 animals (four from each group) before microbiological challenge.

Group *Cff* (Figure 3.1a) displayed EVs that were significantly higher than prior to challenge five days after challenge, reaching maximum values on Day 26 post-challenge. Values remained above negative limits until the end of the trial (Day 40). Within this period, one sample (Day 19) was significantly ($P<0.05$) lower than the two previous and the four following samplings, but not significantly different from values before or on Day 0. From a total of 8 samplings taken after challenge (Day 0), three resulted in negative results, one delivered suspicious results and five were positive. It was notable that there was a great deal of variation, both between animals and between successive samples from individual animals in the EVs that were obtained. In fact, the ICC for this Group was 0.29 meaning that only 29% of the variation could be attributed to animal factors. Isolation of *Cff* was achieved 24 h post-inoculation from all 6 heifers, but it was not recovered thereafter.

Group *Cfv* (Figure 3.1b) displayed suspicious IgA ELISA results (EVs in the range 23-33) on Days 16, 44, 51 and 58 post-challenge. There were no significant differences in mean EVs between samplings made before or after challenge in this group. On none of the sampling days were group means higher

than the positive threshold. Despite this, even though group mean EVs did not fluctutate as much as in Group *Cff* the ICC at heifer level was of 0.03. Hence only 3% of the variation can be explained by an animal factor. The rest is due to a test factor. Isolation of *Cfv* was unsuccessful, even at 24 h after challenge.

The Control group (Figure 3.1c) showed fluctuating EVs that never exceeded the positive cut-off threshold, although the geometric mean for Day 12 was 32.4 (1 and 5 positive and suspicious heifers respectively), just below the positive cut-off. Suspicious mean EVs were obtained on four occasions, the first one on Day -50. Significantly lower values were observed on Day 33. Here again, the ICC was very low: 0.06, thus very low correlation within animals between samplings was observed. No *Campylobacter* spp. were isolated from the Control group on any sampling day.

The mean EV from fresh samples (EV=37.6) was significantly higher ($P \leq 0.05$) than the mean EV for frozen samples (EV=20.7). The ICC of 0.54 suggested that 54% of this variation was due to the actual antibody level and 46% was due to freezing and/or variation between repeated tests.

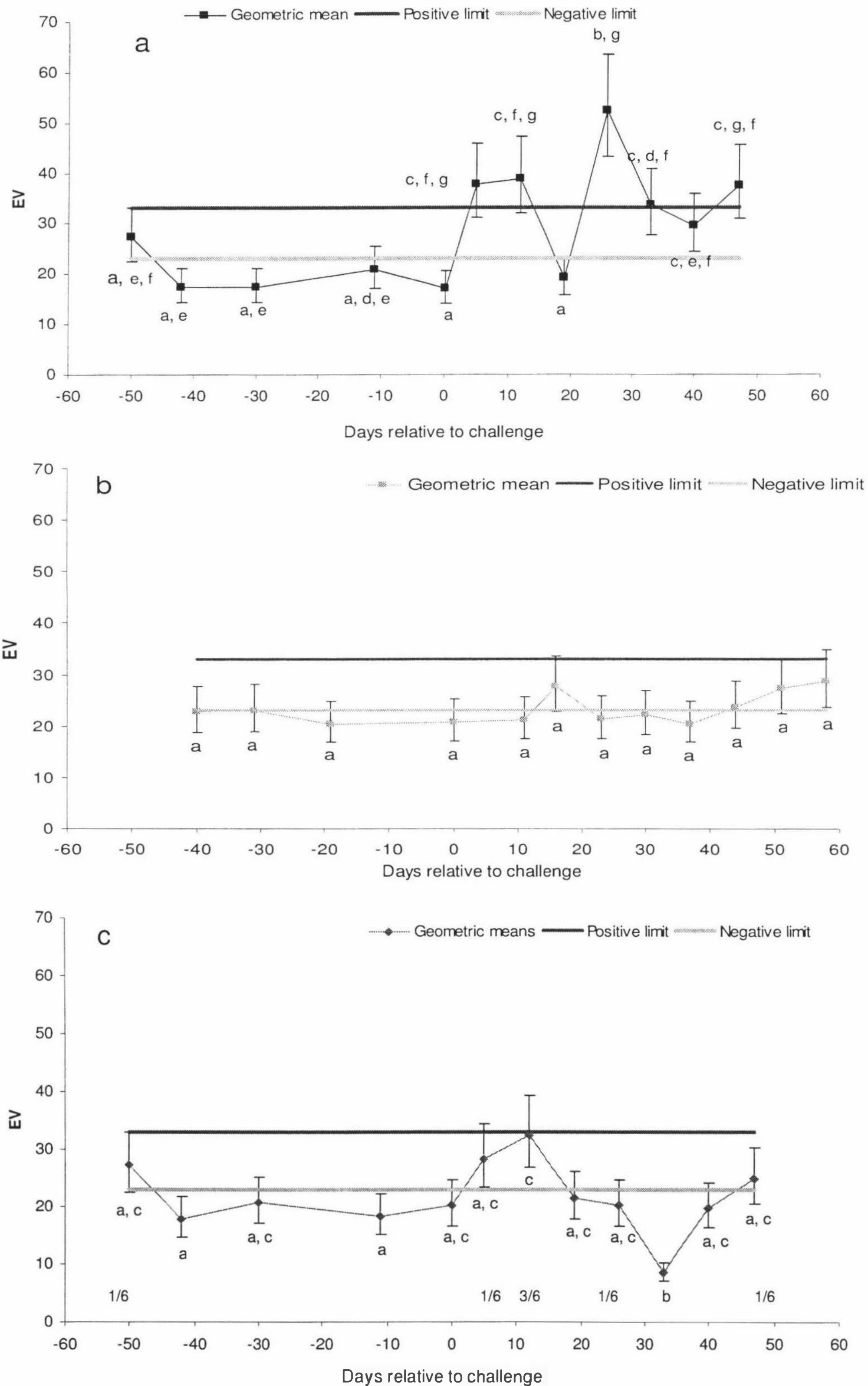
It appears from Figure 3.1 as if the IgA ELISA cross-reacted with IgAs present in the vagina other than IgA secreted against *Cfv*. This could explain the suspicious values that were obtained before challenge in several animals, the positive EVs obtained in animals challenged with *Cff* and the suspicious values obtained for the Control Group.

By serially sampling the same animals, fluctuating EVs were obtained, which exhibited no regular pattern of fluctuation, raising serious concerns about a poor repeatability of results obtained using this test. This concern is supported by the low ICCs per cow found in all three Groups (namely 0.29, 0.03 and 0.06 for Group *Cff*, *Cfv* and Control respectively). Only in the *Cff* group was there a consistent pattern on elevated EVs after challenge, though, again, there was a great deal of within- and between-animal variability.

It also appears that the IgA ELISA test does not produce reliable results when used on frozen samples.

Apparently, the attempt to infect heifers with *Cfv* failed, since they did not produce a humoral immune response. Experimental infection of heifers with strains of *Cfv* has proven difficult in other instances in New Zealand, so this

Figure 3.1: Geometric mean ± SEM of ELISA values (EV) for heifers infected with (a) *C. fetus* subsp. *fetus* (*Cff*), (b) *C. fetus* subsp. *venerealis* (*Cfv*) and (c) Control. Interpretation of thresholds is: negative (EV<23), suspicious (EV 23 to 33), positive (EV>33). Value subscripts with different letters within each graph indicate significant differences (P≤0.05). In figure (c): fractioned values are also given for the number of positive animals over the total number of animals sampled.



result was not entirely unexpected

No isolation of *Campylobacter* was possible in any Group, with the exception of *Cff* isolated from Group *Cff* 24h post-challenge. This raised questions about the sensitivity of the sampling method or the isolation media used and about the infective capacities of the *Campylobacter* strains used.

3.3 Experiment 2: IgA Elisa and microbiological studies of heifers re-challenged with Cfv.

To better understand the results obtained in Experiment 1, a further study was designed to obtain information about:

1) The performance of the IgA Elisa test during experimental re-challenge of heifers with *Cfv*.
2) Evaluate survival of the inoculum during the first 24 h post-challenge.
3) Test the use of a different isolation medium (Lander's) with the same sampling method (vaginal swab) for microbiologic isolation.

3.3.1 Materials and methods.

Heifers from Group *Cfv* (n=6) from Experiment 1 were re-challenged twice (24 h apart) with the same strain of *Cfv* as before, 41 days after the previous experiment had finished. All Groups from Experiment 1 had been kept apart during the period between trials.

Group *Cfv* was re-challenged during oestrus after synchronisation with a double injection of $PGF_{2\alpha}$ (5 mg Lutalyse, Pharmacia Company Limited), 11 days apart. Animals were checked twice-daily 48 h after the second injection for signs of oestrus. All animals had shown oestrus within 24 h of challenge.

The sampling method used was the vaginal swab. Samples for microbiology were taken one week before re-challenge day (Day 0) and at 1, 2, 3, 6, 12 and 24 h after re-challenge. The media used were blood agar and Lander's medium.

Samples for IgA ELISA were taken on Day 0 (before challenge) and 7 days later. Samples for both microbiology and IgA ELISA test were taken at weekly intervals from Day 31 until Day 79 post-challenge. This period of time was given to allow infection to establish.

Starting on Day 149, three further samples were taken at weekly intervals, by vaginal wash, for microbiologic isolation.

Linear regression was used to compare Elisa Values (EV) of the IgA ELISA test within the group. Results obtained for the IgA ELISA test were transformed by the natural logarithm to achieve normality and apply generalized mixed model regression assuming a normal distribution. Variances were adjusted for the correlation between subsequent measurements on the same animal. The null-hypothesis of equal means between sampling days within group was evaluated with 95% confidence ($P \leq 0.05$). All analyses were carried out using SAS (2003) and Microsoft® Excel 2000

3.3.2 Results and preliminary discussion

Concentrations of viable *Cfv* cells used to re-challenge these heifers were 1.36×10^8 and 1.34×10^8 CFU/ml for the first and second challenge respectively.

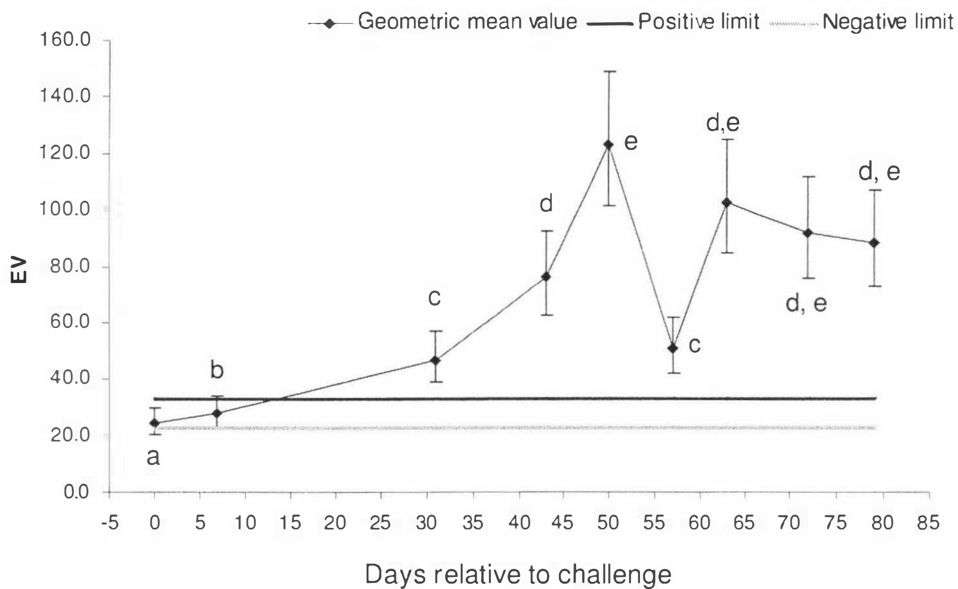
Microbiological isolation of the organism was achieved at 1, 2, 3, 6, and 12 h post-challenge from all heifers. It was isolated once more at 149 days after challenge, from a single animal.

A suspicious mean EV (EV=24.8) was obtained on Day 0 before re-challenge (Figure 3.2). This was in agreement with the last three results obtained from the same Group during Experiment 1. Significantly higher EVs were present from 7 days after challenge (2 positive, 3 suspicious and 1 negative animal respectively), although mean results (mean EV=28.0) were still in the suspicious range of values ($23 \leq EV \leq 33$). Geometric mean EVs became positive by Day 31 (EV=47.0) after challenge, were highest on Day 50 (EV= 122.6) and started to decrease gradually until Day 79 (EV=87.8) staying always above the positive cut-off. After the highest mean value on Day 50, a sudden significant drop occurred on Day 57 (EV=50.7) followed by a significant increase on Day 63 (EV=102.8).

The IgA values in vaginal mucus followed a trend (Figure 3.2) similar to that which has been observed in earlier studies (Corbeil et al., 1975a; Corbeil et al., 1975b; Wilkie et al., 1972; Winter and Caveney, 1978). The highly positive and significant values of IgA ELISA confirmed that the infection attempt was successful in triggering an immune response to an infection with *Cfv*.

From the isolation results it was concluded that the inoculum was viable until at least 12 h after challenge in all animals, that infection established at least in one animal and that, by changing the sampling method to vaginal wash, isolation was possible from that animal more than 4 months after challenge. Hence, the IgA ELISA test indicated an established infection of heifers at Group level with *Cfv*, which was confirmed by long-term isolation of the organism in one of the animals.

Figure 3.2: Geometric means ± SEM of Elisa Values (EV) for heifers re-inoculated with *C. fetus* subsp. *venerealis*. Interpretation thresholds are: negative (EV value < 23), suspicious (EV value 23 to 33), positive (EV value >33). Value subscripts with different letters indicate significant differences (P≤0.05).



Perhaps the most important finding of this study was the ability to isolate *Cfv* after sampling by vaginal wash on Day 149, compared to the inability to isolate it from vaginal swabs from a few hours after challenge. It therefore appears that, for microbiological isolation, the vaginal swab was not sufficiently sensitive to recover *Cfv* by culture. Hence, vaginal washings should be further evaluated.

3.4 Experiment 3: ELISA and microbiological studies of heifers exposed to Cfv after mating by experimentally challenged bulls

Given the results obtained from Experiments 1 and 2, a third study was designed to examine:

1) The performance of the IgA Elisa test from samples taken from virgin heifers after mating by experimentally challenged bulls with *Cfv*.
2) Evaluation of the vaginal wash as a sampling method, together with the use of blood agar or Lander's medium for microbiological isolation of *Cfv*.
3) Evaluation of the preputial wash and the preputial scraping as sampling methods from experimentally challenged bulls with *Cfv* together with the use of blood agar or Lander's medium for microbiologic isolation of the organism.

3.4.1 Materials and methods

3.4.1.1 Heifers

Five heifers (Control Group from Experiment 1) were used. These heifers had been separated from the other two groups of heifers and grazed on a sheep and beef farm until needed for this experiment. They were synchronised with 2 injections of $PGF_{2\alpha}$ (Lutalyse, Pharmacia Company Limited) 11 days apart. Forty-eight hours after the second $PGF_{2\alpha}$ injection, the heifers were introduced to two experimentally challenged bulls (3 and 2 heifers per bull, respectively). This separation was done because of the low proportion of females per male in this trial, to avoid behavioural problems. KaMar heatmount detectors (Livestock Improvement Corporation, Hamilton, New Zealand) were used to assist in detecting that mounting (i.e. mating) had occurred. Mating was observed for two days during daylight hours and the KaMar heatmount detectors were checked every morning for mounting during the night. Two days later, provided that mating had occurred (i.e. had been observed or the KaMar heatmount detector showed mounting), heifers were separated from the bulls.

Vaginal washes for microbiologic isolation were performed 22, 18 and 5 days before mating (Day 0) and 1, 3, 7, 14, 28, 49, 56 and 63 days post-mating.

Samples for IgA ELISA test were also taken but only during the first four

samplings (three prior to mating and one 24h post-mating) due to a discontinuation in the availability of the test. All heifers were pregnancy tested, using ultrasound 60 days after separation from bulls.

Linear regression was used to compare Elisa Values (EV) of the IgA ELISA test within the group. Results obtained for the IgA ELISA test were transformed by the natural logarithm to achieve normality and apply generalized mixed model regression assuming a normal distribution. Variances were adjusted for the correlation between subsequent measurements on the same animal. The null-hypothesis of equal means between sampling days within group was evaluated with 95% confidence ($P \leq 0.05$). All analyses were carried out using SAS (2003) and Microsoft® Excel 2000

3.4.1.2 Bulls

Two virgin bulls were challenged twice (24 h apart) with the same strain of *Cfv* as previously used in heifers. Table 3.2 shows the concentration of viable cells (CFU/ml) in the inocula used for each challenge.

Bulls were turned in with the heifers immediately after receiving their second challenge dose of *Cfv*. This took place 24 h after heifers had received their second dose of $PGF_{2\alpha}$.

The bulls were sampled for microbiological isolation of *Cfv* three times before challenge (Days –22, -14 and –7) and at 1, 3, 5, 7, 14, 28, 49, 56 and 63 days after separation from the females. The sampling methods used were preputial scrapings and preputial washes (see Chapter 2). Both methods were used at all times, except on Day 1 post-separation, where only the preputial scraping procedure was performed. The wash was not used on that day to obviate the possibility of removing the challenge inoculum from the prepuce.

Table 3.2: Concentration of viable *Cfv* cells per ml of the inoculi used for each bull at each challenge expressed in CFU/ml

Bull	Challenge	Inoculum (CFU/ml)
106	1	$1.00x10^8$
	2	$4.75x10^9$
60	1	$1.00x10^8$
	2	$4.75x10^9$

3.4.2 Results and preliminary discussion

3.4.2.1 Isolation, pregnancy rates and IgA ELISA results from females

After mating, a mild purulent exudation was evident as turbidity of the samples collected from heifers, although there was no gross evidence of a purulent vaginal discharge. Three of the five heifers showed several small blisters on the inner aspect of the vulva.

Campylobacter fetus subsp. *venerealis* was isolated from vaginal washes collected from all of the heifers at least once over the 56 days after removal of the infected bulls. The organism was isolated once from three animals (14 days post-mating for two heifers and 28 days post-mating for one heifer), twice from one heifer (3 and 49 days post-mating) and three times from another heifer (3, 28 and 56 days post-mating). These isolations were obtained from subculture plates (blood agar) after the use of Lander's medium. Isolation of *Cfv* from blood agar plates that were directly inoculated with the samples was unsuccessful, due to high level of contamination by other bacteria.

From the five heifers included in this experiment, three became pregnant (two mated to one bull and one mated to the other bull). All of the heifers that became pregnant had *Cfv* isolated only once.

Positive EVs were obtained 22 days before mating (Figure 3.3). This timing coincides with the return of heifers from grazing off in the sheep and beef farm. Those values significantly decreased until Day 1 after mating when they fell below the negative limit value.

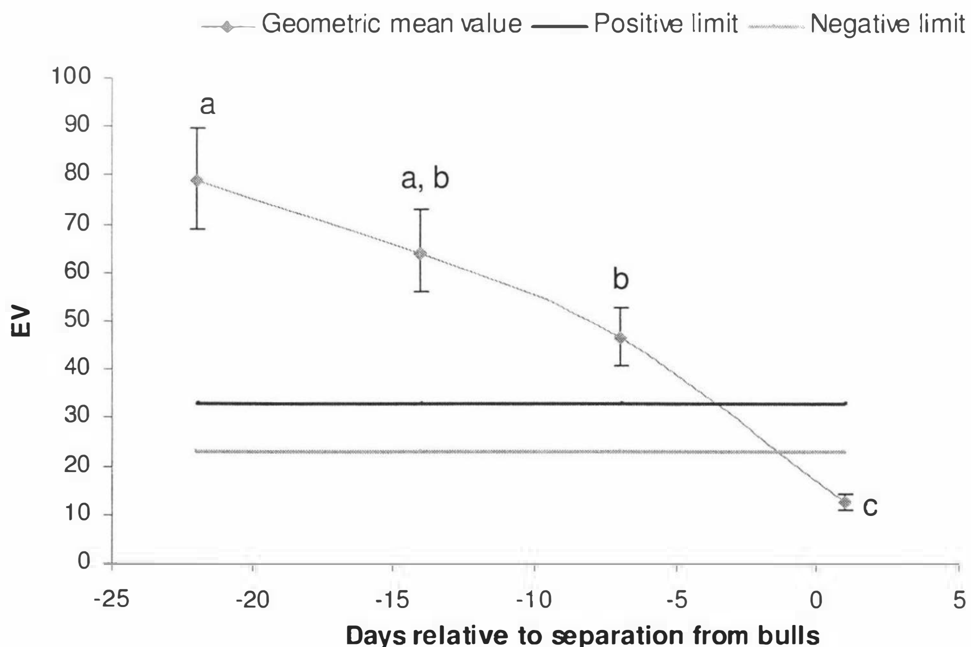
3.4.2.2 Isolation from bulls

Campylobacter fetus subsp. *venerealis* was isolated on Days 1 and 3, 7, 14, 28 and 49 after challenge, from at least one bull on each occasion.

There was no difference in the total number of positive isolations achieved from preputial washes or preputial scrapings (each 7 times in total), but on only two occasions could *Cfv* be isolated by both methods simultaneously. Sensitivities were therefore estimated as 36% for the preputial wash, 64% for the preputial scrape and 86% for both sampling methods used together.

Bacterial contamination was markedly reduced by the use of Lander's transport medium. This medium therefore proved to be very useful, not only for transport, but also for selection and promotion of growth of *Cfv*. Contamination was greatly reduced in the blood agar plates inoculated with an aliquot of this medium after three days of incubation. The blood agar plates directly inoculated with the samples were too contaminated for further examination. The organism was not isolated from bulls beyond 7 weeks after challenge.

Figure 3.3 Geometric means ± SEM of Elisa Values (EV) for heifers mated to experimentally infected bulls with *C. fetus* subsp. *venerealis.* Interpretation thresholds are: negative (EV < 23), suspicious (EV 23 to 33), positive (EV > 33). Value subscripts with different letters indicate significant differences (P≤0.05).



An important point to note from this experiment was that the IgA ELISA test produced positive results in heifers that had never been challenged with *Cfv*, but which had been grazing in a sheep and beef farm until 8 days before the first sampling (Figure 3.3). This again could be interpreted to mean that the IgA ELISA was detecting IgA that was secreted against organisms other than *Cfv*.

The vaginal wash produced positive microbiologic isolations of *Cfv* many times when used with Lander's medium. The vaginal wash in combination with Lander's medium appears to be a reasonably sensitive (20%) sampling method for the isolation of *Cfv*.

Both preputial scraping and preputial washes produced satisfactory rates of isolation of *Cfv*, although poor coincidence in the timing of those isolations. The combination of both methods increased sensitivity to 86% making it evident that this might be the best way to achieve high *Cfv* recovery rates.

Lander's medium was useful as a transport, enrichment and selection medium for the growth of this organism.

3.5 Discussion

3.5.1 IgA ELISA test performance

During these three experiments, there were several indicators that the IgA ELISA test may be cross-reacting with IgAs other than that secreted against *Cfv*. In Experiment 1, heifers were known virgin and had not been challenged with *Cfv* ever before. Nevertheless, group mean and individual animal EVs that, according to the rules of interpretation of the test were in the 'suspicious' range (23-33 units), were obtained before challenge in Groups *Cff* and Control. Furthermore, the high EVs obtained in Group *Cff* after challenge (i.e. with *Cff* rather than *Cfv*) are also indicative of cross-reactivity.

The issue of cross-reactivity of this test with immune responses elicited by infection with *Cff* has been discussed by Hum et al. (1994; 1991) and McFadden et al. (2004a). Hum et al. (1994; 1991) mentioned that *Cff* could be responsible for the false positive reactions that they obtained in known non-infected animals in their experiments, although they discarded its significance. Nonetheless, it might be expected, *a priori*, that this would be the case due to the genomic and phenotypic resemblance seen between the two subspecies of

C. fetus (Harvey and Greenwood, 1983; Roop et al., 1984). Moreover, it must be remembered that the problem of cross-reactivity between the two subspecies of *C. fetus* had already been encountered in the early serological methods of the vaginal mucus agglutination test (Clark, 1971) and the fluorescent antibody technique (Carrol and Hoerlein, 1972). McFadden et al. (2004a) further discussed this possibility in the context of the fact that *Cff* infection is an endemic problem in sheep flocks in New Zealand (Mannering et al., 2004). Taking into consideration the fact that combined production systems, in which sheep and beef cattle run together under extensive range conditions, are common in New Zealand, cross-reaction with sheep-derived *Cff* could explain the positive reactions seen in several unchallenged animals, especially as there was a significant response of the ELISA to *Cff* in the present experiments.

This hypothesis is supported by the observation that Control heifers which had suspicious EVs (i.e. 33>EVs>23) at the end of Experiment 1, had been grazing on a sheep and beef farm for four months, and displayed a significantly elevated group mean EV (EV=79) when brought back to be used for Experiment 3. As discussed by McFadden et al. (2004a), if the protocol followed by Hum et al.(1994; 1991) to obtain their positive and negative cut-off EVs and their positive and negative control values were to be repeated under New Zealand conditions, the values obtained might well be different in the context of a combined seehp/beef system of production (i.e. with an increased likelihood of *Cff* contamination than occurs in Australian systems). This conjecture is discussed in Chapter 5.

During Experiment 2, although infection could only be diagnosed in one animal (isolation at 149 days post-challenge), the pattern of changes in mean EVs (Figure 3.2) closely resembles that of IgA secreted against *Cfv* in vaginal mucus that has been described in earlier reports. Based on those reports, it was expected that IgA would be detectable from 3 to 5 weeks after infection and would remain at high concentrations until up to 40 weeks later (Corbeil et al., 1975a; Corbeil et al., 1975b; Hum et al., 1991; Wilkie et al., 1972; Winter and Caveney, 1978). This pattern, although clearly not present in any of the groups of Experiment 1 (Figure 3.1), was evident in Experiment 2 (Figure 3.2). Andrews et al. (1974) pointed out that even though the IgA could be present for long periods

of time, *Cfv* itself was seldom present in the vagina of cows for more than 6 months after initial infection. Nonetheless, the organism was clearly present on Day 149 in one animal, even though it had probably been cleared from the vaginas of the other heifers.

On the other hand, fluctuations of EV were observed during serial sampling of the same animals over many weeks in all three experiments (Figures 3.1, 3.2 and 3.3). In Experiment 1, ICCs were 0.29, 0.03 and 0.06 from Group *Cff*, *Cfv* and Control respectively. In other words, 71%, 97% and 94% of the variation per cow observed in Group *Cff*, *Cfv* and Control respectively, was due to test variability. This means that a substantial proportion of within-cow variation cannot be explained by the patterns of IgA secretion by that animal, but by the test and the way it behaves on the day it is run. Re-examination of the EVs obtained from the Control Group in Experiment 1 highlights the significance of this point. If these samples were to have been collected from a commercial herd and the veterinarian had sampled the animals on Day 5 or Day 19 (Figure 3.1c), then the herd would have been diagnosed as negative to BVC, because only one heifer was positive. If the same animals had been sampled on Day 12, then the interpretation would have been completely different, because on that particular day 3 of the 6 heifers gave positive results, and the herd would have been classified as positive to BVC. Consequently, results are very difficult to evaluate and appear to be of very limited diagnostic value.

Surprisingly, such fluctuations were recorded by Hum et al. (1994), but no great significance was attributed to them. To illustrate this point, Figure 3.4 shows the values reported by Hum et al. (1994) for five cows mated to a known-infected bull and sampled for IgA ELISA five times at six-week intervals.

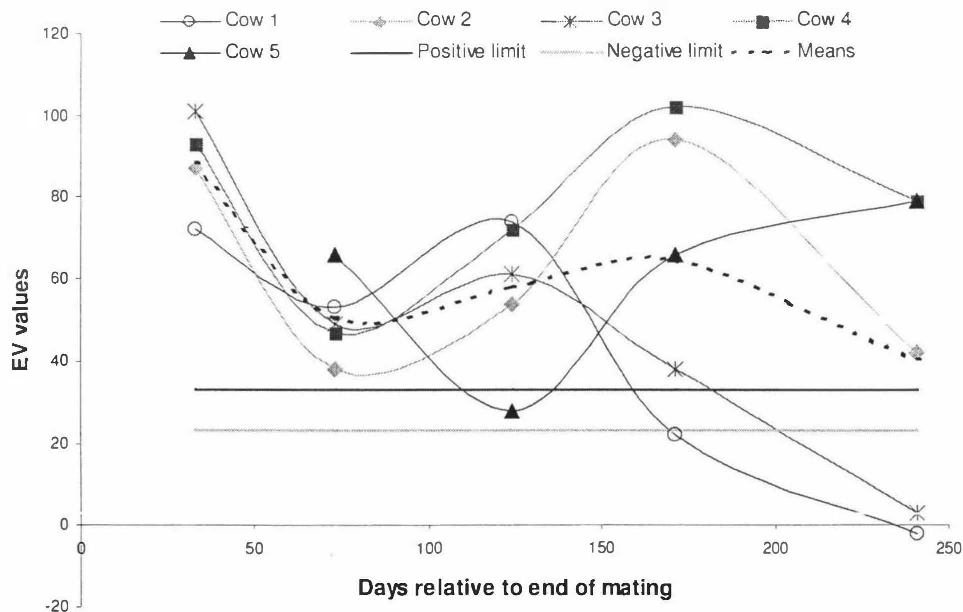
Hum et al. (1994) proposed that three factors might be contributing to such fluctuations. The first of these is the change in the expression of surface antigens of *Cfv*. *Campylobacter fetus* subsp. *venerealis* masks and un-masks surface antigens in order to survive the immune responses generated by the host (Garcia et al., 1995). According to Hum et al. (1994), when the bacterium masks a certain antigen against which IgA has been secreted, a sudden drop in EV occurs. This is followed by a subsequent increase in EV, due to the response of the immune system to the new antigen that is unmasked by the bacterium,

resulting in a new specific IgA. Because of these variations in response, Hum et al. (1994; 1991) considered that the test might be most applicable at the herd rather than animal level. However, the data of McFadden et al. (2004a) and the findings in the present experiments suggest that interpreting the results of the test even at a herd level may be highly inaccurate.

Secondly, Hum et al. (1994) proposed that there might be changes in the volume of mucus secreted into the vagina. This could dilute (during oestrus) or concentrate (during dioestrus) the IgA secreted, generating the fluctuations in titre. This phenomenon also influences microbiological isolation rates (Clark, 1971).

Thirdly, Hum et al. (1994) proposed that the fluctuations could be due to a change in the total amount of antigen coated to the ELISA plate. This is an inherent problem seen with ELISA tests, although properly standardized preparation methods should be able to reduce the effects of this issue on the final results (Crowther, 1995).

Figure 3. 4: Animal and mean EVs obtained from serial sampling five cows after mating with a known infected bull with Cfv (adapted from Hum et al. (1994)



Taken together, therefore, the results reported by Hum et al. (1994), as well as those obtained during the present experiments, indicate a poor

repeatability of the test, which furher suggests that its results are of limited diagnostic value.

3.5.2 Microbiologic isolation methods

Campylobacter fetus subsp. *venerealis* is a difficult organism to culture. From the days of the earliest research on the organism this has been the case (Clark, 1971; Clark and Dufty, 1978; Clark and Monsbourgh, 1979; Lander, 1990b; Roberts, 1979). Despite this, given the low accuracy of serological tests available, it remains the only method available to achieve an unequivocally positive diagnosis of BVC.

There were doubts about the ability of the strain of *Cfv* used to challenge the animals during these experimentsto establish infection, because *Cfv* was never isolated from heifers in Group *Cfv* in Experiment 1. The strain used during these experiments was provided by ESR (Wellington) and had been preserved freeze-dried. Early work by Chin-Fatt (1982) in New Zealand indicated that it was difficult to establish infection in experimentally infected heifers using an Australian lyophilized strain. In his case, infection was successfully achieved in only 2 out of 7 challenged heifers, although it was easier to infect bulls (n=2; infection was established in both). This agrees with the observations made during the present studies, namely that: i) *Cfv* infection was not established in Experiment 1, and the lack of microbiological isolation means that it is unclear how many animals in Experiment 2 developed established infections, ii) isolation from males was possible from Day 1 in Experiment 3.

Reasons for the difficulty in establishing artificial infection with laboratory isolates of *Cfv* are several. Firstly, differences in the virulence of different strains of *Cfv* have been reported (Newsam, 1965a; Newsam and Peterson, 1964). It has also been suggested that strains vary in their ability to cause infertility in individual heifers (Clark, 1971; Te Punga and Boyes, 1958). The reason for this remains unclear. It does seem probable, however, that the laboratory handling could have caused the loss of the organism's protein S-layer, which may have affected its ability to survive in the cow (Blaser and Pei, 1993; Sara and Sleytr, 2000). It is also conceivable that the laboratory handling produced a decrease in the virulence without losing the S-layer. Laboratory

passages are known to decrease virulence of strains, since this is a methodology that is widely used when producing vaccines (Tizard, 1995). It may be that laboratory-maintained strains need a passage through their natural host to regain virulence. This would at least explain why the heifers in Experiment 3 became infected with *Cfv* that had been passed through the bulls' prepuces first. It could even be suggested that, for no foreseeable reason, a strain is able to overcome the immune response of the host given that the establishment of infection of *Cfv* is highly dependant on the balance between the immune response of the host, and the ability of the organism to overcome that immune response (Schurig et al., 1975). Be that as it may, the reason why it is so hard to establish artificial infections in females remains unknown and, given the highly infectious nature of the organism in natural outbreaks, is paradoxical.

On the other hand, the difficulties of recovering the organism from challenged heifer must be attributed in part to the method of sampling. In Experiment 1 and for most of Experiment 2, samples were collected by vaginal swab; a method whose effectiveness was later questioned. Sampling females using the vaginal swab was initially decided upon, given that it is one of the sampling methods recommended by OIE (2004) and because it was used by Tattersfield (2002). Yet this method of sampling females appeared to be ineffective for obtaining positive isolations of *Cff* and *Cfv* (Experiments 1 and 2, first part). Only 6 isolations of *Cff* and none of *Cfv* were obtained from vaginal swabs, from a total of 150 samples taken in Experiment 1, and no isolations of *Cfv* were obtained after 24 h post-challenge in Experiment 2 (40 samples total taken from 24 h post-challenge until 77 days after). A switch to the use of the vaginal wash using 10 ml of sterile PBS (a modification of the method of vaginal lavage proposed by Hum et al., (1993) was therefore made in the second part of Experiment 2 and during Experiment 3), and thereafter isolation rates dramatically improved. In Experiment 3, 15% of the 40 samples taken from the 5 infected heifers produced positive isolations of *Cfv* within 63 days of challenge.

Perhaps the volume of vaginal mucous that is harvested by various sampling methods is of importance in this context. Chin-Fatt (1982) used the aspiration of 12 ml of vaginal mucus into a pipette as sampling method in his

experiments. This method had been used by other researchers with positive results (Clark et al., 1969; Dufty and McEntee, 1969; Schurig et al., 1974). Chin-Fatt (1982) was able to isolate *Cfv* from 72.4% of the 66 samples taken from the two infected heifers until Day 162 after challenge. In the light of these data it seems that, whatever sampling method is used, the most important thing is to achieve the harvest of sufficient volume of vaginal mucus in the sample. In other words, the volume of mucus that a swab can harvest is definitely limited with comparison to 12 ml of vaginal mucus in plastic pipettes (Chin-Fatt, 1982) or 10 ml of mucus plus PBS in the present experiments. Thus, the vaginal wash appears to overcome three issues seen with the vaginal swab. First, there will always be mucus present in the sample and the volume of the sample will not change according to the stage of the oestrous cycle (Clark et al., 1969). Second, the fact that there is a greater volume of sample (more mucus being harvested) is likely to improve the chances of isolating the organism. Third, the mucous will be more liquefied making it easier to take an aliquot of the sample for the inoculation of media. Even if less practical than the vaginal swab, the improvements seen in the isolation rates of these trials should be taken into account if isolation of *Cfv* from cows is an aim.

In the present experiments, therefore, the combination of vaginal swabbing with the use of Skirrow agar was not as effective as the combination of the vaginal washing with the use of Lander's medium. Although larger-scale trials are required to fully evaluate the sensitivity and specificity of the two methods, this observation is important since the usual specimen collected from cows in New Zealand is the vaginal swab.

Preputial washes and preputial scrapings used with Lander's medium produced a total of 7 isolations each. Given the low number of samples included in this work (48 samples total), and the lack of total independence between samplings (because the same two bulls were used and there was a time variable and an age of the bull variable influencing the results), there is a lack of power in these measurements. However, for the sake of completeness, calculations of sensitivity and specificity were done assuming total independence, to see if a trend could be identified that could be used as a basis for further studies. The preputial wash and the preputial scraping were 36% and 64% sensitive for the

isolation of *Cfv*. Both sampling methods used together achieved an 86% of sensitivity. Also, the sensitivity of a single sampling using both methods was 83%. This percentage was increased to 100% with a double sampling using both methods at weekly intervals. Acknowledging the limitations of thee calculations, there are certain inferences that can be drawn: i) using both sampling methods during the same sampling could improve isolation rates, ii) sampling at least twice (7 days apart) using both methods should increase isolation rates. This last observation is supported by the results of early research on BVC (Clark, 1971; Dufty, 1967; Ruckerbauer et al., 1974).

All the isolations from both preputial washes and scrapes from bulls were achieved after the use of Lander's medium. The plates of blood agar directly inoculated with an aliquot of the sample were of no value, due to the heavy growth of contaminants. Lander's medium has been reported to be a useful medium to eliminate contaminants and promote growth of *Cfv* (Lander, 1990a, b). This is supported by the results obtained during Experiment 3.

3.6 Conclusions

From the results of these experiments, it can be concluded that:

1. The disparity between reports on the performance of the IgA ELISA test in New Zealand and in Australia can probably be explained by the cross-reactivity of this test in animals challenged with bacteria other than *Cfv*. The presence of both sheep and beef cattle on farm increases the chances of cattle being challenged with *Cff*, which was seen to apparently produce high EVs for IgA ELISA test (Experiment 1). More work should be done to clarify exactly what the likely factors for the lack of specificity were before recommending the use of this test in New Zealand.
2. The vaginal wash should be the method of choice for microbiological isolation of *Cfv* from females.
3. A combination of preputial washing and preputial scraping is useful for the isolation of *Cfv* from bulls.

Lander's medium should be used as standard procedure for the isolation of *Cfv* from vaginal or preputial wash. It has been seen to reduce contamination and promote growth of the organism from samples from females and males.

Chapter 4: Preliminary research on PCR for the diagnosis of Bovine Venereal Campylobacteriosis

4.1 Introduction, fundaments and history of PCR with relation to C. fetus *species.*

4.1.1 Introduction

Polymerase Chain Reaction (PCR) was first developed in the 1970s and standardized in the 1990s. It mimics the natural process of replication of DNA, limited to the specific region of the genome under study. The technique is widely used in microbiology as well as in many other areas of research.

Correct identification of *Campylobacter* species has been problematic until molecular biology techniques started to be used. Recently however, promising results have been reported for PCR (Blom et al., 1995; Hewson et al., 1985; Hum et al., 1997; Oyarzabal et al., 1997; Pannachio et al., 1993; Wesley et al., 1991). In particular correct identification of *Campylobacter fetus* subsp. *venerealis* (*Cfv*) from the closely related subspecies *Campylobacter fetus* subsp. *fetus* (*Cff*) (Berg et al., 1971; Harvey and Greenwood, 1983; Roop et al., 1984) can be relieved using this technique (Hum et al., 1997; Pannachio et al., 1993).

4.1.2 Fundaments of PCR

In this section, an overview of the fundaments of PCR and the history of the technique in relation to the identification and diagnosis of infections with *Campylobacter* species will be developed.

Polymerase chain reaction limits DNA replication to a specific area of the genome by the use of primers, which are a sequence of nucleotides complementary to a part of the DNA that is specific to the bacterium under investigation. Primers are artificially synthesized and used in pairs, one for each chain of DNA (Dale and von Schantz, 2002).

The necessary reagents for PCR are one (or more) set of primers, the targeted DNA (template), the DNA polymerase enzyme *Taq,* deoxinucleotides (dNTP's: adenine, thymine, guanine and cytosine). For each DNA sequence under investigation, conditions have to be optimised.

A mixture of these components is submitted to a series of cycles (temperatures and time), which will allow replication to take place. The first temperature of the cycle is 94°C (≤1 min) to denature the DNA. Then, the primers need to bind (anneal) to their specific sites. To do this, an optimal temperature has to be determined as too low temperatures produce annealing in the wrong position and too high temperatures will impair annealing. The optimal annealing temperature differs with each PCR reaction, according to the primers used. Finding the optimal annealing temperature for each specific PCR reaction by an iterative process. The normal range of temperatures for annealing is between 40°C and 60°C and the process is rapid (≤ 1 min).

The process of extension follows. For this, the optimal temperature for *Taq* polymerase action is set at 72°C and the enzyme will produce the complementary DNA strands, starting from the position of the primers, at a rate of 1000 nucleotides per minute on a 3'-5' direction. Extension will be interrupted when the temperature is raised again to start a new cycle (Dale and von Schantz, 2002). At each new cycle, many new double stranded molecules of the targeted DNA are generated that have the primers at their start and end points. The aim of the technique is to repeat this cycle as many times as needed to obtain sufficient replications of the targeted DNA (amplified products) to be able to be visualized after separation by agarose gel electrophoresis. The amount of cycles is specific for each PCR reaction as well as the specific length of each cycle.

Because this technique works by identification of a DNA fragment it is very specific to the presence of the targeted DNA, the more specific this target DNA is to the bacterium under evaluation, the more specific the PCR results will be. For example, a specific gene called 16S-rDNA (or 16S-rRNA) that encodes for ribosomal proteins and was found to be ubiquitous, is widely used for primers development. Some of the reasons for this are that it has a conserved function, it is easy to sequence, and a large database (Ribosomal Database Project) has been made available for sequence alignment and identification. It has highly conserved regions, which are common to all bacteria but it also presents hypervariable regions, whose sequence is specific to each species and subspecies. These hypervariable regions change from one strain of bacteria to another, thus the development of primers that specifically target the hypervariable regions of

this gene has proven to be a successful approach to species identification.

4.1.3 PCR for identification of *C. fetus* subsp. *venerealis* strains

In 1993, a field evaluation of a PCR- based test for the diagnosis of bovine campylobacteriosis was published in Australia (Pannachio et al., 1993). The aim of this PCR was to develop primers that would distinguish *C. fetus* from other *Campylobacter* species and another set of primers that would distinguish between *C. fetus* subspecies. Many different sets of primers were evaluated. The test was used on vaginal mucus from cows and heifers. Results were disappointing apparently due to the content of inhibitory substances in the mucus. The use of the PCR on preputial washes required pre-treatment of the samples with phenol/chloroform to remove proteins and contaminants, followed by ethanol precipitation of DNA. The pre-treatment was necessary to clear the sample from other bacteria and chemical compounds, which could inhibit the PCR reaction. The DNA precipitation was necessary to concentrate the DNA present in the treated sample, since it is known that normally, samples carry only a few *Cfv* cells.

Based on this work, Hum et al. (1997) developed a PCR technique for differentiation of isolates previously classified as *Cfv* and *Cff*. This technique uses the primer pairs MG3F, MG4R and VenSF, VenSR (See Chapter 2). The first pair of primers identifies *C. fetus* species, delivering a band of 960 base pairs (bp) and the second pair of primers distinguishes *Cfv* subspecies, delivering a band of 142 bp. A total of 99 bacterial strains classified as either *Cff* or *Cfv* were analysed, and there was an 80.8% agreement between the PCR and biochemical characterisation of the subspecies. The authors attributed the disagreements to a lack of standardized biochemical tests.

This was the first PCR technique that was apparently able to differentiate between *C. fetus* and the rest of the *Campylobacter* species as well as between *Cfv* and *Cff* subspecies. It, therefore, represented a huge step forward. Subsequent studies with these primers have confirmed their value. Newel et al. (2000) and Vargas et al. (2003) evaluated the same PCR technique and obtained the same bands as those reported by Hum et al. (1997). They concluded that although these primers needed to be tested against more species of

Campylobacter for cross-reactivity, the results were encouraging. However, when Wagenaar et al. (2001) validated this PCR with the use of AFLP and biochemical characterisation, the species band obtained with PCR was 750 bp as opposed to the 960 bp previously obtained (Hum et al., 1997; Newell et al., 2000; Vargas et al., 2003). The subspecies band remained the same. It was also reported that 7 out of 54 strains tested by this method did not agree with the results of the biochemical characterisation, although AFLP agreed 100% with the PCR results. Some time later, Muller et al. (2003) agreed with the observations made by Wagenaar et al. (2001) when they also obtained a species band which was 750 bp long.

It is also interesting to note that sometimes only the small band (142 bp) is amplified using this PCR technique. However, a re-run of the same sample, using the same technique, subsequently delivered both bands (D'Anatro, 2004).

A point of note is that the content of *Cfv* cells in a preputial wash tends to be highly diluted because of the large volume used to get the sample. In the report of Pannachio et al. (1993), only 1 ml of the sample was used, being centrifuged at 10,000g for 5 min prior to purification of the DNA for PCR. Although results were encouraging, it must be taken into account that the level of infection was 9,000 to 400,000 bacteria/ml of preputial wash. This would seem to be a high figure since the average concentration of *Cfv* cells in preputial washes collected in the field is between 100 and 20,000 bacteria/ml (Clark, 1971).

These studies demonstrate the possibility of using this PCR technique as a diagnostic test to be applied to clinical samples (preputial washes). It is the aim of following sections to present the results of two preliminary studies on the use of this PCR technique for the diagnosis of bovine venereal campylobacteriosis.

4.2 Experiment 4

4.2.1 Aim

To evaluate the sensitivity of the PCR technique developed by Pannachio et al. (1993) and Hum et al. (1997) by experimentally adding different concentration of *Cfv* cells to preputial washings from bulls, using double

filtration and centrifugation to concentrate those *Cfv* cells.

4.2.2 Materials and methods

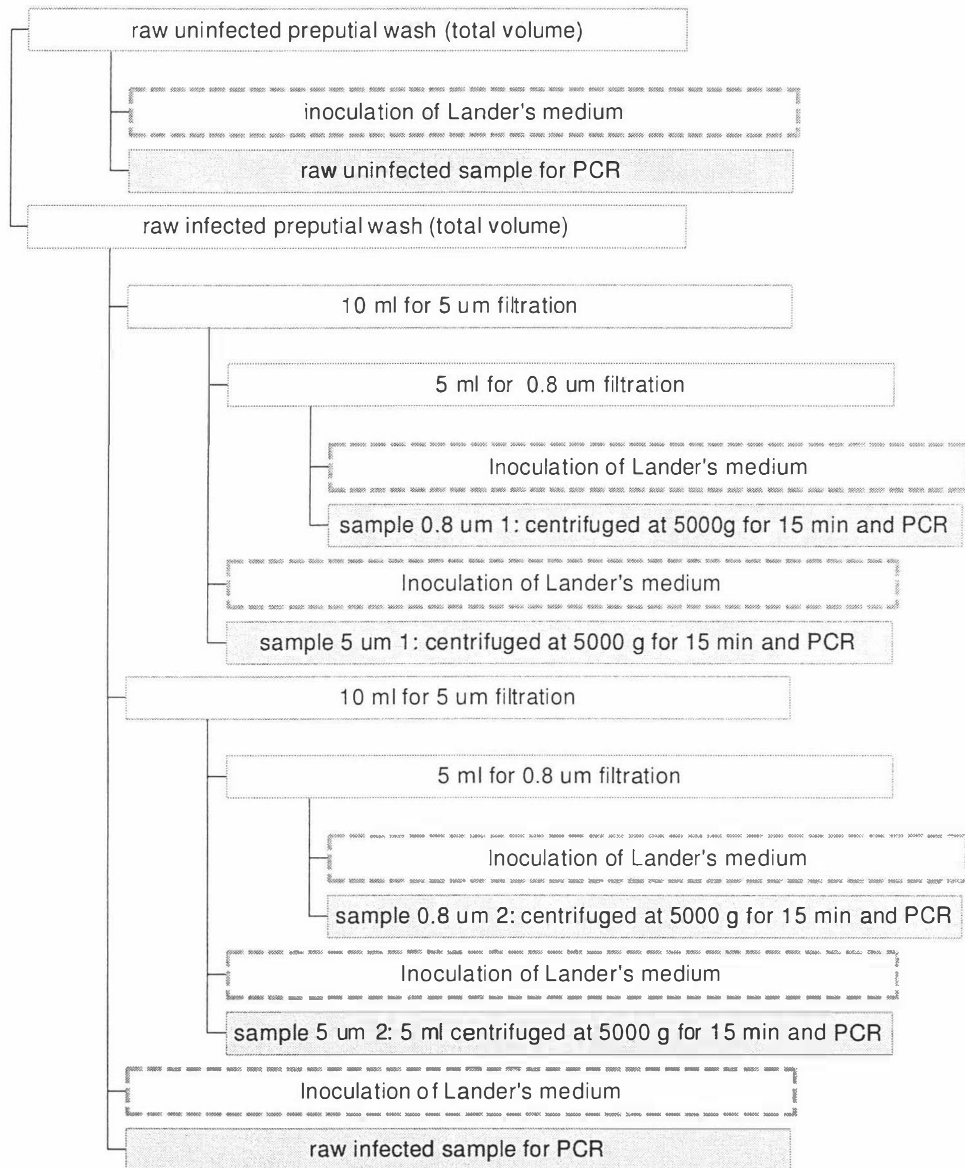
Four preputial washes (30-50 ml, Chapter 2) were obtained from four different bulls. The bulls were virgin, maintained at LATU (Large Animal Teaching Unit, Massey University, Palmerston North) separated from any other stock (n=2) or at Livestock Improvement Corporation Ltd (LIC, Awahuri farm), which ensured they were BVC-free. The level of contamination of the preputial washes differed between sources. The preputial washes from LIC were clear, clean and contained little observable contamination. When the samples came from the bulls at LATU, the samples were cloudy, containing dirt, hairs, grass and other contaminants.

An initial inoculum (Chapter 2) was made by dilution of *Cfv* cells into PBS to a concentration of McFarland Scale 6 (Dilution 1). From this, three consecutive 10x dilutions were made (Dilutions 2 to 4). An aliquot of Dilution 1 was used for assessing the number of CFU/ml of inoculum (see Chapter 2, section 6). From this, the CFU/ml of each of the four 10x dilutions was calculated.

Each preputial wash was experimentally infected with 1 ml of one of the dilutions and the total volume was then divided into two equal aliquots to achieve more repetitions (Figure 4.1). The final concentration of *Cfv* cells/ml for each preputial wash was obtained by dividing the CFU/ml of the dilution used between the total volume of the wash in which it was used.

The treatment of each experimentally infected preputial wash was as follows (Figure 4.1): an aliquot of 10 ml from each sample was taken using a sterile syringe and filtered through a 5 μm pore syringe filter (Sartorius, Minisart, Germany). From the resulting filtrate, an aliquot of 5 ml was taken using a sterile syringe and filtered through an 0.8 μm pore syringe filter (Advantec MFS Inc., USA). Samples were then centrifuged at 5000 g for 15 min in 1.5 ml aliquots, discarding the supernatant, until the total volume was used. The final pellet was reconstituted in the smallest volume possible to maximize the concentration of *Cfv* cells.

Figure 4. 1: Treatment of each preputial wash. White boxes show treatment steps, grey boxes show the final samples submitted for PCR and white dotted boxes show Lander's medium inoculations (um=µm).



A total of 24 samples were submitted for PCR, the 16 treated samples, 4 raw (unprocessed) infected preputial washes and 4 uninfected aliquots (1 from each initial preputial wash).

The pore size that had been referred to in the literature (0.6 µm to 0.65 µm) was very hard to find and importation would have been troublesome. Hence, the choice of pore sizes was based on availability and on a pilot trial that showed a 20% decrease in the number of viable *Cfv* cells after filtration with 0.8 µm pore size filters. The use of a 5 µm pore size filter was chosen to clean the sample from cellular debris and dirt particles without decreasing the number of *Cfv* cells in the sample. The use of the 0.8 µm pore size filter would improve clearing of the sample although decreasing the number of available *Cfv* cells.

Campylobacter fetus subsp. *venerealis* isolation and biochemical characterisation were used for validation of the PCR. Lander's medium broth was used as transport enrichment medium given the good results obtained in previous studies (Chapter 3). The medium was inoculated with 1 ml of the same material that was being submitted to PCR (Figure 4.1) before centrifugation.

This whole procedure was repeated 7 times, so that a total of 14 repetitions of the methodology were achieved.

For statistical purposes, three treatments were defined: i) raw (untreated) infected material, ii) 5 µm filtrates and iii) 0.8 µm filtrates. Within each inoculum dilution, statistical differences between the three were subjected to Chi-square analysis using a 3x2 contingency table. All calculations were done using Microsoft® Excel 2000.

The PCR technique as done as explained in Chapter 2.

4.2.3 Results

The concentration of *Cfv* in the initial inoculi (Dilution 1) ranged between $1.0x10^7$ and $2.5x10^8$. The final concentration in each preputial wash ranged between $6.0x10^2$ and $6.0x10^6$.

4.2.3.1 PCR

A total of 168 PCR reactions were run during this experiment.

An example of the amplified products (bands) obtained during this trial is

shown in Figure 4.2. Specifically, the figure shows the differentiation between *Cff* and *Cfv* obtained by the use of this PCR technique. The subspecies band obtained in *Cfv* samples lies between the 100 and 200 bp bands of the ladder. The species band obtained in all samples lies between the 650 bp and 850 bp bands of the ladder. The PCR technique identified as few as 880 bacteria/ml of preputial wash after filtration and concentration (Table 4.1).

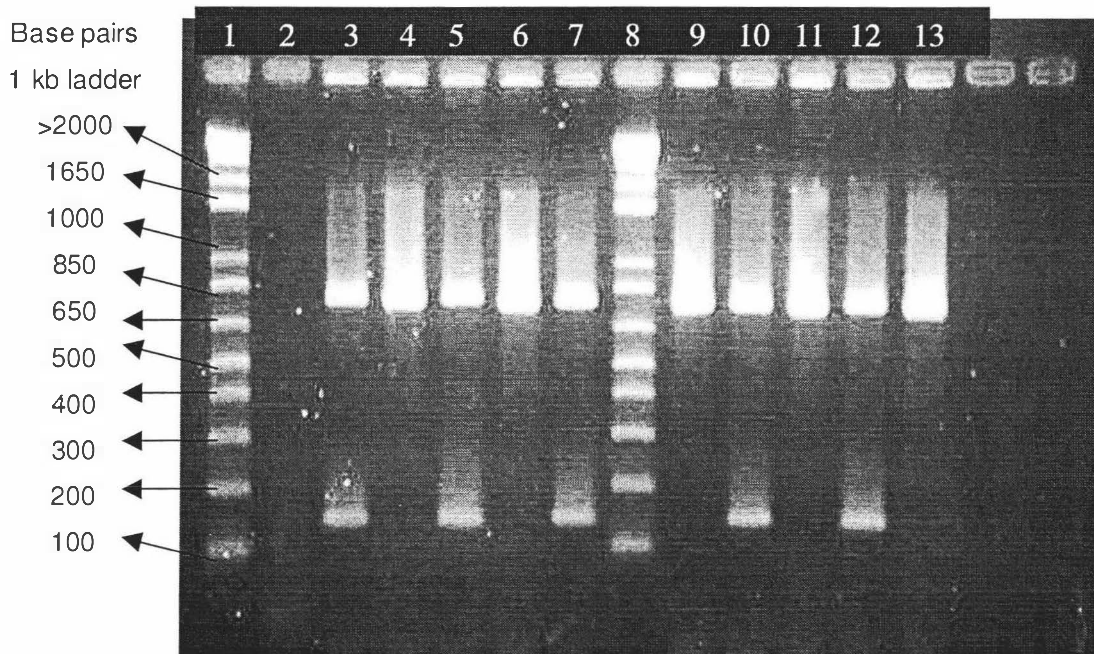
Table 4.1: Percentage of positive results from preputial washes experimentally infected with different concentrations of *Cfv* cells and submitted for PCR. Statistical differences ($P \leq 0.05$) are shown with different superscripts within each dilution.

Type of sample	Dilution 1 $6.0x10^6$-$3.03x10^5$	Dilution 2 $6.0x10^5$-$3.33x10^4$	Dilution 3 $5.6x10^4$-$1.3x10^3$	Dilution 4 $5.6x10^3$ -3.5 10^2
Raw uninfected	0	0	0	0
Raw infected	57.1^a	28.6^a	28.6^a	0^a
5 µm 1	100^b	71.4^b	100^b	57.1^b
5 µm 2	100^b	71.4^b	100^b	42.9^b
0.8 µm 1	100^b	85.7^b	85.4^b	42.9^b
0.8 µm 2	100^b	85.7^b	71.4^b	28.6^b

4.2.3.2 Culture

All samples of raw uninfected material inoculated into Lander's medium were negative to isolation of *Cfv*. All the samples of raw infected and treated material cultured into Lander's medium were positive to isolation of *Cfv*. Growth of organisms other than *Cfv* on the blood agar inoculated with an aliquot of Lander's medium after three day of incubation was almost absent.

Figure 4. 2: Photograph of the bands obtained after submitting to electrophoresis the products of the PCR reaction



Legend: Columns 3, 5, 7, 10 and 12: *Campylobacter fetus* subsp. *venerealis*
Columns 4, 6, 9, 11 and 13: *Campylobacter fetus* subsp. *fetus*
Columns 1 and 8: 1 kilo-base (kb) ladder
Column 2: negative control

4.2.4 Discussion

The size of the bands obtained agree with the sizes reported by Wagenaar et al. (2001) and Muller et al. (2003). Although the amplified product not sequenced, it was clear that the length of the species band was mid-way between the 650 bp and the 850 bp bands of the ladder. This leads to the conclusion that the amplified fragment length in this case is closer to the 750 bp reported by Wagenaar et al. (2001) and Muller et al. (2003) than to the 960 bp reported by Hum et al. (1997), Newell et al. (2000) and Vargas et al. (2003). A possible explanation for this disagreement could be different preparation conditions of the material submitted to PCR or even different conditions of primer preparations.

Even though it was not quantified, it was observed that the initial extraneous contamination of the preputial wash was important to the final results of the PCR. With highly contaminated preputial washes, PCR could achieve positive results only at higher concentrations of inoculum.

The sensitivity of this PCR technique needs to be further assessed. In this

experiment, the lowest concentration of bacteria in preputial wash that was positively identified by PCR was 880 cells/ml. It could be that further processing of the samples by protein precipitation and DNA purification as used by Pannachio et al (1993) could reduce this threshold further. Also, it may be by increasing the number of cycles of the PCR, lower concentrations of bacteria would become identifiable.

The significant differences observed in the percentages of positive results between treated and raw infected material show the importance of such manipulations (Table 4.1). Concentrating the *Cfv* cells in a preputial wash using the majority of the volume of the sample, improved chances of its recovery (Clark, 1971; Pannachio et al., 1993).

The differences in the percentage of positive results in 5 μm samples and 0.8 μm samples, although not significant, confirmed the observation of the pilot study of the decrease of available *Cfv* cells/ml by 20%. Further evaluation of the value of filtration through 0.8 μm filters is probably important.

The differences in the percentage of positive results obtained within the repetitions of the 5 μm treatment (type of sample 5 μm 1 and 5 μm 2) and the 0.8 μm treatment (Type of sample 0.8 μm 1 and 0.8 μm 2; Table 4.1) on the most diluted inoculi (Dilutions 3 and 4), although non-significant, are also not surprising, given the low concentration of *Cfv* cells present in these last two dilutions. Hence, running repeated samples could be an appropriate measure when a low concentration of the organisms is suspected in the sample. The observation of one drop of the raw material under a dark field microscope will give the technician an idea about the concentration of vibrio-like cells available in each sample.

The parallel microbiology results using Lander's medium showed, firstly that concentrations of C*fv* as low as 270 bacteria/ml of preputial wash can give a positive result by the use of this medium. All the preputial washes inoculated with even the most diluted inoculi were positive to microbiological isolation of *Cfv* through this method. Secondly, both the present and the original studies from Lander (1990a; Lander, 1990b) emphasize the value of this medium in eliminating contaminants that would overgrow *Cfv* from the samples.

4.2.5 Conclusions

In the light of the above evidence, it appears that PCR could be a useful technique for the diagnosis of *Cfv* infections in bulls. Further work needs to be done to improve sensitivity of the technique to achieve positive results at very low concentrations of bacteria in preputial samples.

The use of Lander's medium was a good tool for isolating *Cfv* from experimentally infected samples whilst eliminating most.

However, this work was done using artificially infected preputial washes from uninfected bulls. Nonetheless, the work of Pannachio et al (1993) suggested that this PCR could be used on samples from infected animals. Hence it would be of interest to evaluate the same PCR technique for identification of Cfv in samples obtained from infected bulls.

4.3 Experiment 5

In the light of the promising results obtained in Experiment 1, a second experiment was deigned to evaluate the use of PCR on preputial washes from experimentally challenged bulls.

4.3.1 Material and methods

Two 2-years old virgin bulls were bought in from a neighbouring farm and were kept at Massey University (Keebles farm, Massey University, Palmerston North), separated at all times from other cattle.
The bulls were challenged with a strain of *Cfv* and sampled performing preputial washes. The procedures for inoculum preparation, bull challenge and preputial wash sampling were as in Chapter 2.

Bulls were challenged once and sampled 24 and 48h post inoculation. Bulls were then challenged a second time and samples were then taken twice weekly from 3 days after second challenge, for one and half months, making sure that at least 3 or 4 days elapsed between samplings.

Samples (raw material) were taken to the microbiology laboratory and processed as follows: an aliquot of 22 ml from each sample was taken using a sterile syringe and filtered through a 5 μm pore (Sartorius, Minisart, Vivascience, Germany) syringe filter (Sample 5 μm). From the resulting filtrates an aliquot of

11 ml was taken using a sterile syringe and filtered through and 0.8 µm pore (Advantec MFS Inc., USA) syringe filter (Sample 0.8 µm). When the three samples were ready (raw material, 5 µm and 0.8 µm), an aliquot of 1 ml was taken from each and inoculated into Lander's medium (Chapter 2). Samples 5 µm and 0.8 µm were then centrifuged at 5000 g for 15 min in 1.5 ml aliquots. The supernatant was discarded and the final pellet was reconstituted in the minimum volume possible to ensure concentration of any of *Cfv* cell in the samples. The three samples were then submitted to PCR

4.3.2 Results

A total of 84 PCR reactions were run during this experiment, 42 from each bull and 14 from each type of sample (raw material, 5 µm and 0.8 µm).

Positive results were obtained from samples taken from one of the bulls at 24 and 48h after first challenge. All three types of samples were positive 24h post inoculation and only sample 5 µm was positive 48h after inoculation. Campylobacter fetus subsp. venerealis was isolated from this bull by microbiological culture 24 h post-challenge.

4.3.3 Discussion

Preputial washes and isolation of *Cfv* from preputial samples using Lander's medium have been seen to be effective when infection is established in the bull (Chapter 3, Lander (1990a). In this experiment, microbiological isolation of *Cfv* was possible 24h post first challenge from one bull was mirrored by positive PCR results during the 48h after first challenge from the same bull.

4.4 General discussion and conclusions

According to the work presented in this Chapter, the PCR technique developed by Pannachio et al. (1993) and Hum et al. (1997) delivered positive results when used directly on preputial washes taken from bulls and infected *in vitro* with as few as 880 *Cfv* cells/ml of preputial wash. Treatment of samples, through filtration and centrifugation, significantly increase the percentage of positive results showing the importance of such treatment before submitting the sample for PCR.

Polymerase chain reaction also delivered positive results from samples obtained from experimentally challenged animals, even when a culture of the organism was not possible from the same sample.

One of the strengths of molecular biology techniques is that they are based on the use of DNA. Having developed primers capable of differentiating *Cfv* from other *Campylobacter* important progress has already been made. In a disease such as bovine venereal campylobacteriosis, where the number of viable cells harvested during sampling in males cannot be predicted and where proper identification of the organism is so difficult due to its similarities with *Cff*, being able to rely on this PCR technique could be a real advantage for future diagnostic purposes.

Lander's medium was able to produce positive isolations from preputial washes with as few as 270 *Cfv* cells, proving to be effective even under large dilutions of the organism.

Hence there are two powerful tools to be used when trying to identify the presence of *Cfv* in a sample: isolation using Lander's medium and the PCR. If combined wisely with strategic sampling of bulls in herds, including herds with reproductive problems, the use of this technique could result in a prompt and safe diagnosis of BVC in New Zealand.

Nonetheless, these results were encouraging and further research should be done on the best manipulation methods of samples from males to be used alongside this PCR technique for its direct use on clinical samples.

Chapter 5: General Discussion and Conclusions

It is of great importance for dairy (Holmes et al., 2002) and beef (Milligan et al., 1987; Nicol and Nicoll, 1987) farmers alike in New Zealand to maintain good herd reproductive performance, so that calving patterns are aligned with pasture production.

In the dairy industry, the introduction of AI improved reproductive health by eliminating infectious diseases such as bovine genital campylobacteriosis (BVC). Nonetheless, even in the dairy herd, natural service sires remain of some significance since, during the 2003-2004 season, around 25% of animals conceived to natural service (Anonymous, 2005). The magnitude of this figure emphasises the importance of ensuring proper reproductive health of the bulls.

The beef cattle industry in New Zealand is mainly composed of two types of farms: a high proportion of farms with less than 100 cows, and a small proportion of large farms. The latter are mainly located in hill countries and are run under extensive conditions (Anonymous, 1999) where mustering of animals is done only at strategic times of the year (pregnancy testing, drenching, weaning, etc). Most cows in these herds are bred by natural mating. That natural mating management is largely effective is demonstrated by the nationwide study of (McFadden et al., 2004b), which showed that the median in-calf rate for New Zealand's beef herds is 91%. Given the reliance of these systems on natural mating, the reproductive health of bulls has the potential to be a significant limitor upon achieving high reproductive performance in such herds. This is especially so when considering the brevity of most mating periods (between 67 and 87 days; McFadden et al., (2004b), the relatively low bull:cow ratios (1:28, 1:29 and 1:37 for 2-year-old, 3 year-old heifers and mixed-age cows, respectively; McFadden et al., (2004b), and the fact that only 2% of the farmers leave the bulls with the cows after mating finishes (McFadden et al., 2004b)

Bovine venereal campylobacteriosis, caused by *Campylobacter fetus* subsp. *venerealis (Cfv)* has been suspected as the cause of low fertility in several beef herds in the Taihape area (Hughes, 2001) and in a dairy herd in the Gisborne area (Tattersfield, 2002) of New Zealand. However, despite the strong circumstantial evidence that suggests the presence of the disease, attempts made

to microbiologically isolate *Cfv* in these herds have so far been unsuccessful. This has raised concern about the sensitivity of the microbiological isolation methods available in New Zealand.

On the other hand, when an IgA ELISA test that had been developed in Australia (1994; Hum et al., 1991) was used to investigate those problem herds, the results appeared to show that the disease was endemic in them. Subsequently however, further study on the presence of *Cfv* in beef herds in New Zealand, comparing the results of the IgA ELISA test with the fertility of the herds tested, suggested that the performance of the test was unsatisfactory under New Zealand conditions, and that the disease was absent rather than present (McFadden et al., 2004a).

In this context, Morris (1998) has advised caution when applying recommendations generated from Australian beef production systems directly to herds in New Zealand, due to differences between production systems, stocking rates climate, terrain and others. A key difference between the two countries is the running of sheep and beef cattle together (i.e. on the same farm) in New Zealand (McFadden et al., 2004a), which is not generally the case in Australia. Sheep flocks in New Zealand are endemically affected by *C. fetus* subsp. *fetus (Cff)* (Mannering et al., 2004), and the contamination of cattle with this organism has been considered a possible risk factor for false positive IgA ELISA results in cattle (Hum et al., 1994; Hum et al., 1991; McFadden et al., 2004a). The results shown in Experiment 1, in which heifers experimentally challenged with *Cff* delivered positive IgA ELISA results, supports this view. Moreover, several individual animals in that Experiment showed ELISA values (EVs) that were elevated above the negative threshold, even before they had been challenged. Unchallenged animals in the same Experiment (Control group) have exhibited sporadic increases in the EVs above 'suspicious' or 'positive' thresholds. Yet none of these positive results can be explained with reference to the presence of *Cfv* infection. Conversely, all of the heifers used for these experiments had been purchased from a farm that raised both mixed sheep and beef cattle, so they had been grazing alongside sheep before acquisition. Furthermore, when unchallenged animals had been returned to the sheep and beef farm for spring grazing, by the time they were brought back to the experimental farm to be used

in further trial, their IgA ELISA titres had increased to EVs that were well within the positive range. It is therefore not inconceivable that the apparent false positive results in the present work were due to cattle being previously challenged with *Cff*.

McFadden et al (2004a) came to a generally similar conclusion, and suggested that Hum et al. (1994) overestimated the specificity of the IgA ELISA test. The reason for this assertion was that there was no indication of whether EVs of the negative control cows used by Hum et al. (1994) could be affected by a higher exposure to *Cff*, such as occurs in New Zealand. Thus, McFadden et al (2004a) used 206 samples from cows belonging to high-fertility beef herds, that sowed no sign of having BVC, to estimate the false-positive rate under New Zealand conditions. They considered that there were 28% of false positive results; a figure that was considerably higher than the 1.5% published by Hum et al. (1994).

It was therefore also notable that the ELISA results in Experiment 1 suffered from a very low repeatability. Intraclass correlation coefficients in Groups *Cff*, *Cfv* and Control were 0.29, 0.03 and 0.06 respectively, indicating that 71%, 97% and 94% of the variability observed in the results in each group was due to the test itself.

When these low intraclass correlations and the clear evidence (Experiment 1) of cross reactivity to *Cff* are considered together with the results of McFadden et al. (2004a), it appears that many of the problems of interpretation of the IgA ELISA might be explicable in terms of inappropriate cut-points between positive and negative values. To examine this conjecture, the data from the present experiments were used to re-estimate the cut-points for animals managed under New Zealand conditions between positive and negative EVs, this time, including animals that had been challenged with *Cff*.

Taking into account the limitations of using these data for such calculation, given that the Experiments in this thesis were not designed to make quantitative evaluations of ELISA thresholds (i.e. as they were mainly qualitative assessments), two new thresholds were nonetheless estimated. For the purpose of this calculation, Group *Cff* and Control heifers from Experiment 1, and heifers in Experiment 3 prior to Day 0 (since at that point they had never been exposed to

Cfv) were used to generate negative control data. It was assumed that observations were independent, also as none had been exposed to *Cfv*. Using a figure of three standard deviations above the mean value (Hum et al., 1994) for the negative control animals a new limit (Limit A) was calculated to be EV=109. A second threshold (Limit B) was calculated adding 2 standard deviations to the mean (instead of the 3 standard deviations proposed by Hum et al.,1994) and was found to be EV=84. Using these new limits as negative:positive thresholds, the false-positive rate of the test changed from 18.2% (using EV=33) to 4.8% (EV=84) and 1.2%(EV=109), respectively.

Such decreases in the false-positive rates although they look promising, actually result in a concomitant loss of specificity in the test. Thus, the false negative rate increased from 2.3% to 47% to 76%, respectively, for the three thresholds. To quantify these changes in sensitivity and specificity at animal level more accurately, a ROC curve was constructed (Figure 5.1). The 'Gold standards' used to build this curve were:

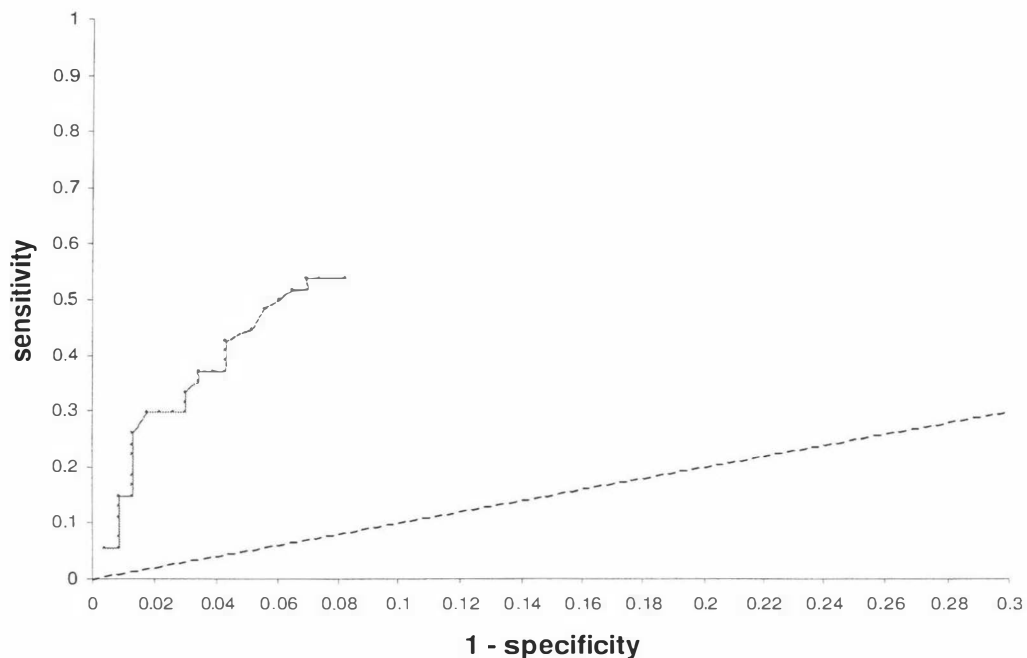
- Heifers from Group *Cff* and Control Group in Experiment 1 and heifers in Experiment 3 prior to mating were considered negative given that they were never exposed to *Cfv*;
- Heifers in Experiment 2, 30 days after challenge were considered positive given their exposure and response to *Cfv*.

Heifers from Group *Cfv* (Experiment 1) were not included in these calculations because they could not be considered free from exposure or positive to infection even though they appeared non-infected based on their IgA ELISA values.

The curve shows changes in sensitivity and specificity with thresholds that ranged between 110 (the point closest to the y-axis) and 33 (the point furthest away from the y-axis, Figure 5.1). It can be appreciated that even when specificity remains high, sensitivity does not improve to a figure that was much above 50% in this cut-off range (a sensitivity of the 50% was obtained at a threshold EV of 44). In terms of the use of the test as a diagnostic method at herd level, with a 50% sensitivity, a 94% specificity and a cut-off value for positive results of EV= 44 (according to the ROC curve) a herd of 100 cows with 20% prevalence of *Cfv*, would not be detected with sufficient confidence even if the

entire herd was sampled. In fact, in a 1000 cow herd, with the same sensitivity, specificity, cut-off and prevalence, one would have to sample 127 animals, and find that at least 12 of these gave positive ELISA results to be only 50% sure that the prevalence of BVC in that herd was 20%.

Figure 5. 1: ROC curve showing sensitivity and specificity of the IgA ELISA test from data in Experiment 1 at different cut-offs (range EV=110 to EV=33 from left to right). The negative controls were data from heifers not challenged with Cfv and the positive controls were data form heifers in Experiment 2.



Thus, given the lack of repeatability of the test, together with the aforegoing calculations of specificity and sensitivity, the IgA ELISA does not appear to be of any value as a diagnostic test for the presence of BVC under New Zealand conditions.

That a cross-reaction with *Cff* would occur is perhaps inevitable, due to the close genomic relationship between the two subspecies of *C. fetus* (Harvey and Greenwood, 1983; Roop et al., 1984). The resulting phenotypic similarities have certainly rendered differentiation between the two subspecies a very difficult task (Chapter 1), especially as it results in the generation of similar immune responses, which has been an issue for all the serologic methods developed for the diagnosis of BVC.

Indeed this cross reactivity has always made serological identifiation of BVC difficult. The fluorescent antibody technique (FA) that was widely used in the 1960s was found to be a satisfactory method for herd screening for the presence of BVC, although its value was undermined by the fact that it too produces false positive results, due to cross reactions with *Cff* (Dufty, 1967; Lein et al., 1968; Mellick et al., 1965; Ruckerbauer et al., 1974).It was largely because of this cross-reactivity that its use was abandoned. Likewise, the vaginal mucus agglutination test (VMAT) was found to have limitations (although it is still widely used at herd level), due to the false positive results obtained as a consequence of: i) cross-reactivity against *Cff* (Clark, 1971); ii) the presence of blood in the samples (Clark, 1971); iii) repetitive sampling (Clark et al., 1970).

When ELISA tests were introduced, they were said to overcome many of the weaknesses of the VMAT (Hewson et al., 1985). ELISA use was also promoted because of its apparent ability to identify infected animals sooner after infection than VMAT. Despite this, the IgA ELISA test (1994; Hum et al., 1991), even though widely used in Australia, appears to produce very unreliable results under New Zealand conditions. This evidence yet again calls into questions the ability of any serological method to properly identify the presence of *Cfv* in a herd, in a situation (such as that in New Zealand) where infection of sheep flocks with *Cff* is endemic (Mannering et al., 2004) and combined sheep and beef production systems are common. In any case, if a serologic diagnostic method is to be used, it definitely should not be used in isolation. Indeed, it could be argued that there is not one single diagnostic method available for BVC that is sufficiently sensitive and/or specific to be used by itself (Andrews and Frank, 1974; Lander, 1990b); except for the microbiological isolation of *Cfv,* which should always be an objective as, whilst its sensitivity is low, it is diagnostic of the presence of the organism.

Microbiological isolation presents its own difficulties however. The sampling method that has been used in New Zealand in recent reports has been the vaginal swab (Tattersfield, 2002). Although this method is presently widely accepted for isolation of *Cfv* (Hum and McInnes, 1993; OIE, 2004; Tattersfield, 2002), most early research on the isolation of *Cfv* from cows used samples obtained by aspiration of vaginal mucus (Chin-Fatt, 1982; Clark et al., 1969;

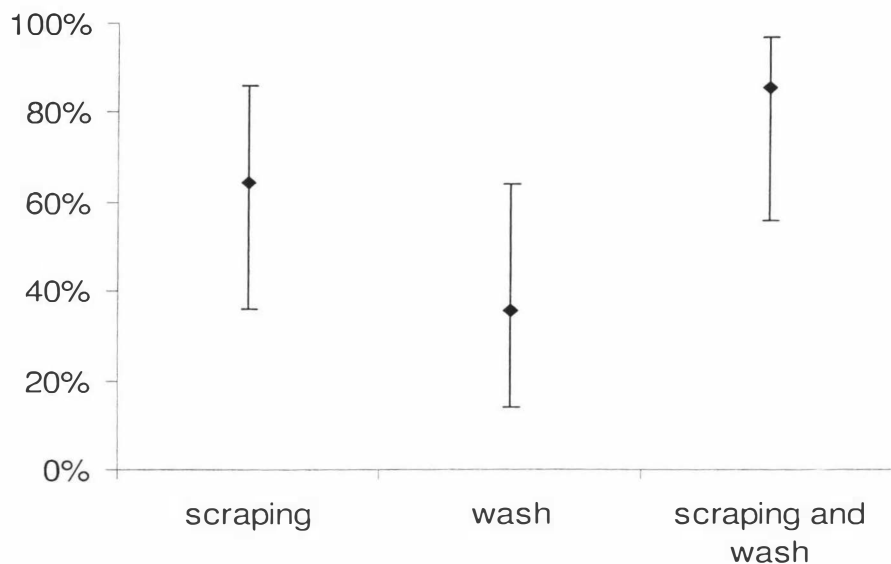
Dufty and McEntee, 1969; Seger and Levy, 1962), achieving isolation rates from 60% (Clark et al., 1969) to 75% (Chin-Fatt, 1982). The vaginal wash has also been proposed as a useful sampling method (Hum and McInnes, 1993).

Comparing the lack of positive cultures achieved by Tattersfield (2002) and the failure to isolate the organism from vaginal swabs collected in Experiments 1 and 2, to the 75% positive isolation rate obtained by Chin-Fatt (1982) using aspiration of vaginal mucus, leads to the view that the problem could be attributed to the low capacity of the vaginal swab to harvest enough viable *Cfv* cells to permit positive isolation of the organism. This view is supported by the higher isolation rates obtained when performing vaginal washes in Experiments 2 and 3. However, it must be remembered that infection rates of cows with *Cfv* in other studies in New Zealand (Chin-Fatt, 1982), was also low (only 2 out of 7 challenged cows established infection). Hence the lack of positive isolation of *Cfv* in Experiment 1 can probably be attributed as much to inadequacies in the methodology of sampling as to the absence of the organism. Moreover, the repeated positive isolation of *Cfv* from vaginal washes in Experiments 3 and 4 leads to the suggestion that this is probably a more appropriate sampling method for microbiological isolation of *Cfv* from cows.

The lack of positive microbiological isolation of *Cfv* from preputial washes obtained from bulls in the most recent publications in New Zealand (Hughes, 2001; McFadden et al., 2004a; Tattersfield, 2002) also raises questions about the sensitivity of the isolation methods used in bulls. However, all of the various methods that have been described to obtain samples from bulls have produced positive isolations of *Cfv* at various times (Chin-Fatt, 1982; Clark and Dufty, 1978; Repiso et al., 2001; Tedesco et al., 1977). Two studies that compared three sampling methods from bulls showed that the preputial scraping apparently resulted in a better sample, because it allowed better isolation rates and less interference by contaminants (Repiso et al., 2001; Tedesco et al., 1977). Results from Experiment 3 indicated that the preputial scraping and the preputial wash both delivered the same total number positive isolations (even though there was little coincidence on positive isolations from both methods on the same day), and that however, the sensitivity increased from 36% with the preputial wash alone, or 64% with the preputial scrape alone, to 86% when both methods were

used together (Figure 5.3). Although this difference was not statistically significant (as only 2 bulls were used), these results certainly infer that it would be a prudent recommendation to collect samples by both methods, rather then relying upon just one.

Figure 5. 3: Sensitivity (± 95% CI) achieved for the isolation of *Cfv* sampling bulls using the preputial scraping, the preputial wash or both methods at the same time together with Lander's medium



A further aspect of the microbiological isolation of *Cfv*, that also appears to be of importance, is the use of selective enrichment media. Historically, it was after the development such media for isolating *Cfv* that samples obtained from the bull became the method of choice for the diagnosis of BVC in a herd (Tedesco et al., 1977). There are many such transport media. Skirrow's agar is useful for the isolation of *Cfv* (OIE, 2004) and is widely used in New Zealand for the isolation of *Cff* from sheep abortions. However, in the present study (Experiment 1), no positive cultures (even from animals challenged with *Cff*) were obtained when Skirrow's agar was used. Hence, it is not inconceivable that the failure to obtain microbiological isolation of *Cfv* in the aforementioned study could be attributed to inadequate sampling or inappropriate media (or both), rather than to a true absence of the organism.

The isolation medium used in Experiments 2 and 3 was Lander's broth.

Developed in 1990 (Lander, 1990b), it is now widely used with very good results (Lander, 1990a; OIE, 2004). In the present study, the medium seemed to be very useful for the isolation of *Cfv*, both by decreasing overgrowing contaminants and promoting the growth of *Cfv* itself.

Taken together, the results of the experiments in this thesis, suggest that the best combination of sample and medium for the isolation of *Cfv* is:

1. Samples from females: the vaginal wash
2. Samples from males: the preputial scraping or the preputial wash, although the combination of both methods is best.
3. Inoculation of Lander's medium with 1 ml of the sample, incubation for three days under microaerophylic conditions. Further subculture of an aliquot of 100 µl of the broth into a blood agar plate, and incubation under microaerophylic conditions for at least 5 days.

Once *C. fetus* has been isolated, it is essential that the two subspecies can be accurately differentiated, before a diagnosis of BVC can be made. The similarity between these two subspecies is such that only molecular biology techniques are able to distinguish between them with a level of accuracy that is needed for diagnostic use in the field. The PCR technique described in Experiments 4 and 5, which was first developed in Australia (Hum et al., 1997; Pannachio et al., 1993), has proven to be accurate in differentiating between isolates of *Cfv* and *Cff*, even though there is yet to be agreement about the size of the amplified bands (Muller et al., 2003; Newell et al., 2000; Vargas et al., 2003; Wagenaar et al., 2001). Preliminary results using this technique for preputial washes from infected bulls (Pannachio et al., 1993) were supported by the present studies, in which as few as 880 *Cfv* cell/ml could be positively identified in artificially-inoculated preputial washes. Moreover, positive identification of *Cfv* cells could be achieved from preputial washes/scrapes obtained 48 h after experimentally infecting bulls.

Hence, it appears that the failure to demonstrate the presence of BVC in recent field investigations (Hughes, 2001; Tattersfield, 2002) could be attributed as much to the methodological inadequacies as to the absence of the disease. The work presented in this thesis identifies the methods that are most likely to yield positive results. It is of some importance to reach a conclusion about whether the

diseases is, or is not present, as the current inability to do so represents a serious limitation to the management of infertility in beef herds.

This importance is emphasised by the work of Hughes (2001) and Tattersfield (2002) who rightly suspect the presence of *Cfv* from the symptoms displayed in the herds they studied. There are many reasons for such a suspicion. Firstly, the chronic decrease in fertility is compatible with endemic BVC. Secondly, the lack of positive results obtained from the diagnosis of other reproductive diseases also indicates that BVC should not be ignored as the cause of the decreased fertility. Moreover, New Zealand possesses the topography and cattle management systems for a disease such as BVC to be present and yet be very difficult to diagnose. Mating management practices compound with this situation. Some beef herds have long breeding seasons, many keep older bulls in from year to year, some leave non-pregnant cows in for the next season, and lending or sharing of bulls is by no means unknown (McFadden et al., 2004b). Likewise, feral cattle of unknown disease status, may have access to hill-country herds grazing near areas of bush. Furthermore, the use of 'experienced' (i.e. older) bulls with maiden heifers, libido testing bulls prior to use (Morris, 1998) (i.e. permitting vaginal intromission) plus rotational use of bulls, have all been recognized as means of spread of *Cfv* infections (Ball et al., 1987; Hartley, 1952).

Hence, because of these geographical and management factors that are compatible with the presence of BVC, together with the presence of herds that display signs that could well be attributed to *Cfv* infections, it is important that it is definitively established whether the organism is, or is not, present in the country. The question of how to do so remains difficult to answer. Firstly, it is clear that the IgA ELISA in its present form is not suitable. Data from the present study, together with the evidence from McFadden et al. (2004a), shows that under the present rules of interpretation, it gives too many false positive to be of any value. Results in Experiment 1 show that cross-reaction with *Cff* is likely to be one of the causes for this, although the presence of IgA secreted against bacteria or substances other than *Cfv* is not ruled out. As explained earlier, if a large-scale study of IgA titres in uninfected animals (including animals that might have been under higher risk of vaginal contamination of *Cff*) were to be

undertaken, cut-off points could be established that are more appropriate to the New Zealand situation. Yet other results from the present study also show that the assay is subject to enormous intra-essay coefficients of variation, meaning that caution is needed to interpret results that fluctuate widely within individual animals on different sampling days. The conclusion is, therefore, that the IgA ELISA does not seem to be useful to show whether BVC is, or is not, present in New Zealand.

Hence, one is forced to attempt the microbiological isolation of the organism. Indeed, it is perhaps both inevitable and appropriate that this should be the case, since in many previous studies, microbiological isolation of *Cfv* has always been regarded as the definitive diagnostic method (Ball et al., 1987; Carrol and Hoerlein, 1972; Clark, 1971; Griffiths et al., 1984; Lander, 1990b; Newsam, 1960; Ruckerbauer et al., 1974; Seger and Levy, 1962). Failure to recover the organism in previous studies (Hughes, 2001; Tattersfield, 2002) can probably be attributed to inappropriate microbiological techniques and the collection of the samples from either the wrong animals or at the wrong times. Results of the present study show that the appropriate means of recovering the organism is by vaginal wash of infected cows and from preputial washes and scrapings from bulls. The preferred medium is Lander's medium. Previous studies have used the vaginal swab (Tattersfield, 2002), a method that did not yield any positive result in these Experiments. Likewise, the Skirrow's medium, in the present studies yielded no positive results.

Given that *Cfv* is a difficult organism to isolate, even with appropriate microbiological methods, samples need to be collected from targeted animals that have higher possibilities of being infected. For bulls, the highest rates of infection is in animals of more than 4 years of age (Philpott, 1968). Younger bulls are not likely to carry infection for long periods (Ladds et al., 1973). The time when bulls are more likely to be infected is during mating, but bulls that serve several times a day have been seen to decrease shedding of *Cfv* cells (Clark, 1971). Sampling bulls after pregnancy testing (8-11 weeks after the end of mating) or sampling bulls younger than 4 years old, might, with luck, yield a positive result, but would not represent the ideal animals. The ideal target would be old bulls, sampled immediately after cessation of mating. Since in the present

study, neither the preputial wash nor the preputial scrape consistently repeated positive results (although positive results were obtained from both), both methods should be used. This would also allow an increase in the sensitivity of the sampling.

Immediate inoculation into Lander's medium, and immediate maintenance of samples under microaerophylic conditions also appear, from the present results, to be required for the isolation of *Cfv*.

At present, PCR is probably best used for the accurate identification of *C. fetus* cultures to the subspecies level. However, the results of the present study show that PCR has the potential to be developed into a method that can be used for identification of the presence of *Cfv* from field samples from bulls. Furthermore, development of the method should focus on improving the concentration methods used in this study (filtration and centrifugation), maybe adding a DNA extraction step before submitting the sample for PCR.

Whether samples can be collected from females that will contribute to determining whether *Cfv* is, or is not, present in New Zealand is less clear. Certainly, samples for IgA ELISA do not currently appear to be of any diagnostic value. Vaginal samples from pregnant cows are unlikely to yield positive results, given the significant drop in isolation rates observed after pregnancy occurs (Clark et al., 1969). Vaginal samples from non-pregnant cows might yield better results, although again, it is more likely that the organism would have been eliminated from most animals if sampling is done as late as during pregnancy testing (Andrews and Frank, 1974). Results of the present study showed that the most consistent isolation of the organism occurred within 48 h of inoculating. Perhaps, therefore, if females were to be used to demonstrate the presence of *Cfv*, the best way to do so would be to perform vaginal washes on recently mated susceptible animals. In other words, maybe test mating of virgin heifers, a method advocated for the diagnosis of this disease in early times (Clark, 1971; Dufty and McEntee, 1969; Dunn et al., 1965; Mellick et al., 1965), although unaesthetic and expensive, could become a useful tool today again.

The easiest sample from which to isolate *Cfv* is the aborted foetus. It is known that, in situations in which it is endemic, BVC causes a small proportion of abortions in the herd (Hartley, 1952) that may not even be considered of

economic importance (Hanley and Mossman, 1977). Unfortunately, abortions are seldom recorded from beef herds. On the other hand, many of the isolations of *Cfv* reported elsewhere have been made from aborted material from cows, mainly from stomach content of aborted foetuses (Campero et al., 2003; Hum, 1987; Hum and McInnes, 1993; Jeffrey and Hogg, 1988; Lawson and Mc Kinnon, 1952; Leaver and Hart, 1960; Roberts, 1971; Varga et al., 1986). As seen before, the problems implicit with mustering of animals in hill country in New Zealand probably poses a great challenge for farmers to check on cattle for abortions (Dewes, 1961; Hanley and Mossman, 1977; Young, 1965) and, consequently, abortions would probably pass unseen by the farmer and not reported to the field veterinarian. Yet given the diagnostic value of aborted foetuses, perhaps specific effort needs to be devoted to the search for such aborted foetuses in the paddocks.

Thus, despite the initial promise that the IgA ELISA would permit a cheap, easy and quick means of determining whether *Cfv* is present in New Zealand, this has not proved to be the case. Answering the question will require the combination of microbiological methods, including the incorporation of advances that have improved culture conditions (e.g. gas sachets to produce the required atmospheric conditions), plus other technological improvements, such as molecular biology techniques, together with reference back to the knowledge of those who studied this disease back in the 1950s. Careful selection of target farms, and target animals will be needed, since it would be easy to waste time and resources in inappropriate sampling regimens. Convincing farmers to put time and effort into finding abortions would, of course, be ideal, although the practical difficulties of doing so are potentially insuperable. At the end of the day, it depends how important the question really is. *Campylobacter fetus* subsp. *venerealis* is an organism that is well adapted to survive without detection for long periods. Hence, sporadic attempts to isolate it are unlikely to succeed. The time will come when a decision will have to be made that if infertility of beef herds is of sufficient economic significance, an effort will be made to finally investigate if BVC is its cause or whether it is not.

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Appendix 1: Media used

Columbia sheep blood agar plates (BA)

Ingredients: tryptone 14.0 g/lt, peptone neutralized 4.5 g/lt, yeast extract 4.5 g/lt, sodium chloride 5.0 g/lt, agar 12.0 g/lt plus 7 % sterile sheep blood (Bridson, 1995).

Manufacturer: Fort Richard Laboratories Ltd, Auckland, New Zealand

Brain Heart Infusion broth (BHI)

Ingredients: Calf brain infusion solids 12.5g/lt, beef heart infusion solids 5.0 g/lt, protease peptone 10.0g/lt, glucose 2.0 g/lt, sodium chloride 5.0 g/lt, disodium phosphate 2.5 g/lt. Final pH 7.4 ± 0.2 (Bridson, 1995)

Skirrow's Agar plates

Ingredients: blood agar base plus vancomycin 5 mg, polymyxin 1,250 IU, trimetoprim 2.5 mg (Bridson, 1995).

Manufacturer: Fort Richard Laboratories Ltd, Auckland, New Zealand.

Lander's medium

Ingredients: Mueller Hinton broth (OXOID Limited), charcoal, lysed horse blood vancomycin 4 mg/ml, polymixin B sulphate 1000 IU, cycloheximide 10 mg/ml, trimetoprim 2 mg/ml, 5-fluorouracile 10 ml/ml, Campylobacter supplement (i.e. sodium pyruvate, sodium metabisulfite and ferrous sulphate, OXOID Limited). The medium was developed by Lander (1990b).

Manufacturer: Fort Richard Laboratories Ltd, Auckland, New Zealand.

Blood Agar/Glycine

Ingredients: a blood agar base (10ml) plus Glycine 2 g, distilled water 200 ml and CBAB 8.8 g.

Manufacture: dissolve CBAB, and glycine into 200 ml of distilled water, cool at 45 °C, ad 10 ml of sheep blood, mix and autoclave.

BHI/Cysteine

Ingredients: BHI (37 g/l) 7.4 g plus Cysteine 0.04 g and agar 0.32 g.

Manufacture: dissolve BHI, cysteine and agar, dispense in 5 ml per universal container and autoclave.

Phosphate buffered saline (PBS)

PBS was made by dilution of one Dulbecco A tablet (OXOID Limited) into 100 ml of distilled water. Final pH= 7.3.

PBS plus Tween 20 (PBST)

PBS plus Tween 20 at 20% dilution.

Manufacturer: Fort Richard Laboratories Ltd, Auckland, New Zealand

Glycerol broth

Ingredients: nutrient broth (i.e. beef extract 3.0 g/lt and peptone 5.0/ g/lt; OXOID Limited) plus glycerol 45 ml and distilled water 300 ml.