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DETECTION OF *CAMPYLOBACTER FETUS* IN BOVINE PREPUTIAL SCRAPINGS USING PCR AND CULTURE ASSAYS

BY

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ABSTRACT

DETECTION OF *CAMPYLOBACTER FETUS* IN BOVINE PREPUTIAL SCRAPINGS USING PCR AND CULTURE ASSAYS

By

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The traditional method for the diagnosis of bovine genital campylobacteriosis is the culture and identification of the causative organism, *Campylobacter fetus* subsp. *venerealis* (Cfv) from the genital tract. This approach is considered relatively insensitive due to the fragility of the bacteria, their specific nutritional and atmospheric requirements and their being easily overgrown by commensal bacteria. The identification of isolates is also problematic due to the limited biochemical activity of the bacteria. With the rapid advances made in the molecular field, assays have become more robust and cost-effective making them feasible for the diagnostic laboratory. The potential speed, sensitivity and specificity offered by these assays provide attractive alternatives for the identification of pathogens which are notoriously difficult to identify.

The first part of this investigation was concerned with the implementation and evaluation of a polymerase chain reaction (PCR) assay for the direct detection of *C. fetus* in bovine preputial specimens. The specificity of a published *C. fetus*-specific primer pair was established by testing *C. fetus* reference and field isolates in addition to a collection of other *Campylobacter* species and organisms which may encountered in the genital tract of cattle. All *C. fetus* isolates tested yielded a single PCR amplicon of approximately 750 bp. No amplicons were generated when any of the other non-*C. fetus* isolates were tested.

Following minor modifications to the assay, the sensitivity of the assay was determined using spiked Weybridge medium. A detection limit of 615 Cfv/mℓ Weybridge medium (or 6,15 cell equivalents per PCR assay) was obtained. Preputial material collected and submitted for laboratory testing may often be contaminated with faeces, urine, semen and/or blood. All of these components are known to be potential PCR inhibitors and the influence of each, on the sensitivity of the PCR assay, was subsequently evaluated. Faeces were identified as a potent inhibitor and contamination of specimens with as little as 1% (w/v) faeces reduced the sensitivity of the assay. Concentrations of up to 50% (v/v) of blood, urine and semen had no effect on the sensitivity of the assay.

Preputial specimens, collected in Weybridge medium, were subsequently pooled and spiked and used to establish the sensitivity of both the PCR and culture methods as well as determine the influence of time on the sensitivity of the assays. Testing was carried out in triplicate on samples collected from different herds which were ascertained to be free of Cfv based on the use of specific selection criteria. The detection limit of the culture method was found to be better than that achieved using PCR only immediately after the samples were

spiked. The detection limit of the culture method decreased with time whilst the detection limit of the PCR assay remain unchanged up to 72 hours post-inoculation.

Ensuing field evaluation involved the testing of 212 clinical samples using both the culture method and the optimized PCR assay. Of the samples tested 4,2% were found to be positive using the PCR assay, whilst only 3,8% were found to be positive by culture. Based upon this evaluation the analytical specificity of the PCR assay was calculated to be 99% and the analytical sensitivity, 85,7%.

The second part of this investigation was concerned with the subspeciation of *C. fetus* isolates. Currently the only test recommended by the *Office International des Epizooties* (OIE) for the subspeciation of isolates, is tolerance to 1% glycine. Doubts over the reliability of this test have led to alternative or supplementary tests being sought. Within the context of this investigation a collection of 40 South African field isolates were subspeciated using a previously described subspecies-specific primer set as well as the traditional 1% glycine tolerance phenotyping test. Additionally, other phenotyping tests (selenite reduction, growth at 42 °C and susceptibility to metronidazole and cefoperazone) were evaluated to determine their suitability for use as an aid in the subspeciation of *C. fetus* isolates.

None of the field isolates yielded a Cfv-specific subspecies PCR amplicon using the published primer set suggesting that all of the isolates were *Campylobacter fetus* subsp. *fetus* (Cff). Based on tolerance to 1% glycine however, only 6 isolates were identified as Cff (glycine tolerant), whilst the remainder were classified as Cfv. The results of the 'sensitive' hydrogen sulphide test indicated that the Cfv isolates were specifically Cfv biovar intermedius. The lack of agreement between the PCR and the phenotyping subspeciation results concur with the findings reported by other researchers. It is consequently concluded that the published VenSF/VenSR subspecies-primer set is unsuitable for the subspeciation of South African field isolates.

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THIS WORK IS DEDICATED IN MEMORY OF

DR JENNIFER LESLIE RANDLES

ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AI	Artificial insemination
APVL	Allerton Provincial Veterinary Laboratory
ATCC	American Type Culture Collection
bp	base pair
bv	biovar
CampE	*Campylobacter* enrichment agar
Cff	*Campylobacter fetus* subsp. *fetus*
Cfv	*Campylobacter fetus* subsp. *venerealis*
Cfv-i	*Campylobacter fetus* subsp. *venerealis* biotype intermedius
CFU	Colony forming unit
CO_2	Carbon dioxide
°C	Degrees Celsius
DNA	Deoxyribose nucleic acid
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dNTPs	Deoxyribose nucleoside triphosphates
g	Gram
x g	Relative centrifugal force
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAT	Fluorescent antibody test
FBP	Ferrous sulphate, sodium metabisulfite and sodium pyruvate
hr	Hour (s)
H_2S	Hydrogen sulphide
KCl	Potassium chloride
L	Litre
mg	Milligram
$MgCl_2$	Magnesium chloride
mℓ	Millilitres

mM	Millimolar
mm	Millimetres
min	Minute
MLST	Multilocus sequence typing
MWM	Molecular weight marker
NCTC	National collection of Type Cultures
nm	Nanometre
OIE	*Office International des Epizooties* (The World Organization for Animal Health)
%	Percent
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline supplemented with 0,02 % Tween
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PVL	Provincial Veterinary Laboratory
KCl	Potassium chloride
®	Registered trademark
RAPD	Random amplification of polymorphic DNA
rDNA	Ribosomal DNA
s	Second
SA	South Africa
subsp.	Subspecies
TBE	Tris borate EDTA
TEM	Transport enrichment medium
TNTC	Too numerous to count
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol hydrochloride
TSI	Triple sugar iron agar
U	Units
µg	Microgram
µ ℓ	Microlitres
µM	Micromolar
µm	Micrometres
V	Volts
VMAT	Vaginal mucus agglutination test

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CHAPTER 1: BACKGROUND, LITERATURE OVERVIEW AND STUDY AIMS AND OBJECTIVES

BACKGROUND

Bovine genital campylobacteriosis caused by *Campylobacter fetus* subsp. *venerealis* (Cfv), is a venereal disease of cattle and is considered to be one of the primary infectious causes of bovine infertility worldwide. In fact it was considered significant enough to be listed in the old classification system of the OIE as a group B notifiable disease. In South Africa, this disease is considered a leading cause of decreased calving percentages. Currently, a definitive diagnosis relies on the cultivation and identification of the causative organism. The success hereof is dependant upon the collection and maintenance of the fragile *C. fetus* bacterium which has limited viability outside of the host. Most of the cattle breeding farms in South Africa tend to be extensive in nature and located far away from diagnostic laboratories. In many cases the consulting veterinarian may need to travel long distances to sample animals and the task of ensuring samples reach the laboratory timeously is a logistical nightmare. Furthermore, in summer the ambient temperatures in most parts of the country readily exceed 30 °C making it difficult to maintain the cold chain. With all these logistical difficulties it is not altogether surprising that, even under optimum laboratory conditions, low isolation rates are reported.

Concerns have also been raised regarding the sensitivity of the culture and identification techniques which are routinely used in veterinary diagnostic laboratories. In an attempt to improve upon existing methodology, alternative diagnostic methods have been sought. Polymerase chain reaction (PCR) assays in particular, present a number of advantages over more traditional methodologies. Apart from exhibiting high specificities and sensitivities, the ability of these types of assays to detect non-viable organisms could also potentially resolve problems encountered in the field with the preservation of organism viability for subsequent bacteriological culture. It was therefore the intention of this project to evaluate a species-specific PCR to screen preputial material directly for *C. fetus* eliminating the need for culture and the dependence on viable bacteria for accurate diagnosis.

Prior to the onset of this investigation the culture methodology being used was reviewed to ensure that all of the OIE recommendations for the cultivation of *C. fetus* were being met.

This was deemed necessary to enable an accurate assessment of the performance of the PCR assay to be made. A transport medium, designed to sustain *C. fetus* during transit, was introduced and supplied to all practitioners wishing to submit samples. Further, the use of a selective culture medium was included in the test procedure, essentially ensuring that all samples were cultured in duplicate. In practice, due to the time and costs involved in preparing and maintaining the necessary transport and culture media, many laboratories make compromises and take short cuts which could potentially impact on test sensitivity.

The subspecies differentiation of *C. fetus* isolates was also addressed within the context of this investigation. Two subspecies exist and under certain circumstances differentiation of isolates to subspecies level is required. A single phenotyping test is customarily used but this has been shown to be unreliable and questions regarding the stability of this phenotypic trait have also caused concern. Molecular research has resulted in the publication of a subspecies-specific PCR test. Due to the close genetic relatedness of the two subspecies the suitability of this assay for testing South African field isolates needs to be established.

LITERATURE REVIEW

1.1 Introduction

Campylobacteriosis, or vibriosis, is a contagious genital disease of cattle caused by the bacterium *Campylobacter fetus* subsp. *venerealis* (Cfv). The disease is characterized by temporary infertility of female cattle, early embryonic mortality, aberrant oestrus cycles, delayed conception and infrequent abortions. Most cows recover from infection and oestrus cycles will recommence after a few months but the herd experiences a decreased pregnancy rate and a prolonged calving season (Monke *et al*., 2002). Infected bulls usually show no clinical signs of infection but become carriers and infect females at service. Artificial insemination (AI), vaccination and the separation of infected from non-infected herds have all found to be effective in controlling bovine campylobacteriosis (Eaglesome & Garcia, 1992).

A second subspecies of *C. fetus* exists, namely *Campylobacter fetus* subsp. *fetus* (Cff). The two subspecies are closely related genomically but differ in the diseases they produce, their habitats and their biochemical characteristics (Brooks *et al*., 2004). *Campylobacter fetus* subsp. *fetus* has been shown to inhabit the intestines of cattle and sheep and is known to cause sporadic abortion in both of these species, but unlike Cfv it is not associated with

bovine infertility (Eaglesome & Garcia, 1992). Differentiation of isolates to subspecies level is imperative for correct diagnosis and treatment of animals (Hum *et al.*, 1997) and for epidemiological surveillance.

1.2 Historical background and classification of the aetiological agent

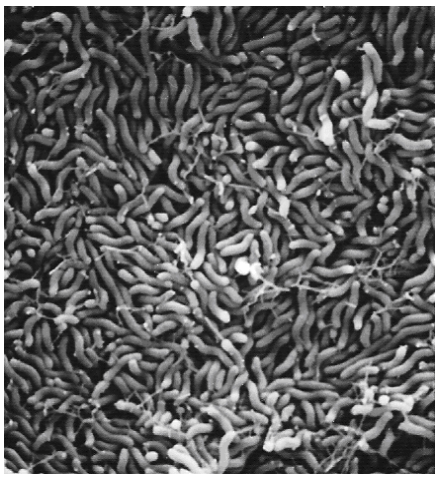
The first documented record implicating *Campylobacter fetus* (formerly *Vibrio fetus*) as a cause of abortion in cattle and sheep was made by McFadyean and Stockman in 1913. Subsequent reports in 1943, 1951 and 1953 again identified the bacterium as a cause of abortion and 'temporary sterility' in both the United States of America (USA) and The Netherlands. It was not until 1954 that it was identified as one of the causes of bovine infertility in South Africa. This initial report, made by van Rensburg (1954), was confirmed by other investigators who documented similar findings in different herds (Irons *et al.*, 2004).

Florent (1959) observed that venereal transmitted enzootic infertility in cattle was caused by a variant of *V. fetus* that he named *V. fetus venerealis,* whereas sporadic abortions in cattle were caused by a *V. fetus* variant of intestinal origin named *V. fetus* subsp. *intestinalis*. Florent further described the biochemical characterization tests that could be used to differentiate the two variants. On the basis of these clinical and biochemical observations the species was split into two subspecies (van Bergen, 2005).

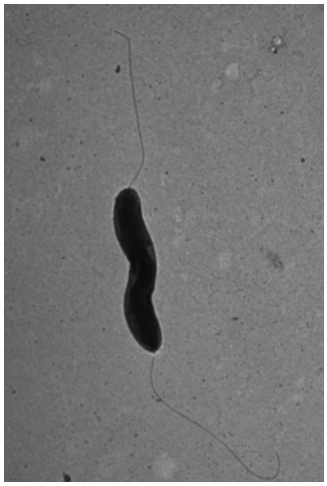
Taxonomic studies carried out and published by Véron and Chatelain (1973) led to the reclassification of both *V. fetus* subspecies into a newly described genus, *Campylobacter. Vibrio fetus intestinalis* became *Campylobacter fetus* subspecies *fetus* (Cff) and *Vibrio fetus venerealis* became *Campylobacter fetus* subspecies *venerealis* (Cfv). Furthermore, a hydrodgen sulphide (H_2S) positive biovar of Cfv, designated *Campylobacter fetus* subsp. *venerealis* biovar intermedius by Véron and Chatelain (1973), was also described. This latter proposal was however never officially adopted largely due to the lack of reference strains, its questionable clinical relevance and its poor original description (van Bergen, 2005). More recently, however, with the availability of extremely sensitive genotyping techniques, multilocus sequence typing (MLST) was applied to a diverse international collection of *C. fetus* isolates. The results indicated that isolates phenotypically classified as biovar intermedius do in fact fulfil the criteria for reclassification as a separate subspecies, for even though they are genetic homologs, there are phenotypic differences (van Bergen, 2005).

1.3 Aetiology

Members of the genus *Campylobacter* are slender, curved, Gram-negative rod-shaped bacteria (0,2 to 0,5 µm by 1,5 to 4,0 µm). Cells may have one or more spirals and often exhibit an S-shaped or gull-wing-shaped formation (see Figure 1). In old cultures, cells often form coccoid or spherical bodies which are non-viable (Ng *et al*., 1985). *Campylobacter* organisms are motile, each cell possessing at least one polar flagellum at one or both ends. Examination using phase-contrast microscopy usually reveals a darting, corkscrew-like motion which is characteristic of *Campylobacter* spp. (Irons *et al.*, 2004).



(A)



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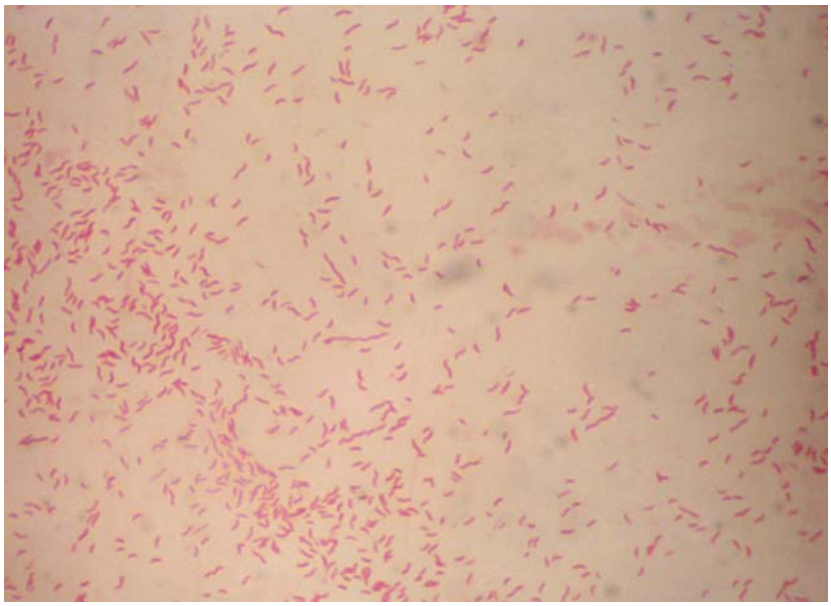

(C)

Figure 1: *Campylobacter fetus* cells as visualised using (A) Scanning electron microscopy and (B) transmission electron microscopy. (C) A Gram stain showing the various morphological forms of *C. fetus*.

Campylobacter spp. are microaerophilic requiring an atmosphere of 10 – 20% carbon dioxide and an oxygen concentration of 5% or less (Timoney, *et al.*, 1988). Bacteria in the genus are non-proteolytic, do not ferment or oxidize carbohydrates and cannot phosphorylate or transport glucose. Energy for growth is derived from the tricarboxylic acid cycle (Garcia, Eaglesome & Rigby, 1983). Consequently only a limited number of morphologic and physiologic criteria are available for classification and identification purposes (Cardarelli-Leite *et al.*, 1996).

Campylobacter fetus is catalase- and oxidase-positive. Optimal growth occurs at 37 °C on serum, blood, thiol, cysteine-heart and brain-heart infusion agars (Timoney *et al.*, 1988). Differentiation of *C. fetus* isolates from other campylobacters is based on susceptibility to different antibiotics and the ability to grow at 25 and 42 °C.

Campylobacter fetus lipopolysaccharides exhibit unique characteristics that contribute to both the biology and virulence of the organism. Based on the variation of the O-antigen two serotypes (A and B) have been identified. All Cfv isolates are serotype A, whereas Cff isolates are serotype A or B (Brooks *et al*., 2002). Unlike other campylobacters, many *C. fetus* isolates possess a proteinaceous surface-layer (S-layer) which forms the outermost component of the cell envelope. This layer effectively evades a variety of immune defences enabling the survival of the bacterium within the host and accounts for the remarkable persistence of *C. fetus* infections (van Bergen, 2005).

1.4 Epidemiology

Campylobacteriosis is of considerable economic importance to the cattle industry worldwide (Hum, 1996) and is considered to be one of the most important infectious causes of poor calving rates in southern Africa. The prevalence of *C. fetus* in sheath wash samples submitted to regional veterinary laboratories in South Africa and Namibia has ranged between 0 and 12% (Irons *et al.*, 2004). The availability of vaccines and the introduction of AI practices have decreased the prevalence of the disease (Schmidt-Dumont, 1984) but many researchers believe that the prevalence of bovine campylobacteriosis is still underestimated as a consequence of the lack of sensitive and reliable diagnostic techniques available for diagnosis (Hum, Quinn & Kennedy, 1994).

Various investigations have been carried out in different countries to assess the prevalence and impact of this disease. The published results surveys are outlined in Table 1. These

results should be interpreted with caution taking into consideration the sensitivity and specificity of the diagnostic methods that have been used.

Table 1: A summary of published data showing the prevalence of *C. fetus* in different countries

Study Area	Sample type(s)	Sample size	Prevalence of *C. fetus* (%) *	Diagnostic method	Reference
Argentina (1966-1981)	nk†	11 300 animals	22	Serological (Fluorescent antibody test)	Villar & Spina (1982)
Australia (1985-1986)	Bulls (preputial suction)	1008 animals 41 herds	87% of herds positive	Serological (Fluorescent antibody test)	McCool *et al.* (1988)
California (United States of America)	Cows	400	47	Serological (Enzyme linked immunosorbent assay)	Akhtar *et al.* (1990a, 1990b)
Columbia	Bulls	nk	15	Bacteriological culture	Griffiths, Gallego, & de Leon (1984)
Egypt	nk	nk	4	nk	Gawade, Nada & Michael (1981)
Nigeria	Cows and bulls	689	Total 2.9 Cfv : 1.9 Cff : 1	Bacteriological culture	Bawa *et al*. (1991)
Malawi (1974-1976)	Bulls (sheath wash) Cows (vaginal mucus)	700	10 - 15% of animals positive	Serological	Klastrup & Halliwell (1977)
Scotland	Bulls	109	0	Bacteriological culture	McGowan & Murray (1999)

Table 1 continued: A summary of published data showing the prevalence of *C. fetus* in different countries

Study Area	Sample type(s)	Sample size	Prevalence of *C. fetus* (%) *	Diagnostic method	Reference
Transkei (former self-governing State in South Africa)	Bulls	87	28.7	Bacteriological culture	Pefanis *et al*. (1988)
Tanzania (1996)	Bulls (sheath scrapes, washes and swabs)	58	Cfv : 5.1	Bacteriological culture	Swai, Hulsebosch & van der Heijden (2005)
United Kingdom (1977-1992)	nk	nk	0.4 – 1.3	nk	Arthur *et al*. (1996)
Zimbabwe	Cows (mucus)	nk	33	Serological (Vaginal mucus agglutination test)	Terblanche (1979)

* Unless otherwise stated, the figures given for prevalence represent the number of animals found to be positive.

†nk : Not known

A global survey involving *Office International des Epizooties* (OIE) member countries was conducted in 2002 by van Bergen, Linnane, van Putten and Wagenaar (van Bergen *et al*., 2005b). Results indicated that a variety of methods are currently being used for the diagnosis of campylobacteriosis and not all of them are approved or considered adequate for identification purposes. The results highlighted the lack of uniformity of testing methods and consequently the under reporting of disease incidence.

1.5 Pathogenesis

Campylobacter fetus subsp. *venerealis* is highly adapted to the genital tract of cattle and sheep and, unlike Cff, does not survive in the bovine intestine (On and Harrington, 2001). In bulls, Cfv is primarily confined to the preputial cavity, in particular the mucosa of the glans penis, the free end of the penis between the glans and the fornix of the prepuce and the distal portion of the urethra (Eaglesome & Garcia, 1992). Infection in bulls is inapparent and is not accompanied by either histological changes or modifications in the characteristics of the semen (Clark, 1971). Individual bulls vary in their susceptibility to infection; some animals become permanent carriers whilst others appear to be resistant to infection (Kahn, 2005). One of the primary factors believed to influence susceptibility is the size (or depth) and the number of crypts in the preputial and penile epithelium. In young bulls (<3-4 years) these crypts are not well developed and infections tend to be transient. Whereas, in older bulls, the preputial and penile epithelial crypts are deeper and the prevailing microaerophilic conditions in these crypts may provide a suitable environment for the persistence of bacteria and the establishment of chronic infections (Garcia *et al*., 1983; Kahn, 2005). These older bulls consequently become reservoirs carrying the infection over from one breeding season to the next (Irons *et al.,* 2004).

In cows and heifers the bacteria localize in the anterior vagina and cervix following infection. In 10-20% of animals, infection does not progress any further. In susceptible animals however, the organisms invade the uterus and uterine tubes with the onset of the progestational phase, resulting in moderate endometritis and salpingitis (Vandeplassche *et al.*, 1963). Endometritis usually persists for several weeks during which time the animal is infertile either as a result of failure of implantation or because of early abortion. On rare occasions the pregnancy may continue to a later stage (5 to 7 months) before placental damage becomes too extensive resulting in foetal death and abortion. *Campylobacter fetus* subsp. *venerealis* infections are usually self-limiting and most cows usually regain fertility within 5 months following elimination of the infection from the uterus and the uterine tubes (Timoney *et al.*, 1988). In severe cases of infection where bilateral salpingitis occurs the animal may become sterile. It has been estimated that up to 10% of infected animals remain carriers of the organism for prolonged periods, and in some cases susceptible animals may become permanent carriers (Irons *et al*., 2004).

Transmission of Cfv between animals primarily occurs during coitus or by means of artificial insemination procedures (the use of contaminated semen collection equipment or poorly

treated semen). In bulls it has been demonstrated that contact with contaminated bedding may lead to infection (Schutte, 1969) and that transmission may also occur between bulls, during mounting, when large numbers of animals are enclosed together (Clark, 1971).

Campylobacter fetus subsp. *fetus* occurs mainly in the intestinal tract of cattle and sheep and may cause sporadic abortion in both these animals (Skirrow, 1994). The pathogenesis of Cff in bovines is very similar to that described for Cfv (Timoney *et al.*, 1988). Unlike infections with Cfv, Cff infection does not result in infertility.

In sheep, Cff is recognised as a significant causative agent of ovine abortions. In sheep once an infection has been introduced into a flock the infection spreads through the flock by the faecal-oral route. Heavy losses, with abortion rates of up to 50% can occur in flocks during the lambing season (van Bergen, 2005). Infection of ewes causes abortion in the last 6 weeks of pregnancy, stillbirths and birth of premature lambs. In ewes the organisms localize in the placentomes after a period of bacteraemia. Placentitis develops and abortion occurs toward the end of the gestation period. Ewes normally develop a vaginal discharge several days before they abort (Timoney *et al*., 1988) and may show signs of fever, depression and diarrhoea (Drost & Thomas, 1996).

In contrast to Cfv, transmission of Cff occurs through the contamination of food and water either with faeces or with aborted foetuses, membranes or discharges. Sheep probably serve as the primary reservoir of Cff whilst other mammals and birds are also believed to act as secondary reservoirs of infection (Garcia *et al.*, 1983). Venereal transmission does not occur (Timoney *et al.,* 1988).

Campylobacter fetus subsp. *fetus* is also recognized as an opportunistic pathogen in humans particularly compromised or immunodeficient patients. Infections are sporadic but a few food-borne outbreaks resulting from the consumption of raw beef, raw milk and cottage cheese have been reported (Mishu, Patton & Tauxe, 1992). The incidence of Cff infections in humans may, however, be underestimated since culture conditions routinely used in medical laboratories are optimised for the recovery of *Campylobacter jejuni* and *Campylobacter coli* and may result in cases going undiagnosed (van Bergen, 2005).

Table 2: An overview of the host preference and clinical importance of the two *C. fetus* subspecies (adapted from Quinn *et al*., 1994).

Species	**Principal host(s)**	**Disease and/or commensal status**
C. fetus subsp. *venerealis*	Cattle	Bovine genital campylobacteriosis: infertility, early embryonic death and occasional abortion.
C. fetus subsp. *fetus*	Sheep Cattle Humans Cattle/Sheep	Ovine genital campylobacteriosis: outbreaks of abortion Sporadic abortions Sporadic infections mainly in immuno-compromised people Commensal in the intestinal tract

1.6 Economic significance

Campylobacteriosis has been referred to as the "quiet profit taker" (Thompson & Blaser, 2000) since infection in a herd can easily be overlooked. Infection is usually not suspected until low calving rates are noted within a herd, by which time extensive losses would already have occurred (van Bergen, 2005). The primary economic losses incurred by the farmer are due to a decrease in calving percentages, delayed calving, culling of infertile animals and abortions (Irons *et al.*, 2004).

Research by Akhtar *et al.* (1993) indicated that infection with Cfv in dairy herds negatively influenced the production of milk in dairy herds but this was not quantified. Hum (2007) indicated that in Australia campylobacteriosis is responsible for significant reproductive wastage in infected beef and dairy herds. It was estimated that during the first year of infection, gross profit margins may be reduced by as much as 66% representing a large economic loss for producers. When the disease becomes established within a herd, profit margins may be as much as 36% below those of non-infected herds (McMillen *et al*., 2006).

Campylobacteriosis is currently classified by the OIE as a "List B" notifiable disease since it is deemed to have socioeconomic and/or public health implications. Consequently the presence of the disease influences the international trade of animals and animal products

(McMillen *et al*., 2006). Freedom from infection with Cfv is a requirement of many countries for the import and export of bovine semen, embryos and cattle, as well as for health certification of bulls in semen production and distribution centres. For this reason, accurate and reliable diagnostic tests for the diagnosis of campylobacteriosis are essential (Oyarzabal *et al*., 1997).

1.7 Diagnosis

Bovine genital campylobacteriosis can be diagnosed from samples taken from bulls, cows or aborted foetuses. Routine control or screening programmes for Cfv usually involves the testing of bull preputial samples and it has been shown that the chance of recovering Cfv is greater when testing specimens collected from bulls than those sampled from cows. It is usually recommended that all the bulls in a herd, or a selection of older bulls should be tested after the breeding season or whilst they are running with the cows (Irons *et al.*, 2004).

The latest *OIE Manual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals* details internationally accepted methods for the diagnosis of campylobacteriosis from bovine samples (OIE, 2008a).

1.7.1 Culture and identification

Sample collection

The quality of the samples submitted for diagnostic examination directly influences the accuracy of the results obtained and the clinical diagnosis. It is therefore necessary to ensure all samples are collected correctly and that aseptic techniques are practised (Irons *et al*., 2004).

In bulls, smegma may be obtained by scraping, suction or by preputial washing (OIE, 2008a). Comparative evaluation of the three sampling techniques by Tedesco, Errico & Del Baglivi (1977) indicated that scraping of the preputial and penile mucosa was the method of choice. The number of Cfv recovered from scraping samples was greater than the recovery rates obtained with either the suction or preputial washing methods. Furthermore, less contamination from background microflora was observed in scraping samples compared with the samples collected using the other two methods. Similar results have been reported by McMillen *et al.* (2006). By comparing quantitative *5' Taq* nuclease assay results the authors

confirmed that specimens collected from infected bulls using the scraping technique yielded higher estimates of Cfv cells than the other two collection methods. The authors commented further that the scraping technique was technically easier to perform than either the suction or washing techniques.

Cows or heifers should ideally be sampled when the animals are close to or are in oestrus (Irons *et al*., 2004). Cervicovaginal mucus may be obtained by swabbing, suction, or by washing the vaginal cavity. The use of a sterile speculum, for each animal sampled, is imperative for ensuring that good quality samples are obtained (OIE, 2008a).

The chances of recovering Cfv and diagnosing campylobacteriosis is greater when testing specimens collected from bulls than those sampled from cows. It is recommended that all the bulls in a herd, or a selection of older bulls should be tested after the breeding season or whilst they are running with the cows (Irons *et al*., 2004). The number of *C. fetus* in preputial samples of infected bulls may range from $<10^2$ to $>2 \times 10^5$ organisms per millilitre (Clark, 1971).

Foetal material, including the placenta, may also be tested to establish the possibility of infection with *C. fetus*. The stomach contents (abomasal fluid), lungs and liver have been shown to be the best samples for the recovery of the bacterium (OIE, 2008a).

Campylobacter fetus does not survive for more than 6-8 hrs in preputial samples. It is therefore critical that samples are collected, sent to the laboratory and cultured within this period. Where this is not possible use of a transport enrichment medium (TEM) should be made (Irons *et al*., 2004). With aborted foetal material however, *C. fetus* has been shown to survive for longer periods of time and experimental studies have demonstrated the recovery of the organisms as long as 72 hrs after abortion (OIE, 2008a).

The modified Weybridge medium described by Lander (1990a) has been evaluated extensively (Hum *et al.*, 1994; Lander, 1990b; Monke *et al.*, 2002) and is widely used due to its ease of preparation and its good performance. It has been shown to be superior to the other TEM's which have been described and used such as the Australian TEM (also known as Clark's medium), Cary-Blair TEM, modified SBL medium and 0,85% saline (Hum *et al*., 1994; Monke *et al.*, 2002). Weybridge medium has been shown to sustain the viability of Cfv during transit whilst concomitantly restricting the growth of commensal and contaminating

microorganisms. Lander (1990b) demonstrated that Cfv could be maintained in Weybridge medium for up to a week and still be successfully cultured.

Bacterial culture and isolation

Culture and identification of *C. fetus* is the most widely used means of diagnosing campylobacteriosis and is considered to be the golden standard (Brooks *et al.,* 2004). The isolation of *Campylobacter* spp. is notoriously difficult however, due to the fragility and the specific nutritional and atmospheric requirements of the bacterium. Specimens require careful and swift handling because of the limited viability of *C. fetus* outside the host (prolonged exposure to atmospheric levels of oxygen have a toxic effect on the bacteria). Furthermore, ubiquitous, faster growing microorganisms present in the sample, such as *Pseudomonas* spp. and *Proteus* spp., often contaminate the samples and make it difficult to detect the presence of *C. fetus* (Eaglesome & Garcia, 1992; Monke *et al.*, 2002).

Upon receipt in the laboratory specimens should be processed immediately. The samples may be cultured directly onto appropriate media or alternatively additional selection procedures may be employed to improve recovery rates. Centrifugation of preputial washings and filtration of samples through membrane filters of 0,65 µm pore size are frequently used (Irons *et al.,* 2004). The value of the latter technique is questionable, since the filters may retain a significant number of campylobacters, in addition to background microflora, potentially resulting in false negative results being obtained (Hum *et al*., 1994).

It is recommended that samples are plated onto at least two agar plates; one selective and the other non-selective (OIE, 2008a). A variety of selective media have been described for the isolation of *C. fetus* including Campylobacter selective agar (CSA), Skirrow's agar and Greenbriar Plus agar. Most of the media described contain different combinations of antimicrobials designed to improve selectivity and recovery of *C. fetus*. An overview of some of the most commonly used selective media is shown in Table 3 below.

Table 3: Selective culture media used for the isolation of *C. fetus* from clinical specimens

		SELECTIVE MEDIA				
		CSA (Dufty, 1967)	**Preston** (Bolton *et al.*, 1983)	**Skirrow's** (OIE, 2008a)	**Clark's** (OIE, 2008a)	**Greenbriar Plus** (Monke *et al*., 2002)
BASAL MEDIUM		Blood agar base	Nutrient broth No. 2	Blood agar base (39 g/L)	Peptone (10 g/L) Sodium chloride (5 g/L) Beef extract (5 g/L) Agar (15 g/L)	Eugon Agar (68,1 g/L)
BLOOD			5% lysed horse blood	5 - 7% lysed, defibrinated horse blood	10% defibrinated sheep blood	5% defibrinated sheep blood
ANTIBACTERIALS	Polymyxin B (IU/L)	1 000	5 000	2 500	1 000	500
	Trimethoprim (mg/L)		5	5		
	Bacitracin (IU/L)	15 000			15 000	7 000
	Vancomycin (mg/L)		10	10		
	Novobiocin (mg/L)	5			5	
	Albamycin (mg/L)					2,5
ANTIFUNGALS	Cyclo-heximide (mg/L)	20	100	50	10	50

One of the antimicrobials frequently incorporated into *Campylobacter* selective media is polymyxin B. Reports by Jones, Davis & Vonbyern (1985) and Hum *et al.* (1994) have indicated that polymyxin B sensitive strains of Cfv have been encountered. This finding has not resulted in alternatives for polymyxin B being sought; consequently the possibility that isolates are being missed and false negative results are being obtained cannot be dismissed.

When evaluating the efficacy of different selective media it is necessary to ascertain the recovery rate of *C. fetus* whilst simultaneously taking into consideration the level of contaminants encountered (Hum *et al.*, 1994). Investigations carried out by Monke *et al.*, 2002 compared the recovery of Cfv using three different culture media namely, blood, Skirrow and Greenbriar Plus agar. Results indicated that Cfv grew equally well on all three media but Skirrow's agar was the most effective at reducing the growth of contaminating microorganisms present in the samples.

From a global survey conducted by van Bergen and colleagues in 2002 it was ascertained that Skirrow's agar was the selective medium most commonly used in diagnostic laboratories (van Bergen *et al*., 2005b). The fact that this medium can be used for the simultaneous isolation of *Brucella abortus* is an additional benefit for many laboratories (Irons *et al*., 2004).

Bacterial identification

Following incubation at 37 °C under microaerophilic atmospheric conditions, *C. fetus* colonies usually become visible on the agar surface after 2 to 5 days. They often show a slight pink colouration, are round, convex, smooth and shiny with a regular edge (see Figure 2). After 3 to 5 days, colonies measure 1 - 3 mm in diameter; growth however, may be impeded by the presence of contaminating microflora (OIE, 2008a).

Figure 2: A 72-hr culture of *C. fetus* colonies on blood agar

Due to the limited biochemical reactivity of campylobacters, identification of isolates is restricted to the use of only a few biochemical tests. *Campylobacter* spp. do not ferment carbohydrates, consequently identification of isolates relies on other biochemical features including antibiotic resistance and the presence of enzymes. Typically the phenotypic characterization of *C. fetus* isolates involves: examining for atmospheric growth requirements (aerophilic, microaerophilic and anaerobic atmospheres); growth at 15, 25, 37 and 42 °C; ability to grow in media containing 1% glycine; reaction of oxidase and catalase; nitrate reduction; hydrogen sulphide production using lead acetate strips and growth on triple sugar iron agar, and susceptibility to nalidixic acid and cephalothin. The limited number of biochemical tests available for the identification of isolates is far from ideal as any isolate exhibiting an atypical characteristic may be incorrectly identified (Vargas *et al*., 2003). Atypical strains of *C. fetus* that grow at 42 °C, do not produce hydrogen sulphide and are resistant to cephalothin have been reported (Edmonds *et al.*, 1985).

For the purposes of accurate phenotyping it is imperative that all biochemical characterization tests are carried out under strictly standardized conditions as described by the OIE manual. (2008a). It was the work of On & Holmes (1991a) which demonstrated the influence of inoculum size on various biochemical tests commonly used for the identification of *Campylobacter* spp. Standardisation of the inoculum size to a density of approximately 10^6 CFU/ mℓ was shown to be necessary for ensuring the reproducibility of test results.

Currently the only method recognized by the OIE for the subspeciation of *C. fetus* isolates is tolerance to glycine: *Campylobacter fetus* subsp. *fetus* is able to grow in the presence of 1% glycine whilst Cfv is unable to (OIE, 2008a). However, the reproducibility of this assay is poor and the test can give ambiguous results (van Bergen *et al.*, 2005c). Furthermore, concerns exist over the reliability of this characteristic as it has been shown that tolerance to glycine can be acquired by transduction or mutation (Chang & Ogg, 1971). Glycine tolerant variants of Cfv have been described (Salama, Garcia & Taylor, 1992; Véron & Chatelain 1973) but it is not known for certain whether these results were accurate or subject to the use of poorly standardised testing methodology. Other biochemical tests have been described for the purposes of subspeciation but these tests, which include: selenite reduction, growth at 42 °C, susceptibility to metronidazole, cefoperazone, basic fuchsin and potassium permanganate, are only indicative and contradictory results have been obtained (Schulze *et al*., 2006).

Improvements in sampling techniques, the use of transport and/or enrichment media and the development of selective media have improved the recovery of *C. fetus* from specimens but nonetheless the culture method is still regarded as insensitive (Irons *et al*., 2004). Consequently a single negative culture result from the laboratory cannot be regarded as adequate for establishing the infection-free status of an animal. The testing of three samples, all yielding negative results are generally recommended (Irons *et al.*, 2004).

1.7.2 Immunological identification methods

Fluorescent antibody tests (FAT) have been used to provide a rapid screening method for the direct testing of diagnostic samples for the presence of *C. fetus* (Campero *et al*., 1993; Eaglesome & Garcia, 1992; Garcia *et al.*, 1983; Lander, 1990b; Seyyal *et al.*, 2000). Figueiredo *et al.* (2002) evaluated a direct FAT for the detection of *C. fetus* in preputial washings. The detection limit of the method ranged between 10^2 and 10^4 CFU/ mℓ and overall was found to be sensitive (92.59%) and specific (88.89%). The major limitations of this technique however, are the inability to distinguish between Cff and Cfv (OIE, 2008a; Garcia *et al*., 1983) and the limited availability of commercial antisera (van Bergen, 2005). Problems with non-specific background staining have also limited the use of this technique to being used as a confirmatory test following bacterial isolation (Figueiredo *et al.*, 2002).

1.7.3 Serological methods

A number of different serological techniques have been developed and evaluated to assess their ability to diagnose campylobacteriosis. The vaginal mucus agglutination, complement fixation and ELISA have all been employed by researchers (Seyyal *et al*., 2000). These tests are designed to screen reproductive material for the presence of *C. fetus* antibodies. Assays evaluating serum antibody levels have largely been unsuccessful as naturally infected cattle develop weak humoral immune responses to *C. fetus* (Ruckerbauer *et al*., 1971).

Vaginal mucus agglutination test

The vaginal mucus agglutination test (VMAT) has been used for testing herds for infection with *C. fetus*; it is not, however, considered to be a suitable test for identifying infected individuals within a herd since it is estimated that agglutinins (IgA) are only detectable in samples from about 50% of infected females. This test is ideally done on vaginal mucus collected 37 to 70 days after infection. In some animals, however, the development of an antibody response may be delayed for up to 3 to 4 months following infection (Clark, 1971).

Further, while some cows may exhibit a prolonged antibody response, up to several years, other animals may become negative within 2 months (OIE, 2008a). Consequently samples of mucus collected from reactors at oestrus may give false negative results, while, in non-reactors, blood in the mucus may lead to false positive results being obtained (Clark, 1971). False positive results may also occur with samples collected from animals with minor inflammatory lesions in the posterior reproductive tract (Eaglesome *et al*., 1983; Hewson, Lander & Gill, 1985).

Enzyme-linked immunosorbent assay (ELISA)

The Enzyme-linked immunosorbent assay, specific for secretory anti-Cfv IgA antibodies in the vaginal mucus following abortion, is a test recommended by the OIE (2008a). These antibodies are long lasting and their concentration remains constant in the vaginal mucus for several months (Hewson, Lander & Gill, 1985; Hum, Stephens & Quinn, 1991). Furthermore, vaccinated animals do not give false positive results with this test since their vaginal mucus will contain only IgG isotype antibodies (Hum, Stephens & Quinn, 1991). Investigations have shown this test method to have a specificity of 98,5% (Hum, Quinn & Kennedy, 1994) and a sensitivity greater than that obtained using the vaginal mucus agglutination test (Hewson, Lander & Gill, 1985). The same researchers made the recommendation that the ELISA is best used as a herd test because of the possibility of obtaining false positive results caused by antibody fluctuations in individual animals.

Attempts to demonstrate antibodies to Cfv in preputial material collected from bulls have largely been unsuccessful (Winter, 1982).

1.7.4 Molecular methods

Due to the inherent problems encountered with the culture and isolation of *C. fetus* and the relative lack of sensitivity which has been achieved, the application of molecular diagnostic techniques to identify *C. fetus* has received much focus since the early 1990's.

Wesley *et al.* (1991) were the first group of researchers to publish work describing the use of a DNA probe specific for *C. fetus*. The 29-mer DNA probe was based on the analysis of the gene encoding the 16S rRNA (rDNA). The probe successfully differentiated *C. fetus* from *C. hyointestinalis*, two species that share a 98% sequence homology in the 16S rDNA. The specificity of this probe was evaluated by a separate group of researchers using PCR and dot

blot hybridization. The probe was shown to react with only Cff and Cfv isolates (Blom *et al*., 1995).

The work of Cardarelli-Leite *et al.* (1996) and Eaglesome, Sampath & Garcia (1995) also focused on exploiting variability within the 16S rDNA to differentiate *C. fetus* from other *Campylobacter* spp. Both endeavours successfully demonstrated the specificity of their respective assays but a notable drawback is that restriction enzyme digestion of the PCR products was required to ultimately differentiate *C. fetus* from other closely related species.

The work of Oyarzabal *et al.* (1997) was aimed at developing a more efficient PCR assay for *C. fetus*, one which would not require multiple steps. Unique primers, based upon the comparison of the 16S rRNA gene sequences of *Campylobacter* spp. to that of related genera, were used. The primer pair selected clearly differentiated *C. fetus* from other campylobacter-like organisms (including *C. hyointestinalis*) but was unable to differentiate to subspecies level. The PCR products were visualized using gel electrophoresis eliminating the need for hybridization or endonuclease digestion of the amplicon. Evaluation of the PCR assay was limited to the identification of presumptive culture isolates and was not extended to the testing of diagnostic samples. No further work evaluating this method has been published to date.

The evaluation of 16S rRNA gene sequences have made it possible to develop a number of PCR assays suitable for the differentiation and identification of *C. fetus*. Alignment of the hypervariable regions within the 16S rDNA has indicated that only a single base mismatch differentiates the two *C. fetus* subspecies; making this region too conserved to enable successful differentiation of the two subspecies (Oyarzabal *et al*., 1997).

Oyarzabal *et al*. (1997) reported that investigations had been carried out to evaluate the feasibility of targeting the 23S rDNA to identify *C. fetus*. There has been, however, no further work published on the use of this particular target. The reason for this is not clear.

It was the work of Hum *et al.* (1997) that first evaluated a multiplex PCR-based assay for identifying and differentiating the two *C. fetus* subspecies. Using two primer pairs (see Table 3) initially developed by Panaccio, Stephens & Widders (1993), Hum *et al.* (1997) tested numerous bacterial strains using both PCR and conventional biochemical typing methods. The PCR assay was shown to produce rapid and reproducible results. Furthermore, this

multiplex PCR yielded two amplification products which could be clearly separated and visualized following gel electrophoresis, negating the need for further analysis of products.

Table 4: Details of the primer pairs used by Hum *et al.* (1997) for the detection and differentiation of *C. fetus* subspecies (Willoughby *et al*., 2005)

Primer	Sequence (5'-3')	Target Gene	Amplicon size	Species detected *
MG3F	ggtagccgcagctgctaagat	Carbon starvation protein gene	750 bp †	*C. fetus*
MG4R	tagctacaataacgacaact			
VenSF	cttagcagtttgcgatattgccatt	Unknown plasmid	142 bp	Cfv
VenSR	gcttttgagataacaataagagctt			

* The presence of an amplicon for MG3F and MG4R indicates that the organism is *C. fetus*, whilst subspeciation relies upon the absence of an amplicon for the primer pair VenSF and VenSR for Cff and the presence of that amplicon to subspeciate as Cfv.

† The original report by Hum *et al.* (1997) and On & Harrington (2001) indicated that the species-specific amplicon was 960 bp. Later reports by Wagenaar *et al.* (2001) and Müller, Hotzel & Schulze (2003), following sequencing of the amplicon indicated that the amplicon to be 750 bp in size. The reason for this discrepancy has not been identified.

Independent investigations (Müller, Hotzel & Schulze, 2003; On & Harrington, 2001; Vargas *et al.*, 2003) have indicated that the PCR method described by Hum *et al.* (1997) is both reliable and sensitive. Good correlation of results, albeit not 100%, was achieved when the PCR method was compared with traditional biochemical methods and more complex genotyping methods including random amplification polymorphic DNA (RAPD-PCR); pulsed field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) (Newell *et al.*, 2000; On & Harrington, 2001; Tu *et al.*, 2005; Vargas *et al.,* 2003; Wagenaar *et al.*, 2001).

Recently concerns have been raised regarding the specificity of the subspecies primers used by Hum *et al.* (1997). A poor degree of correlation between biochemical subspeciation results and PCR results was obtained by Willoughby *et al.* (2005) when a collection of *C. fetus* isolates from the United Kingdom were tested. Based on subsequent epidemiological studies these researchers proposed that the accurate subspeciation of field isolates could not

be accomplished with the subspecies primers used by Hum *et al.* (1997) due to the presence of unusual *C. fetus* strains circulating within the bacterial population. Similar concerns regarding the specificity of the subspecies primers have been expressed by van Bergen (2005). Working with a diverse collection of isolates obtained from several different countries, van Bergen (2005) applied routine phenotyping methods, the PCR protocol described by Hum *et al.* (1997), AFLP and multilocus sequence typing (MLST) to subspeciate isolates. A high degree of correlation between the AFLP and MLST results were observed in comparison to the results obtained using biochemical phenotyping and the PCR described by Hum *et al.* (1997). The highly specialised AFLP and MLST techniques were proven to be superior for subspeciation purposes but have the disadvantage of being exceptionally laborious and time-consuming. Furthermore, these techniques require expensive, specialised equipment which is not readily available in routine diagnostic laboratories (van Bergen, 2005).

The work of van Bergen (2005) also indicated that a number of discrepancies between the phenotyping and PCR subspeciation results were obtained for a collection of nine isolates obtained from South Africa. These results prompted Schulze *et al.* (2006) to propose that the strains from South Africa are evolutionary distinct from isolates obtained from other geographical areas. Further studies on a larger selection of local isolates are consequently warranted.

The subspecies primer set (VenSF and VenSR) amplifies a plasmid-encoded DNA sequence present in Cfv but not Cff. The DNA sequence of the PCR product generated has however not been available in public databases up until recently. Sequencing data obtained and registered by Willoughby *et al.* (2005) indicated that the PCR product has homology with *parA*, a gene encoding a protein which is thought to be associated with DNA segregation during prokaryote cell division. It was also noted that in *C. jejuni* a *parA* homologue is located on a virulence plasmid. Since the presence of this target sequence is believed to be plasmid-encoded and has been shown to correlate with phenotypic subspeciation of *C. fetus* the possibility that plasmid loss or acquisition may influence virulence has been raised. A number of different plasmids are known to occur in Cff and Cfv but there appears to be no obvious difference between the plasmid profiles of the two subspecies. Consequently the relationship between plasmid-encoded *parA* and *C. fetus* subspecies is questionable and further work on plasmid characterisation and carriage in *C. fetus* is needed (Willoughby *et al.*, 2005).

Wang *et al.* (2002) also developed a subspecies specific PCR. The primers were designed from the published *sapB2* (surface layer protein) gene sequence and shown to produce Cff specific amplicons. The original work involved the testing of only a few isolates and subsequent investigations by other researchers (Willoughby *et al*., 2005) have revealed several inconsistencies between traditional phenotyping methods and PCR results using this method. The suitability of the targeted gene sequence has also been questioned as there has been no evidence to indicate that the targeted region of the *sap* gene was subspecies specific (Willoughby *et al*., 2005).

A recent publication (Abril *et al.*, 2007) describes the identification of a new insertion element which was shown by the researchers to be a highly conserved region found exclusively in Cfv strains. The published results are promising but further evaluation, using a more diverse collection of *C. fetus* isolates, is necessary. The original research only evaluated 26 Cfv strains and no consideration was given to Cfv biovar *intermedius* strains.

Limited research exists on the use of PCR to examine diagnostic specimens directly for the presence of *C. fetus*. Eaglesome *et al.* (1995) described the use of PCR followed by restriction enzyme analysis to detect Cfv in experimentally infected semen samples. More recently however, a novel *5' Taq* nuclease assay, based on the primer sets described by Hum *et al.* (1997) has been described (McMillen *et al*., 2006). The assay was shown to be far more sensitive than conventional culture and PCR techniques for the detection of Cfv from smegma and mucus. The assay was able to detect a single Cfv cell in a spiked sample compared to 100 cells in the conventional PCR assay and 2 500 cells from selective culture.

1.8 Treatment and Control

Streptomycin is the antibiotic most frequently administered for the treatment of *C. fetus* infections. Preputial infusions of a dihydrostreptomycin solution together with subcutaneous injections of the antibiotic have been recommended for the treatment of bulls (Eaglesome & Garcia, 1992). The usefulness of treating infected cows is questionable since recovery usually occurs spontaneously within 5 months and the level of immunity acquired affords the animals protection from re-infection. Recovery can be aided however, by intrauterine infusions of streptomycin and penicillin (Drost & Thomas, 1996).

Additional measures which have been found to be effective in controlling bovine campylobacteriosis include: the separation of infected from non-infected herds, artificial

insemination and vaccination (Eaglesome & Garcia, 1992). Artificial insemination (AI) is considered to be the most effective means of controlling campylobacteriosis (Irons *et al*., 2004) as is evidenced in areas where farming practices have changed from natural breeding of animals to monitored AI programmes (Figueiredo *et al.*, 2002). Critical to the success of these programmes are the measures in place at AI centres to ensure the safety and "disease-free" status of the semen that is distributed. Thorough screening and testing of all bulls included in such programmes and treatment of harvested semen are imperative. The treatment of semen with various combinations of antibiotics has been investigated and is now extensively applied. Shisong, Redwood & Ellis (1990) demonstrated that the treatment of artificially contaminated semen with a combination of penicillin, streptomycin, lincomycin and spectinomycin for 40 minutes at 35 °C reduced the number of *C. fetus* bacteria to non-detectable levels (Eaglesome & Garcia, 1992).

Two vaccines are registered for use in South Africa. Onderstepoort Biological Products produces an oil-adjuvanted formalin-inactivated Cfv and Cff bacterin. The other, marketed by Pfizer Animal Health, comprises inactivated cultures of *C. fetus* in combination with *Leptospira* cell components. Initial vaccination of animals should consist of two injections, four weeks apart, with the second being administered shortly before the onset of breeding. A single booster injection given four weeks before each ensuing breeding season will provide the animals with a high degree of protective immunity (Onderstepoort Biological Products, undated). With some bulls, dual vaccination with a double dose of bacterin, four weeks apart, has been found to be curative (Irons *et al*., 2004).

STUDY AIMS AND OBJECTIVES

Problem statement and benefits

Routine bacteriological culture techniques employed in diagnostic laboratories for the detection of *C. fetus*, lack sensitivity and potentially result in a large number of false negative results being reported. The implementation of a diagnostic method with greater sensitivity will improve the diagnostic service by enabling more accurate results to be obtained in a shorter period of time. This improved service would be of direct benefit to cattle owners who need to identify infected animals and effect treatment and/or control procedures immediately, for the purposes of containment. Additionally, the availability of an accurate and sensitive

diagnostic method will enable epidemiologists to establish and monitor the prevalence of *C. fetus* more closely.

Subspeciation of isolates is currently being accomplished using a single phenotypic criterion which is known to be unreliable. In order to accurately discriminate between Cff and Cfv more reliable methods need to be sought and evaluated and if necessary modified.

Study aims and objectives

The primary aim of this investigation was to evaluate and implement a previously described PCR assay for the detection of *C. fetus* directly from bovine preputial specimens (Part A).

The second part of this investigation examined both traditional phenotyping methods and a published multiplex PCR assay for the subspeciation of a collection of South African field isolates (Part B).

The specific objectives of the investigation are outlined as follows:

PART A:

1 Evaluate the specificity of the primer set described by Hum *et al*. (1997) by testing reference and field strains of *C. fetus* together with a collection of other microorganisms frequently isolated from the genital tract of cattle.

2 Optimise the PCR assay for the detection of *C. fetus* in Weybridge medium and evaluate the influence of potential PCR inhibitors (semen, blood, urine and faeces) on the sensitivity of the assay.

3 Spike bull preputial samples and qualitatively evaluate the ability of the PCR method to detect *C. fetus* in the sample matrix. Concurrently determine the *sensitivity* of the PCR method and the bacteriological culture method for the detection of *C. fetus* from spiked samples.

4 Field evaluation of the PCR method: All diagnostic specimens submitted to the laboratory during a fourteen month period will be tested using both the PCR assay and the culture method.

PART B:

1 Optimize the multiplex PCR described by Hum *et al*. (1997) for the subspeciation of *C. fetus* isolates using reference cultures from a recognized international culture collection.

2 Subspeciate a collection of South African *C. fetus* field isolates using standard phenotyping tests (tolerance to 1% glycine, sensitivity to cefoperazone and selenite reduction) and the multiplex PCR assay.

CHAPTER 2: MATERIALS AND METHODS

A PCR assay based on the species-specific primer set described by Hum *et al.* (1997) was evaluated to ascertain it's suitability for the detection of *C. fetus* in clinical specimens. The specificity of the assay was assessed using a panel of pure cultures of different bacteria. Following which the sensitivity and the robustness of the assay was investigated by spiking transport medium and eventually preputial specimens, with different concentrations of Cfv. The final stage of assay validation comprised field evaluation where all clinical specimens received by the laboratory during the course of a 14 month period were tested using both the PCR assay and the traditional culture method.

The second part of this investigation was primarily concerned with the subspeciation of *C. fetus* isolates. The specificity of the multiplex PCR assay described by Hum *et al.* (1997) was evaluated using reference strains before being used to subspeciate a collection of South African field isolates. The *C. fetus* field isolates were also subspeciated using traditional phenotyping tests.

PART A: Evaluation of a *C. fetus* specific PCR for the direct detection of the target organism in bovine preputial specimens

2.1 Establishing the specificity of the species-specific PCR assay

The specificity of the PCR method was determined by testing a collection of reference and South African field *C. fetus* isolates together with other bacterial and protozoal isolates which may be found in the genital tract of cattle. A list of all the organisms tested is given in Table 5 below.

Table 5: Bacterial and protozoal isolates used to evaluate the specificity of the *C. fetus* specific primer set (Mg3F and Mg4R)

Microorganism	Source of Isolate[#]
***Campylobacter fetus* strains**	
C. fetus subsp. *fetus*	NCTC 10842 (France)
C. fetus subsp. *fetus*	5515 (United States of America)
C. fetus subsp. *venerealis*	NCTC 10354 (United Kingdom)
C. fetus subsp. *venerealis*	LMG 6570 (Belgium)
C. fetus subsp. *venerealis* intermedius	10 (South Africa)
C. fetus subsp. *venerealis* intermedius	136 (South Africa)
C. fetus	Field Isolates (40)
***Campylobacter* spp. (excluding *C. fetus* strains)**	
C. coli	ATCC 12102
C. jejuni subsp. *jejuni*	ATCC 29428
C. jejuni	Field Isolates (2)
C. sputorum subsp. *bubulus*	Field Isolates (4)
C. hyointestinalis	Field Isolates (2)
***Non-Campylobacter* species**	
Arcanobacterium pyogenes	ATCC 19411
Arcobacter sp.	Field Isolate
Brevundimonas vesicularis	Field Isolate
Corynebacterium striatum	Field Isolate
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 25922
Histophilus somni	ATCC 700025
Histophilus somni	Field Isolates (2)
Ochrobacter sp.	Field Isolate
Proteus sp.	Field Isolate
Pseudomonas aeruginosa	ATCC 27853
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 12228
Stenotrophomonas maltophilia	Field Isolate
Tritrichomonas foetus	Field Isolates (2)

[#]The number of strains tested, if greater than one, is given in brackets

2.1.1 Preparation of isolates for PCR

All bacterial isolates were grown on Columbia blood agar[1] supplemented with 5% sheep blood, at 37 °C under microaerophilic atmospheric conditions. Appropriate atmospheric conditions were generated in anaerobic jars using *Campylobacter* gas generating kits[1] . The *Tritrichomonas foetus* cultures were maintained in *Trichomonas* medium[1]. Suspensions, corresponding to a 0,5 MacFarland standard, were prepared in phosphate buffered saline (PBS) (pH 7.2) for each of the isolates.

2.1.2 DNA extraction

The DNeasy® blood and tissue kit[2] was used for the extraction of DNA from all test cultures. The protocol was used according to the recommendations of the manufacturer. Briefly; 200 µℓ of each test suspension was centrifuged at 5 000 x g following which the supernatant was discarded. Pellets were resuspended in 180 µℓ of tissue lysis buffer (supplied with the kit) to which 20 µℓ proteinase K was added. Samples were vortexed and then incubated at 56 °C for 10 min. Following incubation, samples were vortexed and then 200 µℓ AL buffer and 200 µl 96% ethanol were added. Samples were vortexed again before being transferred to spin columns and centrifuged at 6 000 x g for 1 min. The flow-through and sample collection tubes were discarded, and the spin columns transferred to new collection tubes. The spin columns were washed in two consecutive steps using 500 µℓ wash buffer 1 followed by 500 µℓ wash buffer 2. Bound DNA was finally eluted by the addition of 200 µℓ elution buffer to the spin column and centrifugation at 6 000 x g for 1 min. Extracts were stored at 4 to 8 °C and tested within 5 days of extraction. For long term storage, DNA extracts were kept at -20 °C.

2.1.3 PCR amplification

Primers

The species-specific primer pair evaluated by Hum *et al*. (1997) was used. The nucleotide sequences of the forward and reverse primers are given in Table 6 below.

[1] Oxoid, Basingstoke, England
[2] Qiagen, GmbH, Hilden, Germany

Table 6: Details of the species-specific primer pair evaluated by Hum *et al.* (1997) and used in this investigation

Primer designation	**Nucleotide sequence**
Forward primer (MG3F)	5'- GGT AGC CGC AGC TGC TAA GAT -3'
Reverse primer (MG4R)	5'- TAG CTA CAA TAA CGA CAA CT -3'

Preparation of PCR reactions

PCR reactions were carried out in 50 µℓ reaction volumes each containing the following:

5 µℓ PCR buffer [100 mM Tris-HCl; 1.5 mM $MgCl_2$; 500 mM KCl, pH 8.3] [3]

2 µℓ 2 mM dNTPs [3]

1 µℓ 10 µM Mg3F forward primer [4]

1 µℓ 10 µM Mg 4R reverse primer [4]

0,2 µℓ *Taq* polymerase [3]

1 µℓ DNA template

Each PCR run included a positive control (comprising 1 µℓ of DNA from Cfv reference strain ATCC 19438) and a negative control (1 µℓ distilled water).

The thermocycling was performed in a BioRad® MJ Mini thermocycler[5]. An initial denaturation (95 °C for 15 s) was followed by 35 cycles of denaturation (15 s at 96 °C), primer annealing (60 s at 60 °C) and primer extension (90 s at 72 °C). The reaction was terminated by a final extension step (180 s at 72 °C) (Schulze *et al*., 2006).

[3] Roche Diagnostics, South Africa
[4] Integrated DNA Technologies, South Africa
[5] BioRad, South Africa

2.1.4 Gel electrophoresis

PCR products were analysed by electrophoresis using 1,5% agarose[3] gels in 1 x Tris-Borate-EDTA (TBE) buffer[6] at 100 V. Following staining in ethidium bromide (0.5 µg/ mℓ), the DNA bands were visualised using a gel documentation system and sized against a 100 bp DNA ladder[7].

2.1.5 DNA sequencing

To confirm the specificity of the species-specific primer set, amplicons from one of the Cfv reference strains (LMG 6570) and one of the field isolates (Reference Bank Number 59) were submitted to Inqaba Biotec for sequencing.

2.2 Optimization of the PCR assay for the detection of *C. fetus* in Weybridge transport medium and evaluation of potential inhibitors on the sensitivity of the assay

All published investigations using the species-specific primer pair originally evaluated by Hum *et al*. (1997) have been limited in their investigations to the testing of bacterial isolates. It was therefore necessary to evaluate and optimize the PCR assay for the detection of *C. fetus* in Weybridge medium which was used as a transport medium throughout this investigation. A series of tests were carried out to ascertain what combination of test variables and/or reagent concentrations gave the best PCR results (ie. the most distinct bands at the lowest bacterial inoculum concentration). Following optimization, the sensitivity of the PCR assay was determined (in triplicate) using bacterial dilutions prepared in the transport medium.

2.2.1 Optimization and determination of the sensitivity of the PCR assay

Preparation of spiked Weybridge samples

From a two day old culture of the Cfv reference strain (ATCC 19438) a bacterial suspension with a turbidity corresponding to a 0.5 McFarland standard was prepared in PBS supplemented with 0,02% Tween 80 (PBS-T). Fifty microlitres of the bacterial suspension was transferred to 4,95 mℓ Weybridge medium to give a 1:100 dilution of the initial suspension. From this dilution five ten fold dilutions were prepared in Weybridge medium.

[6] Sigma-Aldrich, South Africa
[7] Inqaba Biotec, South Africa

From each of the dilutions 100 µℓ was spread plated onto the surface of pre-poured blood agar plates. This was done in triplicate for each of the dilutions. Following 3 days of incubation at 37 °C under microaerophilic conditions, the bacterial colonies were enumerated and the average number of viable cells per millilitre Weybridge medium was determined for each of the dilutions.

DNA extraction

DNA was extracted from 500 µℓ of each dilution as outlined in 2.1.2. Two modifications were, however, made to this protocol, namely: the incubation of samples at 56 °C was carried out for a minimum of at least one hour and final elution of the DNA from the spin column was made in 100 µℓ (instead of 200 µℓ) elution buffer.

PCR amplification

PCR reactions were prepared as described in section 2.1.3. In separate PCR reactions the volume of *Taq* polymerase used was varied (0,2 µℓ, 0,4 µℓ, 0,6 µℓ and 1 µℓ volumes). The volume of extracted DNA added to the PCR reaction mix was also varied (1 µℓ, 2 µℓ and 5 µℓ). Each PCR run carried out included positive and negative controls as outlined in 2.1.3.

For all PCRs, the thermocycling protocol given in section 2.1.3 was followed and amplicons were analyzed by electrophoresis as described in section 2.1.4.

2.2.2 Evaluation of potential inhibitors on the PCR assay

The influence of bovine blood, faeces, urine and semen as potential PCR inhibitors, were evaluated. The 10^3 and 10^4 Cfv-inoculated Weybridge dilutions (prepared in 2.2.1), (the two dilutions immediately above the detection limit of the assay) were spiked with varying concentrations of bovine blood, faeces, urine and semen as illustrated in Figure 3.

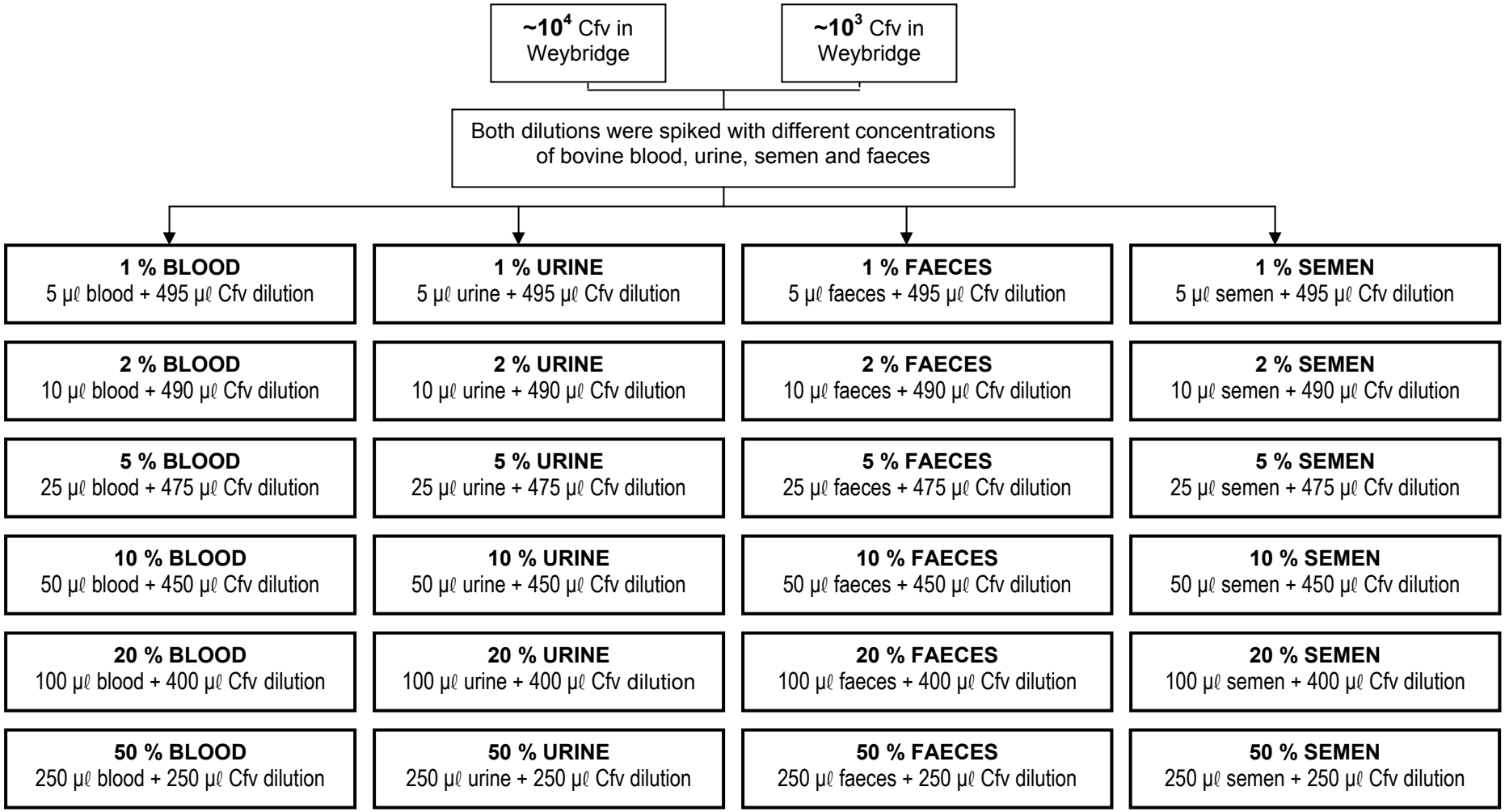

Figure 3: Schematic outline of the spiking of Cfv dilutions in Weybridge medium with different concentrations of blood, urine, faeces and semen.

PCR

DNA was extracted from 500 µℓ of each of the Cfv dilutions prepared in Weybridge medium and spiked with different dilutions of blood, urine, faeces and semen (48 aliquots in total). Extraction, amplification and analysis of PCR products was carried out as outlined in 2.1.2 – 2.1.4 using the modifications stated in 2.2.1 to improve PCR sensitivity. These modifications are summarized as follows:

- During DNA extraction the clinical material was incubated at 56 °C for a minimum of at least one hour;
- Extracted DNA was eluted from the Qiagen® spin column using 100 µℓ elution buffer;
- The volume of *Taq* polymerase incorporated into each PCR reaction was increased to 0,6 µℓ;
- Two microlitres of extracted DNA was used in the PCR.

In each PCR run, extracted DNA from a reference strain (Cfv, ATCC 19438) was used as a positive control whilst sterile distilled water was used as a negative control.

2.3 Evaluating the sensitivity of the PCR and culture methods for the detection of *C. fetus* in spiked bovine preputial specimens

The sensitivity of the PCR method to detect *C. fetus* directly from clinical preputial specimens was evaluated by spiking pooled preputial specimens collected in Weybridge transport medium. The procedure was repeated in triplicate using samples collected from different herds.

2.3.1 Sample selection and collection

Bulls fulfilling at least two of the following criteria were identified and sampled for the purposes of fulfilling this aim of the project:

- Animals should ideally have been vaccinated at least six months prior to intended sampling
- Less than two years of age
- Must have recently been tested and found to be negative for *C. fetus*
- No recent history (last 5 years) of venereal diseases on the farm

Sheath scrapings were performed according to the protocol outlined below (Irons, undated)

Procedure for performing sheath scrapings

- ❒ Restrain bull in a sturdy crush, tie the legs back or apply electro-stimulation.
- ❑ Cut any long hair around the preputial opening and wash and dry the area if it is excessively dirty.
- ❑ Attach a sterile 20 mℓ sterile syringe to the connector-piece of a perspex uterine infusion pipette and pass the dry pipette up the preputial cavity.
- ❑ With one hand guide the pipette whilst using the other hand to apply forwards-and-backwards scraping movements whilst simultaneously aspirating some cellular content from the *glans penis* as well as the fornix area.
- ❑ Withdraw the pipette and add approximately 1 mℓ of the aspirate to Weybridge medium.

Samples were transported to the laboratory immediately following collection. On all three occasions no more than six hours elapsed from the time of sampling commencing and the laboratory processing of the samples. At the laboratory the individual samples were combined and mixed to form a representative pooled sample. From this pooled sample, six 4,5 mℓ aliquots were dispensed and prepared for bacterial inoculation (‘spiking’). The following procedure is diagrammatically represented in Figure 4.

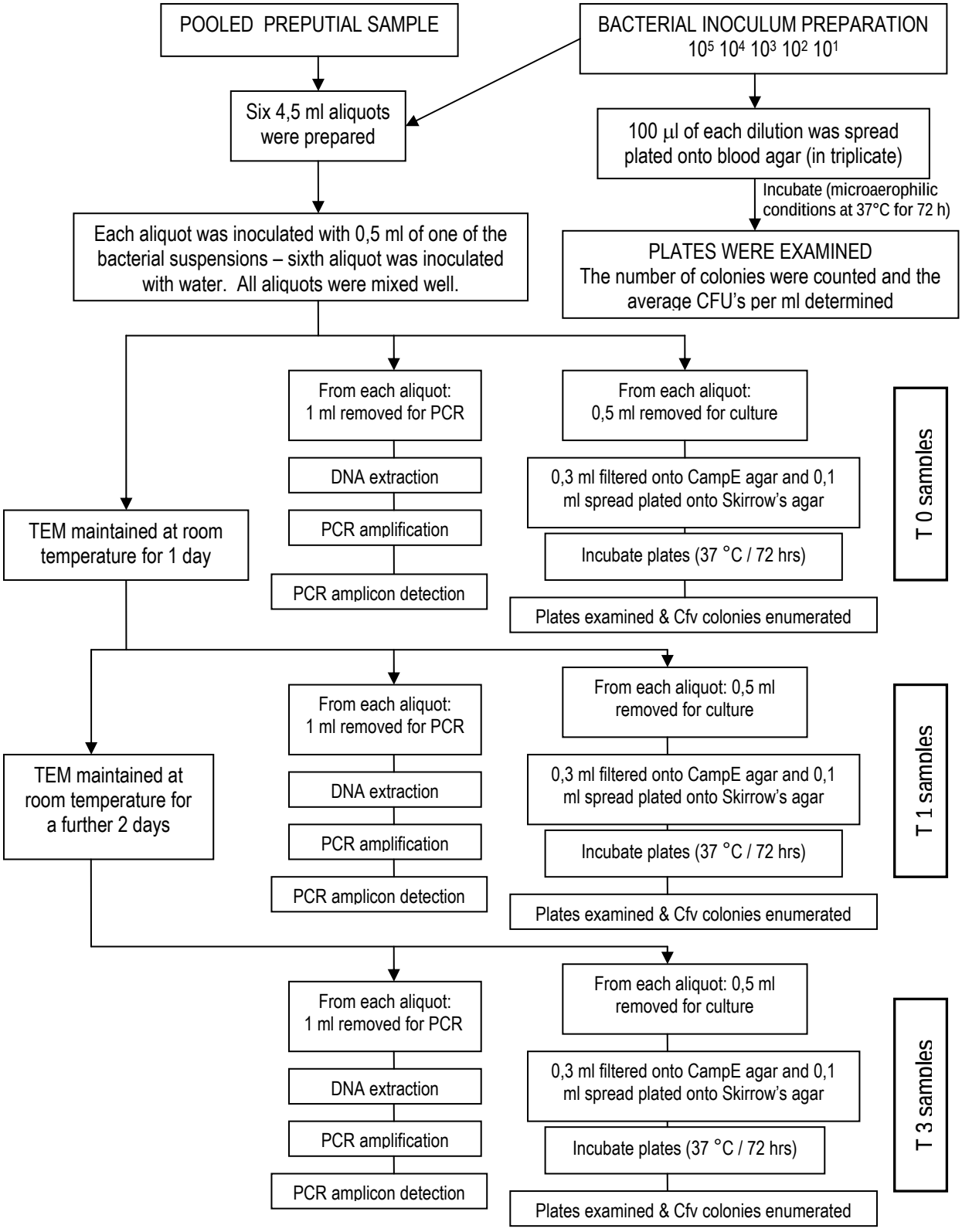

Figure 4: Schematic outline of the spiking of preputial specimens to ascertain the sensitivity of the PCR and culture methods.

2.3.2 Spiking of samples

From a two day old culture of the Cfv reference strain (ATCC 19438) a bacterial suspension with a turbidity corresponding to a 0,5 McFarland standard (approximately 10^8 CFU/ mℓ) was prepared in PBS supplemented with 0,02% Tween 80 (PBS-T). From a 1:100 dilution of this suspension a further four 1:10 serial dilutions were prepared. From each of these four dilutions 100 µℓ was spread plated onto the surface of pre-poured blood agar plates in triplicate. Following 3 days of incubation at 37 °C under microaerophilic conditions, the bacterial colonies were enumerated and the average number of cells per millilitre was determined for each of the dilutions.

2.3.3 Specimen inoculation

Five of the 4,5 mℓ aliquots prepared from the pooled sample were inoculated with 0,5 mℓ of one of the bacterial dilutions prepared. The remaining 4,5 mℓ aliquot of the pooled sample was inoculated with 0,5 mℓ of sterile distilled water and was processed along with all the spiked aliquots as a negative control.

Each of the inoculated samples was mixed well before 1 mℓ from each aliquot was removed for PCR and 1 mℓ for bacterial culture. This was the time = 0 (T 0) samples. The remainder of each of the inoculated sample aliquots were stored at ambient temperature for 24 hrs before a further 1 mℓ was removed from each aliquot for PCR and 1 mℓ for bacterial culture (Time = 1 or T 1 samples). The inoculated aliquots were stored for a further two days before appropriate volumes were again removed for PCR and bacterial culture (Time = 3 days or T 3 samples).

Following the first trial, modifications to the protocol were made to include the removal and testing of aliquots of the spiked samples at T 2 (i.e. 2 days post-inoculation)

2.3.4 Bacterial culture

Approximately 0,5 mℓ of each aliquot removed from the spiked samples were filtered through a 0,65 µm cellulose acetate membrane filter onto the surface of a blood agar plated supplemented with FBP, a *Campylobacter* growth enrichment supplement (CampE agar). A further 0,1 mℓ of each of the aliquots were spread plated onto Skirrow's agar. All plates were incubated under microaerophilic conditions for 3 days before examination. Colonies resembling Cfv (small, smooth and translucent) were enumerated and stained to confirm typical *Campylobacter* microscopic morphology. The sensitivity of the culture method was determined by establishing the lowest Cfv bacterial inoculum at which Cfv was recovered successfully from the spiked sample.

2.3.5 PCR

Aliquots removed for PCR at each time interval were frozen at -18 °C and processed on completion of each trial.

PCR testing was carried out as described in 2.1.2 – 2.1.4, with the following modifications ascertained in 2.2.1 to improve PCR sensitivity. Each PCR run included a positive and negative control as described in section 2.1.3.

The sensitivity of the PCR method was established by viewing the PCR amplification products of each of the spiked aliquots. The lowest Cfv bacterial inoculum concentration at which the *C. fetus* PCR amplicon could be observed was taken as the detection limit of the assay.

2.4 Field evaluation of the PCR assay

All diagnostic specimens submitted to Allerton Provincial Veterinary Laboratory (PVL) during the 14 month evaluation period (June 2007 to August 2008) were tested using both the traditional bacterial culture method and the PCR assay. The aim was to receive and process at least 160 sheath scrapes samples.

2.4.1 Bacterial Culture

All clinical specimens were cultured immediately upon receipt in the laboratory. Based on the information supplied by the private practitioner responsible for collecting and submitting the samples, it was confirmed that all samples were processed within 36 hrs of collection. Each specimen was cultured onto two agar plates, one selective (Skirrow's agar) and one non-selective (CampE agar). A 0,1 mℓ volume of each sample was spread-plated onto a Skirrow's agar plate whilst a further 0,3 mℓ of each sample was filtered through a 0,65 µm cellulose acetate membrane filter onto a CampE plate. Plates were set up in anaerobic jars within 20 minutes of plating out. Microaerophilic conditions were created in the jars using gas generating kits[1]. The plates were incubated at 37 °C for a minimum of 3 days before examination.

Following incubation, all plates were examined for the presence of small (1-3 mm diameter), smooth and shiny colonies having a slightly grey to pink appearance (OIE, 2008a). Suspect colonies were Gram stained and if slender, curved Gram negative rods were observed, the colonies were re-streaked onto Columbia blood agar plates to purify and bulk up growth for further testing. Preliminary tests used to establish whether the

suspect isolates belonged to the genus *Campylobacter* included: catalase and oxidase production; growth at 25 °C; anaerobic growth at 37 °C; no hydrogen sulphide production on TSI agar. All biochemical and other characterization tests were carried out as outlined in Appendix 2.

2.4.2 PCR

Two millilitre aliquots of each clinical specimen were stored at -20 °C until processing. The PCR protocol outlined in 2.1.2 – 2.1.4, with modifications given in 2.2.1, was used to process aliquots of all clinical specimens.

Each PCR included a positive and negative control as described in 2.1.3. In addition to this, for each submission a composite sample, comprising equal volumes of each of the samples in the submission, was prepared. The composite sample was inoculated with a 10^5 suspension of Cfv in Weybridge medium (1:10 ratio) and processed along with all the samples comprising the submission.

PART B: SUBSPECIATION OF *C. FETUS* ISOLATES

2.5 Evaluating the specificity of the subspecies PCR assay

The specificity of the subspecies primer set described by Hum *et al.* (1997) was evaluated using six international *C. fetus* reference cultures. Details of these cultures are given in Table 7.

Table 7: International reference cultures used for PCR specificity testing

Bacterium	**Strain and Source of isolate**
C. fetus subsp. *fetus*	NCTC 10842 (France)
C. fetus subsp. *fetus*	5515 (United States of America)
C. fetus subsp. *venerealis*	NCTC 10354 (United Kingdom)
C. fetus subsp. *venerealis*	LMG 6570 (Belgium)
C. fetus subsp. *venerealis* intermedius	10 (South Africa)
C. fetus subsp. *venerealis* intermedius	136 (South Africa)

2.5.1 DNA extraction

DNA extracts prepared in Part A, section 2.1.2 were used for this purpose.

2.5.2 PCR amplification

Subspecies primers

The subspecies-specific primer pair evaluated by Hum *et al.* (1997) was used. The nucleotide sequences of the forward and reverse primers are given in Table 8.

Table 8: Details of the two primer pairs evaluated by Hum *et al.* (1997) and used in this investigation to subspeciate isolates

Primer pairs		Nucleotide Sequence
Species-specific primer pair	**Forward primer (MG3F)**	5'- GGT AGC CGC AGC TGC TAA GAT -3'
	Reverse primer (MG4R)	5'-TAG CTA CAA TAA CGA CAA CT -3'
Subspecies-specific primer pair	**Forward primer (VenSF)**	5'- CTT AGC AGT TTG CGA TAT TGC CATT -3'
	Reverse primer (VenSR)	5'- GCT TTT GAG ATA ACA ATA AGA GCTT -3'

Preparation of multiplex PCR reactions

PCR reactions were carried out in 50 µℓ reaction volumes each containing the following:

5 µℓ PCR buffer [100 mM Tris-HCl; 1.5 mM $MgCl_2$; 500 mM KCl, pH 8.3][3]

2 µℓ 2 mM dNTPs [3]

1 µℓ 10 µM Mg3F forward primer [4]

1 µℓ 10 µM Mg 4R reverse primer [4]

0,2 µℓ *Taq* polymerase [3]

1 µℓ DNA template

The thermocylcing was performed in a BioRad® MJ Mini thermocycler. An initial denaturation (95 °C for 180 s) was followed by 30 cycles of denaturation (20 s at 95 °C), primer annealing (20 s at 52 °C) and primer extension (120 s at 72 °C) (Hum *et al*., 1997). PCR product detection was carried out as described in section 2.1.4.

2.6 Comparison of traditional phenotyping methods and the multiplex PCR for the subspeciation of *C. fetus* isolates.

2.6.1 Multiplex PCR for the subspeciation of *C. fetus* isolates

A collection of 40 South African field isolates were subspeciated using the multiplex PCR previously evaluated (section 2.4). A Cfv and a Cff reference strain were used as controls, with each of the PCR runs carried out.

2.6.2 Biochemical characterization tests

The following phenotyping tests were carried out on the six *C. fetus* reference isolates and the collection of 40 field isolates using 72-hr bacterial cultures:

- Antibiotic susceptibility: filter disks impregnated with cephalothin (30 µg), cefoperazone (30 µg), nalidixic acid (30 µg) and metronidazole (5 µg)
- Hydrogen sulphide production (in triple sugar iron agar and brain heart infusion broth supplemented with L-cysteine)
- Selenite reduction
- Tolerance to 1% glycine
- Atmospheric requirements (anaerobic growth and growth in air)
- Temperature dependence (growth at 25 °C and 42 °C)

Details of all test procedures are given in Appendix 2. All phenotyping tests, with the exception of atmospheric and temperature requirements, were carried out in triplicate. Replicate tests were carried out on separate occasions using different batches of freshly prepared media. Both Cff and Cfv reference strains were inoculated and tested with each batch of tests carried out.

CHAPTER 3: RESULTS

PART A: EVALUATION OF A *C. FETUS* SPECIFIC PCR FOR THE DIRECT DETECTION OF THE TARGET ORGANISM IN BOVINE PREPUTIAL SPECIMENS

3.1 Establishing the specificity of the species-specific PCR assay

The PCR results assessing the specificity of the subspecies primer set, Mg3F and Mg4R, are given in Appendix 3. All *C. fetus* isolates yielded a single PCR amplicon of approximately 750 bp. No amplicons were observed with any of the other *Campylobacter* strains or the collection of genital-associated microbes which were tested.

The specificity of the primer pair was further demonstrated by DNA sequencing and alignment. PCR amplification products from one of the reference Cfv strains (LMG 6570) and a field isolate (Reference Bank Number 59) were sent to Inqaba Biotec for sequencing. Forward and reverse sequences for each isolate were assembled before a BLAST search was carried out in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). The accession numbers of isolates used to compare sequencing data are shown in Table 9. Both sequences showed 100% similarity with the three sets of sequencing data.

Table 9: Accession numbers of sequencing data of 3 isolates used to show 100% similarity with the DNA sequences obtained following sequencing of PCR amplicons of Cfv strain LMG 6570 and a field isolate

Accession Number	Description
CP000487.1	*Campylobacter fetus* subsp. *fetus* 82-40, complete genome
AY158814.1	*Campylobacter fetus* subsp. *fetus* 82-40, complete genome
AY158813.1	*Campylobacter fetus* subsp. *fetus* carbon starvation protein gene, partial cds

3.2 Optimization and sensitivity of the PCR assay for the detection of *C. fetus* in Weybridge transport medium and evaluation of potential inhibitors on the sensitivity of the assay

A volume of 0,6 µℓ *Taq* polymerase (1 IU/µℓ) was found to yield the best PCR results providing clear, sharp bands. No notable improvements were observed when a higher volume of enzyme was used. Similarly, the increase in DNA template from 1 µℓ to 2 µℓ made significant improvement to the PCR results with little difference being noted when the volume was increased from 2 µℓ to 5 µℓ. Consequently an enzyme volume of 0,6 µℓ and a template volume of 2 µℓ were used for all further assays.

The sensitivity of the optimized PCR assay was ascertained by testing a series of Cfv dilutions prepared in Weybridge medium. Three Cfv-inoculated dilution series were prepared and tested. The results hereof are given in Table 10.

Table 10: Evaluation of the sensitivity of the PCR assay using suspensions of Cfv prepared in Weybridge medium

Bacterial suspension in PBS-T	Bacterial count (Cfv / mℓ)	Number of Cfv cells subjected to extraction	Number of Cfv 'DNA units' added to the PCR reaction	Presence of a 750 bp PCR amplicon
First Series:				
~10^1	4,5	2,25	0,045	-
~10^2	45	22,5	0,45	-
~10^3	450	225	4,5	+
~10^4	4 500	2 250	45	+
~10^5	45 000	22 500	450	+
~10^6	450 000	225 000	4 500	+

Table 10 continued: Evaluation of the sensitivity of the PCR assay using suspensions of Cfv prepared in Weybridge medium

Bacterial suspension in PBS-T	Bacterial count (Cfv / mℓ)	Number of Cfv cells subjected to extraction	Number of Cfv 'DNA units' added to the PCR reaction	Presence of a 750 bp PCR amplicon
Second Series:				
~10^1	6	3	0,06	-
~10^2	60	30	0,6	-
~10^3	600	300	6	+
~10^4	6 000	3 000	60	+
~10^5	60 000	30 000	600	+
~10^6	600 000	300 000	6 000	+
Third Series:				
~10^1	7,95	3,975	0,0795	-
~10^2	79,5	39,75	0,795	-
~10^3	795	397,5	7,95	+
~10^4	7 950	3 970	79,5	+
~10^5	79 500	39 700	795	+
~10^6	795 000	397 000	7 950	+

The detection limit of the PCR assay was taken as the number of organisms in the last dilution to give a positive PCR result. The average detection limit, as determined from the experimental data given in Table 9 was 615 Cfv/ mℓ Weybridge medium or 6,15 cells per PCR reaction.

Having established the detection limit with the third Cfv dilution series the two dilutions above the detection limit were selected and used to evaluate the influence of potential inhibitors on the PCR reaction. The PCR results illustrating this are summarized in Table 11.

Table 11: PCR results for Cfv-inoculated Weybridge medium spiked with different concentrations of potential PCR inhibitors

Bacterial suspension in PBS-T	Cfv count (per mℓ)	PCR RESULTS																					
		No Inhibitor	Inhibitor added																				
			Faeces			Blood						Urine						Semen					
			1%	2%	5%	1%	2%	5%	10%	20%	50%	1%	2%	5%	10%	20%	50%	1%	2%	5%	10%	20%	50%
~10^3	79,5	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
~10^4	7 950	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Significant inhibition of the PCR reaction was noted for samples spiked with faeces. Conversely, blood, urine and semen had no impact on the PCR results at the concentrations tested even when the test aliquots were spiked with as much as 50% (per total volume) of the inhibitor.

3.3 Evaluating the sensitivity of the PCR and culture methods for the detection of *C. fetus* in spiked bovine preputial specimens

The sensitivity of the PCR assay compared to the culture method were determined by spiking pooled preputial samples collected from animals in herds identified to be free from infection. Testing was carried out in triplicate.

First run

Five bulls, all less than 2 years of age, stationed at a local AI research station were sampled for testing purposes. Bacterial culture and PCR results for the pooled and spiked specimens are summarized in Tables 12 and 13.

Table 12: Culture results for the first pooled and spiked, preputial specimen

Bacterial suspension in PBS-T	Actual Cfv count in PBS-T (per mℓ)	Cfv count in spiked sample (per mℓ)	Number of Cfv colonies recovered					
			T 0		T 1		T 3	
			CampE plate	Skirrow's plate	CampE plate	Skirrow's plate	CampE plate	Skirrow's plate
~10^1	7,7	0,77	0	*	0	*	0	*
~10^2	77	7,7	0	*	0	*	0	*
~10^3	770	77	0	*	0	*	0	*
~10^4	7 700	770	0	*	0	*	0	*
~10^5	77 000	7 700	1	*	0	*	0	*

* *C. fetus* was not recovered

Severe problems were experienced with *Pseudomonas* colonies growing on the culture plates. All Skirrow's plates were overgrown with *Pseudomonas* colonies preventing the detection and recovery of any *C. fetus* colonies. Contamination on the CampE plates was minimal with only a few plates yielding *Pseudomonas* colonies. Despite the reduction in *Pseudomonas* contamination on the CampE plates the recovery of *C. fetus* from the samples was exceeding low with only a single colony being recovered from an aliquot inoculated with 77 000 Cfv/mℓ.

Table 13: PCR results for the first pooled and spiked, preputial specimen

Bacterial suspension in PBS-T	Actual Cfv count in PBS-T (per mℓ)	Cfv count in spiked sample (per mℓ)	Number of Cfv cells subjected to extraction	Number of Cfv 'DNA units' added to the PCR reaction	PCR results		
					T 0	T 1	T 2
~10^1	7,7	0,77	0,385	0,0077	-	-	-
~10^2	77	7,7	3,85	0,077	-	-	-
~10^3	770	77	38,5	0,77	+	+	-
~10^4	7 700	770	385	7,7	+	+	-
~10^5	77 000	7 700	3 850	77	+	+	-

The PCR results for all of the T 3 samples were negative. This prompted the collection and testing of T 2 samples in both of the subsequent test runs. A detection limit of 770 Cfv/mℓ or 0,77 Cfv per PCR was obtained.

Second and third runs

The preputial scrapings used for these experiments were collected by a private practitioner. The bulls which were sampled had previously been tested for both *C. fetus* and *T. foetus* and found to be negative. No fertility problems had been reported in either herd, and no evidence of campylobacteriosis or trichomoniasis had been found in, at least, the last five years. Additionally, some of the animals had been vaccinated against campylobacteriosis. Bacterial

culture and PCR results for the pooled and spiked specimens are summarized in Tables 14 and 15 (Run 2) and 16 and 17 (Run 3).

Table 14: Culture results for the second pooled and spiked, preputial specimen

Bacterial suspension in PBS-T	Actual Cfv count in PBS-T (per mℓ)	Cfv count in spiked sample (per mℓ)	Number of Cfv colonies recovered							
			T 0		T 1		T 2		T 3	
			CampE plate	Skirrow's plate	CampE plate	Skirrow's plate	CampE plate	Skirrow's plate	CampE plate	Skirrow's plate
~10^1	6	0,6	0	2	0	0	0	0	0	0
~10^2	60	6	0	11	0	1	0	0	0	0
~10^3	600	60	0	58	0	29	0	6	0	R
~10^4	6 000	600	0	TNTC	0	291	0	R	0	R
~10^5	60 000	6 000	0	TNTC	0	TNTC	0	TNTC	0	R

Key for Tables 14 and 16

TNTC: Too numerous to count (> 300 colonies)

R: Recoverable (*C. fetus* colonies were cultured but could not be accurately counted due to the presence of contaminant bacteria).

No Cfv colonies were recovered from any of the CampE plates. Overall very few contaminant colonies (bacteria originating from the preputial specimen) were present on the Skirrow's plates making it possible to clearly identify any Cfv colonies present. The number and extent of growth of the contaminant colonies increased with time and the culture plates inoculated with T 3 samples had significantly more contaminant colonies than the T 1 and T 2 samples. In all cases though, it was still possible to re-isolate Cfv. The detection limit of the culture method at T 0 and T 1 time intervals was 6 Cfv/mℓ. The detection limit increased to 60 Cfv/mℓ by T2 and T3.

Table 15: PCR results for the second pooled and spiked, preputial specimen

Bacterial suspension in PBS-T	Actual Cfv count in PBS-T (per mℓ)	Cfv count in spiked sample (per mℓ)	Number of Cfv cells subjected to extraction	Number of Cfv 'DNA units' added to the PCR reaction	PCR results			
					T 0	T 1	T 2	T 3
~10^1	6	0,6	0,3	0,006	-	-	-	-
~10^2	60	6	3	0,12	+	-	+	+
~10^3	600	60	30	1,2	+	+	+	+
~10^4	6 000	600	300	12	+	+	+	+
~10^5	60 000	6 000	3 000	120	+	+	+	+

Positive PCR results were obtained at all time intervals tested with a detection limit of 60 Cfv/mℓ or 0,12 Cfv per PCR.

Table 16: Culture results for the third pooled and spiked, preputial specimen

Bacterial suspension in PBS-T	Actual Cfv count in PBS-T (per mℓ)	Cfv count in spiked sample (per mℓ)	Number of Cfv colonies recovered							
			T 0		T 1		T 2		T 3	
			CampE plate	Skirrow's plate	CampE plate	Skirrow's plate	CampE plate	Skirrow's plate	CampE plate	Skirrow's plate
~10^1	10	1	0	1	0	0	0	0	0	0
~10^2	100	10	0	5	0	0	0	0	0	0
~10^3	1 000	100	0	28	0	0	0	8	0	0
~10^4	10 000	1 000	0	271	0	127	0	121	0	R
~10^5	100 000	10 000	8	TNTC	0	TNTC	0	TNTC	0	R

The recovery of Cfv from filtered samples (CampE plates) was again very low as shown in Table 16 with Cfv only being recovered on one plate, that being at T 0 with a sample suspension of 10 000 Cfv/mℓ. The results of all three runs indicate that the filtration of specimens retains most, if not all of the Cfv in the specimens and therefore this method is considered to be highly insensitive. *Campylobacter fetus* subsp. *venerealis* was successfully recovered on the Skirrow's plates. At T 0 Cfv was recovered from all suspensions plated out. The detection limit decreased with time but colonies were still recoverable 3 days post-inoculation. Levels of background contaminants were minimal. The number and influence of these colonies on the recovery of Cfv increased with time.

Table 17: PCR results for the third pooled and spiked, preputial specimen

Bacterial suspension in PBS-T	Actual Cfv count in PBS-T (per mℓ)	Cfv count in spiked sample (per mℓ)	Number of Cfv cells subjected to extraction	Number of Cfv 'DNA units' added to the PCR reaction	PCR results			
					T_0	T_1	T_2	T_3
~10^1	10	1	0,5	0,01	-	-	-	-
~10^2	100	10	5	0,1	+	+	+	Weak
~10^3	1 000	100	50	1	+	+	+	+
~10^4	10 000	1 000	500	10	+	+	+	+
~10^5	100 000	10 000	5 000	100	+	+	+	+

Positive PCR results were obtained at all time intervals tested with a detection limit of 10 Cfv/mℓ or 0,1 Cfv per PCR. The detection limit remained unchanged from the time of inoculation with Cfv up to 3 days post-inoculation.

3.4 Field evaluation of the PCR assay

During the investigation period, 212 samples were received and processed using both the optimized PCR assay and the traditional culture method. The complete results hereof are given in Appendix 4. Table 18 provides a summarized representation of the overall PCR and culture results obtained from the field evaluation.

Table 18: Comparison of the bacterial culture and PCR results obtained for the screening of *C. fetus* in clinical specimens

	Culture positive	Culture negative	TOTAL
PCR positive	7	2	9
PCR negative	1	202	203
TOTAL	8	204	212

Using the culture method as the gold standard, the analytical sensitivity and specificity of the PCR assay was calculated as follows:

Analytical sensitivity (ASe)

$$ASe = \frac{\text{True positives}}{\text{True positives} + \text{False negatives}}$$

$$ASe = 7 / (7+1)$$

$$ASe = 85.7\%$$

Analytical specificity (ASp)

$$ASp = \frac{\text{True negatives}}{\text{True negatives} + \text{False positives}}$$

$$ASp = 202 / (202 + 2)$$

$$ASp = 99\%$$

PART B: SUBSPECIATION OF *C. FETUS* ISOLATES

3.5 Evaluating the specificity of the subspecies PCR assay

The multiplex PCR was evaluated using six reference *C. fetus* isolates representing both subspecies as well as the Cfv intermedius biotype. A single PCR amplicon of approximately 750 bp was obtained with all six cultures. A second amplicon of approximately 180 bp was obtained with both of the Cfv isolates but not the Cfv-intermedius or Cff reference isolates.

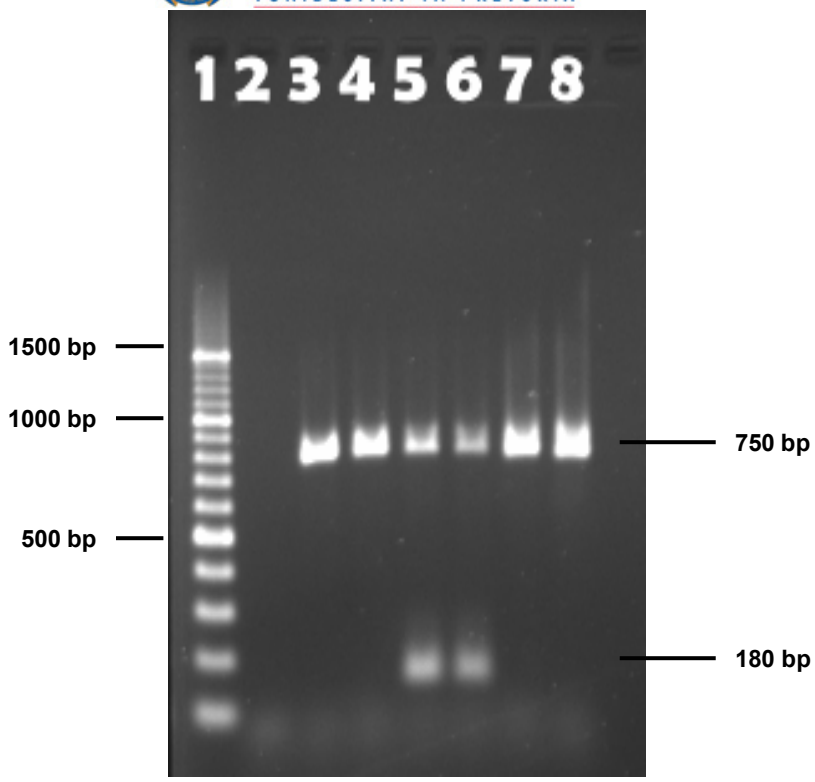

Figure 5: Gel showing the PCR amplicons obtained following testing of the C. fetus reference cultures using the multiplex PCR evaluated by Hum *et al*. (1997). Lane 1: MWM; Lane 2: Negative control; Lane 3: Cff (NCTC 10842); Lane 4: Cff (Strain 5515); Lane 5: Cfv (NCTC 10354); Lane 6: Cfv (LMG 6570); Lane 7: Cfv-i (Strain 10); Lane 8: Cfv-i (Strain 136).

3.6 Comparison of traditional phenotyping methods and the multiplex PCR for the subspeciation of *C. fetus* isolates

The results of the biochemical tests are summarized in Appendix 5. Based on tolerance to 1% glycine and the ability to produce hydrogen sulphide (in L-cysteine supplemented medium and detected using lead acetate paper), isolates were classified as being Cff, Cfv or Cfv-intermedius. Five of the 40 field isolates tested were able to grow on blood medium supplemented with 1% glycine and were consequently identified as being Cff. All of the remaining isolates were unable to grow on the glycine-enriched media. By virtue of the fact that all of these isolates produced hydrogen sulphide in L-cysteine supplemented medium, they were all classified as being Cfv biovar intermedius.

The multiplex PCR results are summarized in Appendix 6. Identical results were obtained for the entire collection of South African field isolates tested; a species-specific amplicon of approximately 750 bp was obtained but no subspecies-specific amplicons (182 bp) were generated with any of the isolates tested.

A comparison of the phenotyping and PCR subspeciation results are also given in Appendix 6.

3.7 Evaluation of supplementary phenotyping methods for the subspeciation of *C. fetus* isolates.

Selenite reduction, susceptibility to cefoperazone and metronidazole and growth at 42 °C were investigated as potential supplementary tests for the subspeciation of *C. fetus* isolates. The results obtained are summarized in Appendix 5. None of the tests which were evaluated showed 100% correlation with the subspecies differentiation made based on glycine tolerance.

CHAPTER 4: DISCUSSION

PART A: EVALUATION OF A *C. FETUS* SPECIFIC PCR FOR THE DIRECT DETECTION OF THE TARGET ORGANISM IN BOVINE PREPUTIAL SPECIMENS

In veterinary diagnostic laboratories the culture and isolation of *C. fetus* is the traditional method for the diagnosis of bovine campylobacteriosis. The inherent limitations of this methodology prompted this investigation to identify and evaluate a more sensitive means of detecting *C. fetus*. Molecular techniques, particularly PCR, are becoming more practical and affordable for diagnostic laboratories to incorporate into their test portfolio. The specificity and sensitivity of assays, combined with the speed at which samples can be processed and results obtained, are appealing attributes (Willoughby, 2003).

The multiplex PCR initially evaluated by Hum *et al*. (1997) is the most extensively described assay for the identification and subspeciation of *C. fetus*. In the multiplex format many investigators have used the PCR for the direct identification and subspeciation of bacterial isolates. No publications to date have reported on the use of this PCR for the direct screening of clinical specimens. The ability to screen clinical specimens directly could potentially improve the overall sensitivity and accuracy of diagnostic results as many of the shortcomings conventionally encountered with the culture method would be eliminated. As a possible screening assay only the species-specific primer set was evaluated; that is a uniplex as opposed to a multiplex PCR format was used. It was reasoned that as a result of the reportedly low prevalence of *C. fetus* in South Africa (Irons *et al.,* 2004) and the improved test sensitivity achieved with uniplex test formats (Schulze *et al*., 2006) it would be more feasible to screen samples using only the *C. fetus* specific primer set.

The specificity of the species-specific uniplex PCR was demonstrated by testing a collection of reference and field *C. fetus* isolates as well as bacteria taxonomically related to *C. fetus* or known to exist in similar microbiological niches. The *C. fetus* isolates represented a geographically diverse collection which was sourced from different veterinary laboratories across the country. Further, some of the field isolates were recovered from culture collections and represent a genetic gene pool that existed up to two decades ago. All the *C. fetus* isolates

yielded a single PCR amplicon of approximately 750 bp in size when tested with the uniplex PCR (Appendix 3). The results indicate that the test has an analytical specificity of 100%. It is interesting to note that initial publications (Hum *et al*., 1997; On & Harrington, 2001; Vargas *et al.*, 2003) reported the generation of a PCR amplicon of about 960 bp in size. The amplicon has however been more recently reported and sequenced as 750 bp (Wagenaar *et al*., 2001; Müller, Hotzel & Schulze, 2003; Willoughby *et al*., 2005). The reason for the discrepancy of almost 200 bp in size has not been explained.

Initial attempts using the published PCR protocol to detect *C. fetus* in Weybridge TEM indicated that refinements were necessary as the detection limit attained was very high. Following minor experimental modifications, including an increase in the volume of DNA template and *Taq* polymerase included in the PCR reaction mix, the detection limit was lowered to approximately 0,6 cell equivalents per assay.

During sample collection preputial material can quite easily become contaminated with urine, semen, faeces and/or blood. All four of these components have been documented as containing components which may interfere with the PCR test (Wilson, 1997). The influence of different concentrations of each of these individual components was evaluated. Sample 'contamination' of up to 50% with either urine, semen or blood, had no impact on the detection limit of the PCR assay. Faecal contamination however was shown to have a major inhibitory effect on the assay even at concentration levels as low as 1%. This indicates that the DNA extraction protocol does not remove all the inhibitory agents present in faeces, resulting in the inclusion of these components in the PCR reaction mix. Faecal contamination of field samples is often obvious from visual inspection; consequently making it possible to identify problematic samples upon receipt. As discussed further below, the inclusion of an internal control in the assay would, to a great extent, be the most feasible way of ensuring that false negative results are not reported as a result of PCR failure due to inhibitors present in the samples. The possibility of using alternative extraction protocols to overcome the problem of faecal inhibitors can also be explored as means of overcoming this potential problem. In practice, however, faecal contamination of preputial specimens is rarely a problem unless really poor sampling technique is used by the practitioner in the field.

The spiking of preputial material was carried out in triplicate using material collected by different practitioners and from animals in different herds. Sampling criteria were instituted to ensure as far as possible that only animals negative for *C. fetus* were sampled. The first run of spiked preputial material clearly illustrates the advantage of PCR over bacterial culture as a means of

detecting *C. fetus* (refer Tables 12 and 13). The presence of *Pseudomonas* in the preputial material dramatically impeded the recovery of Cfv. *Pseudomonas* colonies, resistant to the cocktail of antibiotics incorporated into the TEM and Skirrow's medium, readily grew on the culture plates, obscuring any Cfv colonies which were plated out. Using the PCR assay, Cfv was detected at 770 Cfv/mℓ (or 0,77 cell equivalents per assay) for both T 0 and T 1 samples. Surprisingly, no amplicons were obtained when any of the T 3 samples were tested. In view of the fact that PCR amplicons were visualised when the T 3 samples from both subsequent test runs were analyzed, it is hypothesized that the failure to detect Cfv is a result of the presence of *Pseudomonas* in the pooled sample. Certain strains of *Pseudomonas* such as *Pseudomonas aeruginosa*, are known to produce the enzyme DNase (Gilardi, 1985). The activity hereof could have resulted in the degradation of DNA present in the sample. Problems resulting from the contamination of preputial cultures by *Pseudomonas* spp. have been reported (Monke *et al*., 2002). It is interesting to note however, that none of the diagnostic samples tested during field evaluation exhibited such extensive *Pseudomonas* contamination as was noted with this test run, suggesting that in practice this type of contamination may be a rare occurrence.

In the subsequent two runs the detection limit of the culture method was better than the PCR only at T 0. Due to logistical reasons, clinical samples seldom reach the laboratory the day they are collected. With the use of a TEM, samples typically arrive the day after collection (T 1) or two days later (T 2), depending on the reliability and efficiency of the courier service available to the private practitioner. The detection limit of the culture assay decreased with time, with a concomitant increase in the number of background/contaminating organisms. Despite the observed decrease in detection limit with time, Cfv colonies were still recoverable 72 hrs post-inoculation. With the second and third test runs the sensitivity of the PCR did not appear to be affected by time, with the detection limit remaining unchanged between T 0 and T 3 samples.

It is evident from all three of the spiked preputial test runs that the filtration of samples is highly ineffective at recovering Cfv. *Campylobacter fetus* subsp. *venerealis* was only recovered on two occasions from plates inoculated with filtered aliquots. In the first test run a single colony was isolated from the CampE plate which had been inoculated with 0,3 mℓ of a suspension containing 7 700 Cfv/mℓ. Whilst in the third test run 8 Cfv colonies were recovered following the filtration of 0,3 mℓ of a suspension containing 10 000 Cfv/mℓ. Sample filtration was first introduced as a means of improving the recovery of *Campylobacter* spp. from samples containing large numbers of contaminating bacteria. Membrane filters provide a means of physical selection by permitting the movement of slender, motile bacteria such as

campylobacters through the pores whilst impeding the passage of larger or non-motile bacteria (Kulkarni *et al*., 2000). The use of a physical means of selection as opposed to a chemical means (the inclusion of antibiotics in the medium) also allows for the cultivation of strains which are sensitive to antibiotics (Steele & McDermott, 1984). For these reasons, the primary plating on selective media in combination with a filtration method is considered to be the optimal method for recovering *Campylobacter* spp. from clinical samples (Nachamkin, 1999). The results which have been published on the efficiency of sample filtration are varied (Dufty, 1967; Lander, 1990b; Hum *et al*., 1994) but in general it is acknowledged that sample filtration is not as sensitive as culturing with selective media. It is therefore advised that filtration should be used to complement the direct culture on selective media and not be used as a replacement (Nachamkin, 1999). Based on the experimental results obtained during this investigation, these views are fully supported.

Field evaluation of the PCR assay was carried out over a 14 month period using diagnostic specimens submitted to Allerton PVL. All bovine sheath scrapes submitted in Weybridge transport medium were tested using both the culture method and the optimized PCR assay. Of the 212 samples received and tested, 4,2% were found to be positive using the PCR assay whilst 3,8% were found to be positive using the culture method (refer Table 18). A high degree of correlation was observed between the PCR and the culture results. Discrepancies between results were only noted on two occasions. From a submission of five sheath scrapings (Laboratory Reference Number A2007/07_219), three of the samples were found to be positive using PCR but *C. fetus* was only successfully isolated from one of the samples. PCR testing of all the samples in the submission was repeated with the same results being obtained. Amplicons from the PCR positive, culture negative samples were submitted for sequencing. Results verified that the sequence of the PCR products correlated with published *C. fetus* sequences. Unfortunately, repeat samples from these animals could not be obtained to verify the results. This discrepancy is possibly a demonstration of the higher sensitivity of the PCR assay; illustrating the advantages of the PCR in detecting *C. fetus* in low numbers where the culture could possibly fail especially in the presence of large numbers of contaminant bacteria or where non-viable bacteria are present.

The converse situation was observed with the second anomalous result (Laboratory Reference Number A2008/05_079). With this particular submission three positive results were obtained using PCR but *C. fetus* was isolated from four of the samples within the submission. Once again, the PCR testing was repeated with no change in test results. The failure of the PCR to detect *C. fetus* directly in the clinical sample, even on repeat testing, is harder to explain. It was

noted however, that very few *C. fetus* colonies (2-3) were observed on the primary culture plates and in fact, the identity of the colonies was confirmed using PCR as it was feared that bacterial re-streaks would not grow. Although no evidence of gross faecal contamination was observed, the presence of trace amounts cannot be ruled out. The possibility also exists that the number of *C. fetus* cells in the sample was exceedingly low. Due to the small volume of sample aliquoted for DNA extraction and the subsequent 'dilution' of possible DNA units in the PCR reaction mix, the possibility does exist that the target DNA was diluted out before the PCR was run. In spite of the two anomalous results obtained it is important to note that neither of the tests failed to detect a positive herd.

To eliminate the possibility of reporting false negative PCR results, a spiked sample, comprising aliquots from all the samples in a submission, was prepared and tested alongside the test samples for each submission handled. In order for all of the negative results in a submission to be considered valid, the spiked sample had to have yielded an amplicon. Conversely, failure to obtain a positive result with the spiked composite sample would have indicated that the PCR failed and that the negative sample test results were invalid. During the course of the field evaluation, all negative PCR results were passed based on the detection of an amplicon in the corresponding spiked composite sample.

A far more expedient approach to validating negative PCR results would be the incorporation of an internal control into the assay. This would in fact serve a dual purpose as it would enable problems due to the presence of inhibitors to be identified, as well as being able to establish whether sufficient test material has been collected and tested. The latter point has a direct (and frequently underestimated) bearing on the validity of test results as without an internal control it is not possible to ascertain whether a negative result is in fact due to the absence of the target organism or whether insufficient material has been collected and tested. By simultaneously amplifying a suitable gene target ("house-keeping gene") it would be possible to ascertain, by virtue of observing a separate control PCR band, whether sufficient material has been tested. In the event that a negative or weak control band is observed a negative PCR result can be more accurately interpreted (Hoorfar *et al*., 2003; Rosenstraus *et al*., 1998). Beta-actin, beta-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA have all been used as house-keeping genes (Thellin *et al*., 1999) and the suitability of one of these will be investigated.

The PCR facilities used during this investigation were designed to ensure complete separation off all stages of testing. Extreme care was taken to eliminate cross contamination of samples and possible contamination of samples with amplified products. In diagnostic labs where the sample throughput is high and the risks associated with PCR amplicons contaminating samples is great the use of uracil DNA glycosylase to control carryover contamination is recommended (Longo, Berninger & Hartley, 1990). With the implementation of this PCR method as a routine assay the uracil DNA glycosylase system will be incorporated into the assay.

PART B: SUBSPECIATION OF *C. FETUS* ISOLATES

Tolerance to 1% glycine is currently the only internationally accepted phenotypic test prescribed for the differentiation of the two *C. fetus* subspecies (OIE, 2008a). Based on the results of the glycine tolerance test a collection of South African *C. fetus* field isolates were subspeciated. Of the forty isolates tested, six were shown to be tolerant to glycine and were classified as Cff. All the remaining isolates were classified as Cfv by virtue of their inability to grow on blood medium supplemented with 1% glycine. All of the glycine intolerant field isolates were shown to produce hydrogen sulphide when tested using the 'sensitive' hydrogen sulphide test. Based on the description initially made by Véron and Chatelain (1973) these isolates are more specifically classified as Cfv biovar intermedius.

Together with appropriate reference cultures the entire collection of field isolates was subspeciated using the multiplex PCR initially evaluated by Hum *et al.* (1997). All the field isolates yielded species-specific amplicons but no subspecies-specific amplicons. The VenSF and VenSR subspecies-specific primer set used in the assay targets a segment of the Cfv genome. The generation of an amplicon of approximately 182 bp is indicative that the test organism is Cfv. *Campylobacter fetus* subsp. *fetus* is identified by exclusion. Based on the PCR test results obtained all the field isolates should be classified as Cff.

The lack of correspondence between the phenotyping and genotyping subspeciation results was somewhat startling. Previous reports have only intimated minor incongruencies when comparing these two subspeciation approaches (Hum *et al*., 1997; Müller, Hotzel & Schulze, 2003). A recent investigation, published by Willoughby *et al.* (2005), alluded to greater problems with the multiplex PCR when they tested a collection of United Kingdom isolates. These investigators found that only three isolates, out of a group of nineteen, which were initially phenotyped as Cfv, gave the same subspeciation results when subspeciated using this specific multiplex PCR

assay. After further analysis the researchers proposed that the discrepancies were the result of an unusual Cfv clone circulating within the cattle population in the United Kingdom.

Unfortunately the initial evaluation of the multiplex PCR by Hum *et al.* (1997) did not take biovar intermedius strains into consideration. In fact, many of the phenotyping and genotyping investigations which have been carried out in this field have seemingly ignored the existence of this particular group of bacteria. This is somewhat understandable since the initial description of this group of isolates was very vague and reference isolates weren't readily available (van Bergen, 2005). As a consequence of the delayed acknowledgement of this group, it now makes it difficult to evaluate the results of prior investigations carried out.

Extensive genotyping studies have been carried out by van Bergen and various collaborators (2005a, 2005c) using field isolates gathered from countries across the globe. This work involved the testing of several isolates which had been phenotyped as Cfv biovar intermedius. Interestingly, seven of these isolates originated from South Africa. When tested using the multiplex PCR, six of the isolates were identified as Cff and one as Cfv. Amplified Fragment Length Polymorphism (AFLP) was ultimately employed to examine all of the isolates under investigation. Analysis of a specific AFLP region was found to provide a reliable means to differentiate Cff, Cfv and Cfv biovar intermedius. The AFLP results for the group of South African isolates ultimately concurred with the phenotyping results intimating that the isolates were in fact Cfv biovar intermedius.

Based on the phenotyping results (glycine tolerance and hydrogen sulphide production) and the outcome of the PCR results it is believed that all of the *C. fetus* field isolates tested in this investigation, with the exception of the 6 identified as Cff, are in fact Cfv biovar intermedius. Due to the seemingly large proportion of Cfv biovar intermedius isolates in South Africa and the unpredictable results obtained with this group of organisms when using the multiplex PCR, the PCR assay is deemed unsatisfactory for subspeciating South African field isolates.

There are risks associated with basing subspeciation results on a single phenotypic criterion, especially a characteristic such as glycine tolerance. With the subspeciation results given above, the assumption was made that no glycine tolerant Cfv isolates are present and similarly that no glycine sensitive Cff isolates existed.

For the most part the glycine tolerance test was found to provide reproducible results. However, due to the importance of this test it is recommended that testing is done in duplicate in order to

eliminate the possibility of an aberrant, incorrect result being reported. Furthermore, it is recommended that hydrogen sulphide testing be carried out. Although this may have little clinical significance at the present time, the data may prove extremely useful for future investigations looking to examine the epidemiology of isolates within the country and the development of new genotyping techniques.

Within the context of this investigation attempts were made to evaluate other phenotyping tests to subspeciate *C. fetus*. Selenite reduction, growth at 42 °C and susceptibility to the antibiotics cefoperazone and metronidazole have previously been found to correlate with glycine tolerance (On & Harrington, 2001) and have been suggested as supplementary subspeciation tests. *Campylobacter fetus* subsp. *fetus* isolates have been shown to reduce selenite, grow at 42 °C and are generally found to be resistant to cefoperazone and metronidazole. In contrast, Cfv isolates have been shown to be sensitive to cefoperazone and metronidazole, do not reduce selenite and do not grow at 42 °C. The value of these criteria has varied between investigations and it was considered pertinent within the scope of this investigation to ascertain how South African test isolates would react.

Of the four test methods selenite has probably been used most frequently, alongside the glycine tolerance test, as an aid for subspeciation (Müller, Hotzel & Schulze, 2003). Investigations by Schulze *et al.* (2006) reported that 100% of the Cfv isolates examined in their study were all incapable of reducing selenite; whilst the majority of Cff isolates produced weak to strong reactions. In this investigation, both of the Cff reference cultures yielded strong, unequivocal positive results whilst the Cfv and Cfv biovar intermedius reference cultures were unable to reduce selenite. Of the six Cff field isolates tested, only five were shown to reduce selenite (86%). Several of the isolates identified as Cfv biovar intermedius however, were also able to reduce selenite. Working on the assumption that the glycine-based subspeciation of isolates is accurate, there appears to be no consistency between the selenite reduction results and the subspecies. In general, the selenite reduction test was found to unreliable with variable results being obtained with replicate tests. Weak test reactions were also frequently obtained making interpretation difficult.

The test results obtained for the growth of isolates at 42 °C and susceptibility to metronidazole, did not correspond well with the glycine tolerance results either. The only set of test results which partially corresponded with the glycine results was the susceptibility to cefoperozone. Ninety seven percent of the Cfv isolates tests were found to be sensitive to cefoperazone, whilst four of the six Cff isolates (67%) were found to be resistant. Schulze *et al*. (2006) also reported

a high degree of correspondence between these two tests; all of the Cfv isolates they tested were sensitive to cefoperazone whilst 86% of the Cff isolates were resistant. This test could be a useful aid in assessing the accuracy of glycine subspeciation results, but ultimately the discrepancies between cefoperazone susceptibility and glycine tolerance cannot be overlooked. Furthermore, the sensibility of relying on an antibiotic susceptibility result is also questionable due to the fact that this trait can be acquired by transduction or mutation (Vargas *et al*., 2003).

In order to resolve the subspeciation dilemma it appears that more specialised genotyping techniques are going to be needed. These techniques are largely beyond the resources of routine diagnostic laboratories requiring technical expertise and expensive hardware. Thus for the time being the only option available to diagnostic laboratories is the glycine tolerance test.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

Within the scope of this investigation a PCR assay, based on the *C. fetus* specific primer set initially evaluated by Hum *et al.* (1997), was optimized and used to screen preputial specimens collected in TEM for the presence of *C. fetus*. The assay was shown to have a high analytical specificity (100%) and high analytical sensitivity being able to detect as few as 0,6 cell equivalents per assay. The results of the subsequent field evaluation indicated that the PCR assay was as effective as the culture method for detecting *C. fetus* in clinical specimens.

The diagnosis of campylobacteriosis by PCR technology offers a number of advantages over the conventional culture and identification of the causative agent. As evidenced with the first spiked sample run which was performed, the presence of and overgrowth by contaminating microflora is a problem which is often encountered with preputial specimens. In extreme cases the presence of *C. fetus* may be completely obscured resulting in the reporting of false negative test results. With the exception of possible bacterial DNAse activity on aged specimens, the PCR is, however, able to successfully identify *C. fetus*, irrespective of the number of background organisms. Even more significant, from the point of diagnosing campylobacteriosis, is the fact that the PCR is able to detect the target organism as long as 72 hours after sample collection. Comparatively, the culture method performs poorly showing decreased sensitivity with time. Isolation of Cfv at 72 hours was only successful when the initial Cfv concentration were high; even then recovery was subjective, being dependant upon the type and extent of background contamination.

The rapid turnover time attained using PCR is another distinct advantage which was very evident during this investigation. Depending on the number of samples received per submission it is possible to finalise results within one working day. With the culture method between three and twelve working days are required to isolate, purify and complete the phenotyping tests. The time required to prepare the different media required for the phenotyping of isolates also warrants mentioning.

The PCR has been used successfully to detect *C. fetus* in preputial specimens. Testing of diagnostic specimens will, however, continue being done in parallel using both the PCR and culture assays in order to accumulate sufficient data to enable the diagnostic sensitivity and specificity of the PCR assay to be determined as recommended by the OIE (2008b).

The generation of further field data will also determine whether a positive culture, negative PCR result is of diagnostic significance or whether it was just an anomalous result obtained during the course of this study.

Prior to the implementation of the PCR assay as a routine diagnostic test, the reproducibility of the assay will also be evaluated according to OIE guidelines. Three suitable laboratories will be identified to participate in ring trials whereby a panel of samples will be compiled and submitted to each laboratory for testing. The same assay, reagents and control samples will be used in each of the laboratories and the results will be assembled and analyzed to ascertain reproducibility (OIE, 2008b).

As discussed in a preceeding chapter, the implementation of an appropriate internal control is one of the immediate plans intended for improving the PCR assay. Future work will also involve the evaluation of PCR performance in sample matrices other than preputial samples. Apart from preputial material, foetal material and vaginal discharges are occasionally submitted to the laboratory for *C. fetus* testing. Preliminary work has already been conducted on abomasal fluid collected from bovine foetuses (results not shown). Bacterial culture and PCR were carried out on abomasal fluid from two separate cases. On both specimens, culture yielded a pure growth of *C. fetus* whilst DNA extraction followed by PCR testing as described above, yielded distinct, PCR bands. The testing of additional samples is planned.

The subspeciation of *C.fetus* isolates has proved to be a far more challenging endeavour. From the representative collection of *C. fetus* field isolates assembled and tested in this investigation it appears that South Africa has an unusually large proportion of biovar *intermedius* strains. The multiplex PCR of Hum *et al*. (1997) fails to differentiate this group, resulting in the incorrect subspeciation of isolates. All of the Cfv biovar intermedius isolates tested in this investigation failed to yield a subspecies-specific amplicon resulting in their erroneous classification as Cff. It is evidently clear from these results that the subspecies-specific primer set used in this assay is unsuitable for subspeciating South African field isolates. There is a distinct need for further genetic analysis on Cff, Cfv and Cfv biovar *intermedius* strains. The identification of unique genomic regions could be used to develop more specific assays for the specific subspeciation of isolates.

Abril *et al.* (2007) recently described the discovery of an insertion element which is believed to exist only in Cfv. These researchers did not evaluate biovar *intermedius* as part of their investigation and it is consequently not known how these strains will react with the PCR assay

that was developed. It will be interesting to observe how South African field isolates respond when tested using this assay and it is certainly planned to take the collection of field isolates which has been assembled and subspeciate them using this assay.

A unique collection of local field isolates has been assembled from across the country and using this as a platform, further genetic analyses are planned. The ultimate aim is to develop a quick and reliable means of differentiating *C. fetus* subspecies. Once this challenge has been tackled numerous avenues exist for further investigations into the epidemiology of *C. fetus* in South Africa and the genetic evolution of this bacterium in the country.

APPENDIX 1: REAGENTS AND MEDIA

CAMPYLOBACTER ENRICHMENT MEDIUM (CampE)

A non-selective enrichment medium for the isolation of *Campylobacter* spp. It is essentially a blood agar medium supplemented with a *Campylobacter* growth supplement (Oxoid, 1992).

Materials:

39 g Columbia blood agar base (Oxoid CM331)
2 vials Campylobacter growth supplement (Oxoid SR0232E)
50-70 mℓ defibrinated sheep blood

Preparation:

1. Add 39 g Columbia blood agar base to 1 L distilled water and allow to stand for 10 minutes.
2. Bring to the boil to dissolve the agar completely.
3. Autoclave at 121 °C for 15 minutes and then cool in a water bath set at approximately 50 °C.
4. Aseptically add 50-70 mℓ defibrinated sheep blood to the basal medium.
5. Add the contents of two vials *Campylobacter* growth supplement to the basal medium.
6. Mix well before dispensing approximately 12 mℓ into 60 mm Petri dishes.

COLUMBIA BLOOD AGAR

(Oxoid, 1992)

Materials:

39 g Columbia blood agar base (Oxoid CM331)

50 - 70 mℓ sheep blood

Preparation:

1. Add 39 g Columbia blood agar base to 1 L distilled water and allow to stand for 10 minutes.
2. Bring to the boil to dissolve the agar completely.
3. Autoclave at 121 °C for 15 minutes and then cool in a water bath set at approximately 50 °C.
4. Aseptically add 50-70 mℓ sheep blood.
5. Mix gently before dispensing approximately 18 mℓ into 90 mm petri dishes.
6. Store the plates at 4-8 °C in sealed plastic packets for up to 8 weeks.

For the preparation of blood agar plates supplmented with 1% glycine:

- A 10% solution of glycine is prepared by dissolving 1 g glycine (BDH, South Africa) in 10 mℓ distilled water.
- Following filter sterilization 1,65 mℓ of the glycine solution is added to each Petri dish.
- Exactly 15 mℓ of blood agar is dispensed into each Petri dish and mixed well to ensure an even distribution of glycine throughout the medium.

(OIE, 2008a)

HYDROGEN SULPHIDE BROTH

(Kirkbride, 1990)

Materials:

Brain heart infusion broth (Oxoid CM 225)
Agar technical (Oxoid LP0013)
L-cysteine (Sigma C1276)

Preparation:

1. Dissolve 3,7 g of the BHI basal medium together with 0,16 g agar technical in 100 mℓ of distilled water.
2. Autoclave at 121 °C for 15 minutes and then cool in a water bath set at approximately 50 °C.
3. Dissolve 0,02 g L-cysteine in a small volume of distilled water.
4. Filter sterilize the cysteine solution into the cooled broth medium using a 0,22 µm syringe filter (Millipore, South Africa).
5. Mix well before dispensing into 4 mℓ volumes.
6. Store the tubes of media at 4-8 °C. Discard unused media after 10 days.

SELENITE BROTH

(Schulze *et al*., 2006)

Materials:

Nutrient broth no. 2 (Oxoid CM067)

Sodium selenite pentahydrate (Merck 1.06607.0100)

Preparation:

1. Dissolve 25 g of the nutrient broth basal medium into 1 L of distilled water.
2. Autoclave at 121 °C for 15 minutes and then cool in a water bath set at approximately 50 °C.
3. Dissolve 1 g of sodium selenite pentahydrate in a small volume of distilled water.
4. Filter sterilize the solution into the cooled nutrient broth medium using a 0,22 µm syringe filter (Millipore, South Africa).
5. Mix well before dispensing into 5 mℓ volumes.
6. Store media at 4-8 °C. Discard unused media after 10 days.

SKIRROW'S AGAR

(OIE, 2008a)

Materials:

39 g Columbia blood agar base (Oxoid CM331)
50 mg Cycloheximide (Sigma C7698)
2 x Skirrow's selective supplement (SR0069)
50-70 mℓ defibrinated sheep blood

Preparation:

1. Add 39 g Columbia blood agar base to 1 L distilled water and allow to stand for 10 minutes.
2. Bring to the boil to dissolve the agar completely.
3. Autoclave at 121 °C for 15 minutes and then cool in a water bath set at approximately 50 °C.
4. Reconstitute 2 vials of Skirrow's selective supplement using 2 mℓ sterile distilled water for each vial.
5. Dissolve 50 mg cycloheximide in 2 mℓ of sterile distilled water.
6. Aseptically add the selective supplement to the basal medium followed by 50-70 mℓ of defibrinated sheep blood.
7. Filter the cycloheximide solution into the basal medium using a 0,22 µm filter membrane (Millipore, South Africa).
8. Mix gently before dispensing approximately 18 mℓ into 90 mm Petri dishes.
9. Store the plates at 4-8 °C in sealed plastic packets for up to 3 weeks.

TRIPLE SUGAR IRON (TSI) MEDIUM

(Schulze *et al*., 2006)

Materials:

Triple Sugar Iron Medium (Oxoid CM 277)

Preparation:

1. Dissolve 65 g of the basal medium in 1 L of distilled water.
2. Autoclave at 121 °C for 15 minutes and then cool in a water bath set at approximately 50 °C.
3. Dispense media into sterile test tubes and allow to solidify at an angle.
4. Store the tubes of media at 4-8 °C. Discard unused media after 10 days.

WEYBRIDGE TRANSPORT MEDIUM

(Lander, 1990a)

Materials:

21 g Mueller Hinton broth (Oxoid CM405)
70 mℓ haemolysed horse blood
5 g bacteriological charcoal (Sigma C7606)
40 mg Vancomycin (Fluka 94747)
20 mg Trimethoprim (Sigma T7883)
10 000 IU Polymyxin B sulphate (Sigma P4932)
100 mg Cycloheximide (Sigma C7698)
500 mg 5-fluorouracil (Sigma F6627)
0,25 g Sodium pyruvate (Sigma P2256)
0,25 g Sodium metabisulphite (Sigma S9000)
0,25 g Ferrous sulphate ($FeSO_4 .7H_2O$) (Sigma F8048)
In lieu of Sodium pyruvate, Sodium metabisulphite and ferrous sulphate – two bottles of Campylobcter growth supplement (Oxoid SR0232E) can be added.

Preparation:

1. Add 21 g of the Mueller Hinton broth medium and 5 g bacteriological charcoal to 900 mℓ of distilled water.
2. Mix well and autoclave.
3. Once the basal medium has cooled add the lysed horse blood.
4. Dissolve the vancomycin, polymyxin B and cycloheximide in a small quantity of distilled water and filter sterilize into the basal medium using a 0,22 µm filter membrane (Millipore, South Africa).
5. Preparation of trimethoprim: Dissolve 0,02 g trimethoprim in a minimum amount of distilled water with a few drops of lactic acid added. Adjust the pH to 7.0. Filter sterilise into the basal medium.
6. Preparation of 5-fluorouracil: Dissolve 0,5 g of 5-fluorouracil in 25 mℓ distilled water containing 1 mℓ 2 M sodium hydroxide. Incubate at <56 °C for approximately 2 hrs. Adjust the pH to 7.0. Filter sterilize the antibiotic into the basal medium
7. Stir the mixture well before dispensing 4 mℓ volumes into sterile containers.
8. Store media at 4-8 °C. Discard unused media after 3 weeks.

APPENDIX 2: BIOCHEMICAL CHARACTERIZATION TESTS

Tests were inoculated directly from 72 hr cultures or suspensions thereof prepared in PBS. The turbidity of the suspensions were initially standardized using a 0,5 McFarland standard and then futher diluted (1:100) in PBS to obtain the recommended inoculum size (Schulze *et al*., 2006).

Atmospheric requirements:

Tests isolates were streaked onto three blood agar plates. Each of the plates were incubated at 37 °C under different atmospheric conditions namely – anaerobic, microaerophilic and aerobic.

Temperature dependance:

Test isolates were streaked onto three blood agar plates. Each of the plates were incubated under microaerophilic conditions at different temperatures, namely: 25 °C, 37 °C and 42 °C.

Antibiotic susceptibility:

Antibiotics evaluated include: Cephalothin (30 µg discs)
Cefoperazone (30 µg discs)
Nalidixic acid (30 µg discs)
Metronidazole (5 µg discs)

All discs were purchased from Oxoid[1].

Bacterial suspensions were swabbed onto the surface of blood agar plates in such a manner that following incubation a confluent lawn of bacterial growth would develop. A single disc of each of the antibiotics being tested, were placed onto the surface of the blood plate before the plates were incubated under microaerophilic conditions for 72 hrs.

Production of hydrogen sulphide:

The ability of the isolates to produce H_2S was evaluated using two different methods.

- *TSI medium:* The medium was inoculated with a standardized suspension of the isolate. Tests results were read after 72 hrs of incubation. Blackening of at least 75% of the tube was regarded as positive; a trace of blackening (usually at the inoculum-slant interface only) was regarded as being weakly positive and the absence of blackening was regarded as negative (On & Holmes, 1992).

- *Lead acetate:* Brain heart infusion broth supplemented with 0.02% L-cysteine was inoculated with a standardized suspension of the isolate. A piece of lead acetate paper (Fluka 06728) was suspended from the top of the test tube. Following 72 hrs incubation, the lead acetate strips were examined for the development of a black precipitate. An uninoculated control was incubated alongside all tests to eliminate the possibility of auto-blackening of test paper strips (Branson, 1972)

Catalase:

The presence of the catalase enzyme was demonstrated by suspending part of a bacterial colony in a drop of 3% hydrogen peroxide. The development of bubbles indicated the presence of the enzyme (Véron and Chatelain, 1973).

Oxidase:

The presence of cytochrome c was demonstrated using commercial oxidase sticks (Oxoid BR64A). Positive test results were indicated by the development of a purple colour within half a minute of being touched against a bacterial colony.

Glycine tolerance:

Blood agar plates containing 1% glycine were inoculated with 20 µℓ of the prepared bacterial suspensions. Blood agar plates without glycine were inoculated and incubated in parallel with the test plates. Following incubation for 72 hrs, plates were examined for growth (OIE, 2008a).

Selenite reduction:

Approximately 0,1 mℓ of the test suspension was inoculated into a bottle of selenite broth. Tests were incubated for up to 5 days. The ability to reduce selenite was based on reddening of the selenite broth (Schulze *et al*., 2006).

APPENDIX 3: PCR RESULTS OBTAINED USING THE SPECIES-SPECIFIC PRIMER SET (Mg3F and Mg4R)

Reference bank number	Microorganism	Source of Isolate	PCR Result (Mg3F and Mg4R species-specific primers)
Campylobacter fetus isolates			
39	Cff	NCTC 10842 (ATCC 27354)	+
40	Cff	5515 (ATCC 33247)	+
41	Cfv	NCTC 10354 (ATCC 19438)	+
42	Cfv	LMG 6570	+
43	Cfv-i	Unknown	+
44	Cfv-i	Unknown	+
1	*C. fetus*	Unknown	+
2	*C. fetus*	Unknown	+
3	*C. fetus*	Bovine foetus	+
4	*C. fetus*	Unknown	+
8	*C. fetus*	Bovine preputial specimen	+
9	*C. fetus*	Bovine preputial specimen	+
10	*C. fetus*	Bovine preputial specimen	+
11	*C. fetus*	Bovine preputial specimen	+
17	*C. fetus*	Unknown	+
22	*C. fetus*	Bovine preputial specimen	+
26	*C. fetus*	Bovine foetus	+
28	*C. fetus*	Unknown	+
29	*C. fetus*	Unknown	+
30	*C. fetus*	Unknown	+
31	*C. fetus*	Unknown	+
34	*C. fetus*	Bovine faeces	+
35	*C. fetus*	Bovine faeces	+
36	*C. fetus*	Bovine foetus	+
37	*C. fetus*	Bovine preputial specimen	+
45	*C. fetus*	Bovine foetus	+
46	*C. fetus*	Bovine foetus	+
47	*C. fetus*	Bovine preputial specimen	+
48	*C. fetus*	Bovine foetus	+

Reference bank number	Microorganism	Source of Isolate	PCR Result (Mg3F and Mg4R species-specific primers)
49	*C. fetus*	Bovine foetus	+
50	*C. fetus*	Bovine preputial specimen	+
51	*C. fetus*	Bovine preputial specimen	+
52	*C. fetus*	Bovine preputial specimen	+
53	*C. fetus*	Bovine preputial specimen	+
59	*C. fetus*	Bovine foetus	+
60	*C. fetus*	Bovine preputial specimen	+
61	*C. fetus*	Bovine preputial specimen	+
62	*C. fetus*	Bovine preputial specimen	+
65	*C. fetus*	Bovine preputial specimen	+
66	*C. fetus*	Unknown	+
70	*C. fetus*	Bovine preputial specimen	+
71	*C. fetus*	Bovine preputial specimen	+
72	*C. fetus*	Bovine preputial specimen	+
73	*C. fetus*	Bovine preputial specimen	+
74	*C. fetus*	Bovine preputial specimen	+
75	*C. fetus*	Bovine preputial specimen	+
Non-*Campylobacter fetus* isolates			
5	*C. jejuni*	Avian intestine	-
6	*C. sputorum* subsp. *bubulus*	Bovine preputial specimen	-
12	*C. sputorum* subsp. *bubulus*	Bovine preputial specimen	-
16	*C. jejuni*	Unknown	-
18	*C. coli*	ATCC 43478	-
19	*C. jejuni*	ATCC 29428	-
21	*C. sputorum* subsp. *bubulus*	Bovine preputial specimen	-
32	*C. hyointestinalis*	Bovine faeces	-
33	*C. sputorum* subsp. *bubulus*	Bovine preputial specimen	-
38	*C. hyointestinalis*	Unknown	-
Non-*Campylobacter* isolates			
7	*Brevundimonas vesicularis*	Bovine preputial specimen	-
13	*Brevundimonas vesicularis*	Bovine preputial specimen	-

Reference bank number	Microorganism	Source of Isolate	PCR Result (Mg3F and Mg4R species-specific primers)
14	*Stenotrophomonas maltophilia*	Bovine preputial specimen	-
20	*Arcobacter skirrowii*	Bovine intestine	-
23	*Corynebacterium striatum*	Bovine preputial specimen	-
24	*Histophilus somni*	Bovine vaginal aspirate	-
25	*Histophilus somni*	Bovine preputial specimen	-
27	*Ochrobacter* sp.	Bovine faeces	-
54	*Histophilus somni*	ATCC 70025	-
55	*Staphylococcus aureus*	ATCC 25923	-
56	*Escherichia coli*	ATCC 25922	-
57	*Pseudomonas aeruginosa*	ATCC 27853	-
58	*Staphylococcus epidermidis*	ATCC 12228	-
63	*Tritrichomonas foetus*	Bovine preputial specimen	-
64	*Tritrichomonas foetus*	Bovine preputial specimen	-
67	*Arcanobacterium pyogenes*	ATCC	-
68	*Proteus* sp.	Unknown	-
69	*Enterococcus faecalis*	ATCC 29212	-

Key:

+ Positive
- Negative

APPENDIX 4: FIELD EVALUATION OF CLINICAL SPECIMENS

LABORATORY REFERENCE NUMBER	DISTRICT	ANIMAL IDENTIFICATION	PCR RESULT	CULTURE RESULT
A2007/07_219	Mooi River	03771	-	-
		R50	+	-
		03368	+	+
		0451	+	-
		0568	-	-
A2007/09_508	Mtubatuba	SIM 17	-	-
		14/04 Brangus	-	-
		26/05 Brangus	-	-
		40/05 Brangus	-	-
		1/04 Brahman	-	-
A2007/09_317	Estcourt	P69	-	-
		T35	-	-
		01	-	-
		R99	-	-
		V302	-	-
		V79	-	-
A2007/10_392	Estcourt	RB	-	-
		GB	-	-
A2007/10_356	Underberg	1	-	-
		2	-	-
A2007/10_405	Ladysmith	3AY	-	-
		TRS 5563	-	-
		HR 11803	-	-
		HLF	-	-
		HR88	-	-
		IM	-	-
		LR 00	-	-
		DJ Simm	-	-
		72	-	-
		AY5	-	-
		DJ Red Brangus	-	-

LABORATORY REFERENCE NUMBER	DISTRICT	ANIMAL IDENTIFICATION	PCR RESULT	CULTURE RESULT
		HVT	-	-
		AY	-	-
		TRS 803	-	-
		598	-	-
		16	-	-
		LSS 17	-	-
		517	-	-
		537	-	-
		Polled 20	-	-
		562	-	-
		352	-	-
		LSS 07	-	-
A2007/10_567	Estcourt	BM	-	-
		M99.02	-	-
		M01.66	-	-
A2007/10_684	Estcourt	PG H	-	-
		PG 2	-	-
		PG 5B	-	-
		PG 59	-	-
		PG Y	-	-
A2007/11_047	Estcourt	W01	-	-
		W02	-	-
		W03	-	-
		TR1	-	-
		M03	-	-
		M04	-	-
		NR1	-	-
A2007/11_094	Underberg	Symons	-	-
		Hereford	-	-
A2007/12_275	Estcourt	Jersey Bull	-	-
A2007/12_188	Ladysmith	366	-	-
		500	-	-
A2008/02_174	Ladysmith	B01	-	-
		B18	-	-

LABORATORY REFERENCE NUMBER	DISTRICT	ANIMAL IDENTIFICATION	PCR RESULT	CULTURE RESULT
		B20	-	-
		04125	-	-
		04197	-	-
		Friesland	-	-
		Jersey	-	-
		David	-	-
A2008/02_270	Estcourt	460 05	-	-
		472 05	-	-
A2008/02_539	Ladysmith	B18	-	-
		Jersey	-	-
		Jeff	-	-
		B01	-	-
		04197	-	-
		Red bull	-	-
		B20	-	-
		04215	-	-
A2008/03_054	Greytown	ANG002	-	-
		ANG003	-	-
		ANG004	-	-
		ANG009	-	-
		ANG007	-	-
		ANG001	-	-
		ANG006	-	-
		ANG005	-	-
		404	-	-
		434	-	-
		JSFC1502	-	-
		BRAH32	-	-
		BRAH58	-	-
		BRAH63	-	-
		Sussex 5	-	-
		Hereford 4	-	-
		Sussex 2	-	-
		Sussex 1	-	-
		Heifer 6	-	-

LABORATORY REFERENCE NUMBER	DISTRICT	ANIMAL IDENTIFICATION	PCR RESULT	CULTURE RESULT
A2008/03_164	Greytown	08	-	-
		01	-	-
		07	-	-
		05	-	-
		06	-	-
		03	-	-
A2008/03_239	Ladysmith	Jersey	-	-
		B18	-	-
		B20	-	-
		04215	-	-
		B01	-	-
		Red	-	-
		Jeff	-	-
		04197	-	-
A2008/03_433	Mooi River	AA 98 Pagate	-	-
		AA Zama	-	-
		AA 48 Max	-	-
		AA 583	-	-
A2008/03_434	Estcourt	42	-	-
		0404	-	-
A2008/04	Howick	1	-	-
		2	-	-
		3. Shark	+	+
		4	-	-
		5	-	-
A2008/04_023	Estcourt	34	-	-
		23	-	-
		8	-	-
A2008/05_0079	Newcastle	1	+	+
		2	-	-
		3	+	+
		4	+	+
		5	-	-
		6	-	+
A2008/05_100	Estcourt	0625	-	-

LABORATORY REFERENCE NUMBER	DISTRICT	ANIMAL IDENTIFICATION	PCR RESULT	CULTURE RESULT
		0633	-	-
		06040	-	-
		06110	-	-
		06114	-	-
		0648	-	-
A2008/05_117	Greytown	# 2	-	-
		# 1	-	-
		# 3	-	-
A2008/05_184	Dundee	Brahman	-	-
		Brangus	-	-
A2008/05_185	Dundee	CR213	-	-
		3070	-	-
		CR3-47	+	+
A2008/05_195	Estcourt	Simbra	-	-
		BLES	-	-
		3045	-	-
		Brahman	-	-
A2008/05_386	Utrecht	1	-	-
		2	-	-
		3	-	-
		4	-	-
		5	-	-
		6	-	-
		7	-	-
		8	-	-
		9	-	-
		10	-	-
		11	-	-
		12	-	-
		13	-	-
		14	-	-
		15	-	-
A2008/05_425	Vryheid	GG0014	-	-
		1702	-	-
		0410	-	-

LABORATORY REFERENCE NUMBER	DISTRICT	ANIMAL IDENTIFICATION	PCR RESULT	CULTURE RESULT
		530	-	-
		1903	-	-
		0329	-	-
A2008/05_446	Dundee	FLA 1-99	-	-
		49	-	-
		1	-	-
		60	-	-
		2	-	-
		299	-	-
		57	-	-
		FS (9)	-	-
		217	-	-
		JM1	-	-
		MI1	-	-
A2008/05_467	Dundee	2001-17	-	-
		Stoekbul	-	-
		0365	-	-
		962000	+	+
		0282	-	-
		0318	-	-
		552	-	-
		99110	-	-
		HVR5302	-	-
		SB4902	-	-
		JAV 7/01	-	-
		Rondloper	-	-
A2008/06_240	Dundee	U.70.03	-	-
		U.92.03	-	-
A2008/07_178	Mtubatuba	143/04 Brahman	-	-
		HR 15/04	-	-
		FJB 18/02	-	-
		TCC 02/02	-	-
		HVR 237/04	-	-
		89/04	-	-
		D3 29/2	-	-

LABORATORY REFERENCE NUMBER	DISTRICT	ANIMAL IDENTIFICATION	PCR RESULT	CULTURE RESULT
		GG 118 Brangus	-	-
		3TU Bonsmara	-	-
		GG306 Brangus	-	-
		GG24 Brangus	-	-
		GG204 Brangus	-	-
A2008/07_275	Piet Retief	1	-	-
		2	-	-

Key:

+ Positive
- Negative

APPENDIX 5: BIOCHEMICAL CHARACTERIZATION OF C. *FETUS* ISOLATES

The first six *C. fetus* cultures listed in the table below (Reference bank numbers 39-44) are the reference cultures. Test results have been shaded in grey. All subspeciation tests were carried out in triplicate.

Reference bank number	Source	Catalase	Oxidase	Motility	H_2S production		Growth in/at				Antibiotic Susceptibility				Selenite reduction	Tolerance to 1% glycine	Biochemical subspeciation
					TSI	Lead acetate	Air	Anaerobic atmosphere	25 °C	42 °C	Cephalothin (30 µg)	Cefoperazone (30 µg)	Nalidixic acid (30 µg)	Metronidazole (5 µg)			
39	NCTC 10848	+	+	+	- - -	+ + +	-	+	+	+ - +	S S S	R R R	R R R	R R R	+ + +	+ + +	**Cff**
40	ATCC 33247	+	+	+	- - -	+ + +	-	+	+	- - +	S S S	R R R	R R R	R R R	+ + +	+ + +	**Cff**
41	NCTC 10354	+	+	+	- - -	- - -	-	+	+	- - -	S S S	R R R	R R R	S S S	- - -	- - -	**Cfv**
42	LMG 6570	+	+	+	- - -	- - -	-	+	+	- - -	S S S	S S S	R R R	R R R	- - -	- - -	**Cfv**
43	10	+	+	+	-	+	-	+	+	+	S	S	R	R	-	-	**Cfv-i**

Reference bank number	Source	Catalase	Oxidase	Motility	H_2S production		Growth in/at				Antibiotic Susceptibility				Selenite reduction	Tolerance to 1% glycine	Biochemical subspeciation
					TSI	Lead acetate	Air	Anaerobic atmosphere	25 °C	42 °C	Cephalothin (30 µg)	Cefoperazone (30 µg)	Nalidixic acid (30 µg)	Metronidazole (5 µg)			
					- -	+ +				- -	S S	S S	R R	S R	- -	- -	
44	136	+	+	+	- - -	- + + +	-	+	+	- - -	S S S	S S S	R R R	R R R	- - -	- - +	**Cfv-i**
1	Allerton PVL 1991	+	+	+	- - -	+ + +	-	+	+	+	S S S	R R R	R R R	R R R	+ + +	+ + +	**Cff**
2	Allerton PVL 1996	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S S S	W W W	- - -	**Cfv-i**
3	Allerton PVL 2006	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	R S R	- - -	- - -	**Cfv-i**
4	Western Cape PVL	+	+	+	- - -	+ + -	-	+	+	-	S S S	S S S	R R R	R R R	- - -	+ + -	**Cff**
8	Ellisras PVL 2006	+	+	+	- - -	- + + +	-	+	+	-	S S S	S S S	R R R	R R R	+ + +	- - -	**Cfv-i**
9	Ermelo PVL 2006	+	+	+	- -	+ +	-	+	+	-	S S	S S	R R	S R	+ -	- -	**Cfv-i**

Reference bank number	Source	Catalase	Oxidase	Motility	H_2S production		Growth in/at				Antibiotic Susceptibility				Selenite reduction	Tolerance to 1% glycine	Biochemical subspeciation
					TSI	Lead acetate	Air	Anaerobic atmosphere	25 °C	42 °C	Cephalothin (30 µg)	Cefoperazone (30 µg)	Nalidixic acid (30 µg)	Metronidazole (5 µg)			
					-	+					S	S	R	S	-	+	
10	Ermelo PVL 2006	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S R S	- - -	- - -	**Cfv-i**
11	Allerton PVL 2006	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S S S	W W -	- - -	**Cfv-i**
17	Allerton PVL 1990	+	+	+	- - -	+ + +	-	+	+	-	S S S	R R R	R R R	R R R	W W +	+ + +	**Cff**
22	Allerton PVL 2007	+	+	+	- - -	+ - +	-	+	+	-	S S S	S S S	R R R	R R R	- - -	- - -	**Cfv-i**
26	Ellisras 2007	+	+	+	- - -	+ + -	-	+	+	-	S S S	S S S	S S S	S R R	+ + +	- - -	**Cfv-i**
28	Onderstepoort Veterinary Institute	+	+		- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S R R	- - -	- - -	**Cfv-i**
29	Onderstepoort Veterinary	+	+	+	- -	+ +	-	+	+	-	S S	S S	R R	S S	- -	- -	**Cfv-i**

Reference bank number	Source	Catalase	Oxidase	Motility	H_2S production		Growth in/at				Antibiotic Susceptibility				Selenite reduction	Tolerance to 1% glycine	Biochemical subspeciation
					TSI	Lead acetate	Air	Anaerobic atmosphere	25 °C	42 °C	Cephalothin (30 µg)	Cefoperazone (30 µg)	Nalidixic acid (30 µg)	Metronidazole (5 µg)			
	Institute				-	+					S	S	R	S	-	-	
30	Onderstepoort Veterinary Institute	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	R R R	+ - -	- - -	**Cfv-i**
31	Onderstepoort Veterinary Institute	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S R R	- - -	- - -	**Cfv-i**
34	Allerton PVL 2007	+	+		- - -	+ + +	-	+	+	-	S S S	R R R	R R R	R R R	+ + +	+ + +	**Cff**
35	Allerton PVL 2007	+	+	+	- - -	+ + -	-	+	+	-	S S S	R R R	R R R	R R R	+ + +	+ + +	**Cff**
36	Ermelo PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S S S	- - -	- - -	**Cfv-i**
37	Ermelo PVL 2007	+	+	+	- - -	+ + +	-	-	+	-	S S S	S S S	R R R	R S R	W W W	- - -	**Cfv-i**
45	Ermelo PVL 2007	+	+	+	- -	+ +	-	+	+	- -	S S	S S	R R	S S	+ +	- -	**Cfv-i**

Reference bank number	Source	Catalase	Oxidase	Motility	H_2S production		Growth in/at				Antibiotic Susceptibility				Selenite reduction	Tolerance to 1% glycine	Biochemical subspeciation
					TSI	Lead acetate	Air	Anaerobic atmosphere	25 °C	42 °C	Cephalothin (30 µg)	Cefoperazone (30 µg)	Nalidixic acid (30 µg)	Metronidazole (5 µg)			
					-	+					S	S	R	S	+	-	
46	Ermelo PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	R S R	- - -	- - -	**Cfv-i**
47	Allerton PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	R R R	+ + +	+ - + +	**Cff**
48	Allerton PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S S S	- - +	- - -	**Cfv-i**
49	Allerton PVL 2007	+	+		- - -	+ + +	-	+	+	-	S S S	S S S	R S S	R S S	- - -	- - -	**Cfv-i**
50	Western Cape PVL 2007	+	+	+	- - -	+ + +	-	+	-	-	S S S	S S S	R R R	S S S	- - -	- - -	**Cfv-i**
51	Vryburg PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S R R	- - -	- - -	**Cfv-i**
52	Vryburg PVL 2007	+	+	+	- -	+ +	-	+	+	-	S S	S S	R R	S S	- -	- -	**Cfv-i**

Reference bank number	Source	Catalase	Oxidase	Motility	H_2S production		Growth in/at				Antibiotic Susceptibility				Selenite reduction	Tolerance to 1% glycine	Biochemical subspeciation
					TSI	Lead acetate	Air	Anaerobic atmosphere	25 °C	42 °C	Cephalothin (30 µg)	Cefoperazone (30 µg)	Nalidixic acid (30 µg)	Metronidazole (5 µg)			
					-	+					S	S	R	S	-	-	
53	Vryburg PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	R R R	+ + +	- - -	**Cfv-i**
59	Ermelo PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	R R R	- - -	- - -	**Cfv-i**
60	Western Cape PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S R S	- - -	- - -	**Cfv-i**
61	Ermelo PVL 2007	+	+	+	- - -	+ - +	-	+	+	-	S S S	S S S	R R R	S S R	- - -	- - -	**Cfv-i**
62	Vryburg PVL 2007		+	+	- - -	+ + +	-	+	+	-	S S S	S S S	R R R	S S S	- W W W	- - -	**Cfv-i**
65	Vryburg PVL 2007		+		- - -	- + +	-	+	+	-	S S S	S S S	R R R	S S S	- - W W	- - -	**Cfv-i**
66	Vryburg PVL 2007	+	+	+	- - -	+ + +	-	+	+	-	S S S	R R R	R R R	R R R	+ + +	- - -	**Cfv-i**

Reference bank number	Source	Catalase	Oxidase	Motility	H_2S production		Growth in/at				Antibiotic Susceptibility				Selenite reduction	Tolerance to 1% glycine	Biochemical subspeciation
					TSI	Lead acetate	Air	Anaerobic atmosphere	25 °C	42 °C	Cephalothin (30 µg)	Cefoperazone (30 µg)	Nalidixic acid (30 µg)	Metronidazole (5 µg)			
70	Ermelo PVL 2008	+	+	+	- - -	- W + W	-	+	+	-	S S S	S S S	R R R	S S S	+ + -	- - -	**Cfv-i**
71	Allerton PVL 2008	+	+	+	- - -	+ W +	-	+	+	+	S S S	S S S	R R R	S S R	- - -	- - -	**Cfv-i**
72	Ermelo PVL 2008	+	+	+	- - -	+ W W	-	+	+	-	S S S	S S S	R R R	S S S	- - -	- - -	**Cfv-i**
73	Allerton PVL 2008	+	+	+	- - -	+ + +	-	+	+	-	S S S	S S R	R R R	S S S	- - -	- - -	**Cfv-i**
74	Allerton PVL 2008	+	+	+	- - -	+ + W	-	+	+	+	S S S	S S S	R R R	S S S	- - -	- - -	**Cfv-i**
75	Allerton PVL 2008	+	+	+	- - -	+ + +	-	+	+	+	S S S	S S S	R R R	S S S	- - -	- - -	**Cfv-i**

Key:

+	=	growth
-	=	no growth
R	=	resistant
S	=	sensitive
W	=	weak

APPENDIX 6: SUBSPECIATION OF *C. FETUS* ISOLATES USING PHENOTYPING CHARACTERIZATION TESTS AND A PCR ASSAY

Reference bank number	Source of isolate	Date of isolation	Animal / Sample origin	PCR results		PCR subspeciation result	Biochemical subspeciation result
				C. fetus primer pair	Sub-species primer pair		
39	NCTC 10842 / ATCC 27374 France	Unknown	Ovine	+	-	**Cff**	**Cff**
40	ATCC 33247 5515 United States of America	Unknown	Human	+	-	**Cff**	**Cff**
41	NCTC 10354 / ATCC 19438 United Kingdom	Unknown	Bovine	+	+	**Cfv**	**Cfv**
42	LMG 6570 Belgium	Unknown	Bovine	+	+	**Cfv**	**Cfv**
43	10 South Africa	Unknown	Unknown	+	-	**Cff**	**Cfv-i**
44	136 South Africa	Unknown	Unknown	+	-	**Cff**	**Cfv-i**
1	Allerton PVL (KwaZulu Natal)	1991	Unknown	+	-	**Cff**	**Cff**
2	Allerton PVL (KwaZulu Natal)	1996	Unknown	+	-	**Cff**	**Cfv-i**
3	Vetdiagnostix (KwaZulu Natal)	2006	Bovine foetus	+	-	**Cff**	**Cfv-i**
4	Western Cape PVL (Western Cape)	Unknown	Unknown	+	-	**Cff**	**Cff**
8	Ellisras PVL	2006	Bovine preputial	+	-	**Cff**	**Cfv-i**

Reference bank number	Source of isolate	Date of isolation	Animal / Sample origin	PCR results		PCR subspeciation result	Biochemical subspeciation result
				C. fetus primer pair	Sub-species primer pair		
	(Limpopo)		specimen				
9	Ermelo PVL (Mpumalanga)	2006	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
10	Ermelo PVL (Mpumalanga)	2006	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
11	Allerton PVL (KwaZulu Natal)	2006	Bovine preputial specimen	+	-	**Cff**	**Cfv-I**
17	Allerton PVL (KwaZulu Natal)	1990	Unknown	+	-	**Cff**	**Cff**
22	Allerton PVL (KwaZulu Natal)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
26	Ellisras PVL (Limpopo)	2007	Bovine foetus	+	-	**Cff**	**Cfv-i**
28	Onderstepoort Veterinary Institute (storage)	Unknown	Unknown	+	-	**Cff**	**Cfv-i**
29	Onderstepoort Veterinary Institute (storage)	Unknown	Unknown	+	-	**Cff**	**Cfv-i**
30	Onderstepoort Veterinary Institute (storage)	Unknown	Unknown	+	-	**Cff**	**Cfv-i**
31	Onderstepoort Veterinary Institute (storage)	Unknown	Unknown	+	-	**Cff**	**Cfv-I**
34	Allerton PVL (KwaZulu Natal)	2007	Bovine faeces	+	-	**Cff**	**Cff**
35	Allerton PVL (KwaZulu Natal)	2007	Bovine faeces	+	-	**Cff**	**Cff**
36	Ermelo PVL (Mpumalanga)	2007	Bovine foetus	+	-	**Cff**	**Cfv-i**

Reference bank number	Source of isolate	Date of isolation	Animal / Sample origin	PCR results		PCR subspeciation result	Biochemical subspeciation result
				C. fetus primer pair	Sub-species primer pair		
37	Ermelo PVL (Mpumalanga)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
45	Ermelo PVL (Mpumalanga)	2007	Bovine foetus	+	-	**Cff**	**Cfv-i**
46	Ermelo PVL (Mpumalanga)	2007	Bovine foetus	+	-	**Cff**	**Cfv-i**
47	Allerton PVL (KwaZulu Natal)	2007	Bovine preputial specimen	+	-	**Cff**	**Cff**
48	Allerton PVL (KwaZulu Natal)	2007	Bovine foetus	+	-	**Cff**	**Cfv-i**
49	Allerton PVL (KwaZulu Natal)	2007	Bovine foetus	+	-	**Cff**	**Cfv-i**
50	Western Cape PVL (Western Cape)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
51	Vryburg PVDD (North West Province)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
52	Vryburg PVDD (North West Province)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
53	Vryburg PVDD (North West Province)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
59	Ermelo PVL (Mpumalanga)	2007	Bovine foetus	+	-	**Cff**	**Cfv-i**
60	Western Cape PVL (Western Cape)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
61	Ermelo PVL (Mpumalanga)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
62	Vryburg PVDD (North West province)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
65	Vryburg PVDD (North West Province)	2007	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**

Reference bank number	Source of isolate	Date of isolation	Animal / Sample origin	PCR results		PCR subspeciation result	Biochemical subspeciation result
				C. fetus primer pair	Sub-species primer pair		
66	APVL (KwaZulu Natal)	1995	Unknown	+	-	**Cff**	**Cfv-i**
70	Ermelo PVL (Mpumalanga)	2008	Unknown	+	-	**Cff**	**Cfv-i**
71	Allerton PVL (KwaZulu Natal)	2008	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
72	Ermelo PVL (Mpumalanga)	2008	Unknown	+	-	**Cff**	**Cfv-i**
73	Allerton PVL (KwaZulu Natal)	2008	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
74	Allerton PVL (KwaZulu Natal)	2008	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**
75	Allerton PVL (KwaZulu Natal)	2008	Bovine preputial specimen	+	-	**Cff**	**Cfv-i**

Key:
+ Positive
- Negative

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