DIAGNOSIS OF BOVINE CAMPYLOBACTERIOSIS

by

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ABSTRACT

Seven virgin heifers and two bulls, five and six years old respectively, were challenged with *Campylobacter fetus* subsp. *venerealis*, isolate FD15. The challenge dose consisted of approximately 10^9 organisms per ml of phosphate buffered saline (PBS)pH7.2.

Cervicovaginal mucus was collected from heifers beginning one week after exposure using the technique described by Hoerlein and Kramer.[31] These were cultured on a solid selective medium.[14 23] Of the seven heifers, two became infected as determined by consistent recovery of the organism from cervical mucus samples. Seventy-four samples from both heifers were cultured; eight samples from one heifer, D07, were discounted on the assumption that she overcame her infection. Of the sixty-six samples, forty-eight yielded the organism on culture, giving a recovery rate of 72.4%.

Preputial samples were collected from the two bulls by a pipette[3] using the technique described by Dufty.[23] These were cultured directly and following millipore filtration on a solid selective medium.[14 23] They were also examined by immunofluorescence using a similar technique to that of Dufty[22] and Schutte.[65] Of the thirty-two samples examined by both methods, twenty-six (81%) were positive on immunofluorescence examination, twenty-two (69%) were positive on culture, and twenty-seven (84%) were positive to both tests.

It is suggested that the techniques used for sample collection and examination by culture and immunofluorescence provide an effective method for herd diagnosis of the disease.

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INTRODUCTION

Although the clinical signs associated with genital tract infection by *Campylobacter fetus* subsp. *venerealis* in cattle have been well documented, definitive diagnosis has been a difficult task. The major problems have been the organism's susceptibility to normal atmospheric levels of oxygen, its fastidious growth requirements, and overgrowth by contaminating organisms. Indeed, many of the early reports of diagnosis of the disease have been largely based on the detection of the organism by microscopy.

The attention of many workers in this field has been directed to overcoming these problems thereby increasing diagnostic efficiency. This has resulted in the development of better and more selective media for bacteriological culture, the use of microaerophilic conditions for culturing the organisms, the use of millipore filtration to reduce contamination, and the development of enrichment media to preserve the viability of the organism during transportation.

Diagnosis of the disease in bulls can now be efficiently carried out by cultural and immunofluorescence examinations of preputial samples, either singly or preferably in combination. Using this approach, diagnostic efficiency rates of over 90% have been achieved.[11 22 64]

Diagnosis of the disease in females is best carried out by cultural examinations of cervical and/or vaginal mucus samples collected from non-pregnant animals. Success rates of 78%,[14] 83%,[37] and 54%[31] have been achieved using this method. The use of the vaginal mucus agglutination (VMA) test is considered valuable for herd diagnosis.[9]

The objectives of the investigation reported in this thesis were to attempt to artificially infect virgin heifers and bulls with *C. fetus* subsp. *venerealis*, isolate FD15, to determine the duration of the infection in heifers as shown by recovery of the organism from cervical mucus, and to assess the diagnostic efficiency of cultural examination in the female and of cultural and immunofluorescence examination in the male.

I. A REVIEW OF BOVINE CAMPYLOBACTERIOSIS

INTRODUCTION

The pathogenicity of the genus now known as Campylobacter was first recognised in 1913 when McFadyean and Stockman reported a 'vibrio-like' organism associated with abortions in pregnant ewes.[43] They subsequently produced abortions in cows by intravenous innoculations with the same organisms. In 1918, Smith[73] recovered a microaerophilic 'spirillum' from aborted calves which he suspected was similar to that described by McFadyean and Stockman.[43] This organism was designated *Vibrio fetus* on morphological grounds.[74] The genus Vibrio was chosen because of the predominance of comma-shaped cells, typical of other members of the genus, over spirilloid ones, particularly in young cultures. Little attention was directed to these organisms until their pathogenic role in temporary sterility in cattle was established.[58]

It became evident that more than one type of *Vibrio* could be isolated from the genital tract of cattle. Evidence presented then suggested that catalase positive strains should be considered pathogenic whereas catalase negative strains, which were not related antigenically to the former, should be considered saprophytes.[6 46] Two distinct types were recognised within the same species, *V.fetus var. venerealis* associated with infertility and *V.fetus var. intestinalis* associated with sporadic abortions; as well as these, some intermediate types were also recognised.[26 27]

In addition to cattle and sheep, *V.fetus* has been isolated from a

wide range of animals including pigs, poultry and birds.[72] They have also been recovered from cases of human enteritis.[35]

TAXONOMY

Originally the organism was placed in the genus Vibrio largely on morphological grounds.[74] However, this criterion alone is now considered of dubious taxonomic value.[20] *V.fetus* differs greatly in phenotypic respects from the type species of the genus Vibrio, which is *V.cholerae* and their guanine and cytosine (G+C) content of the deoxyribonucleic acid (DNA) component also differs greatly.[78]

For these reasons, a new genus, *Campylobacter*, has been created with *C.fetus* having been proposed as the type species.[66] In the Approved Lists of Bacterial Names,[70] the term Campylobacter has now been accepted as the generic name for the microaerophilic 'vibrios' on the grounds that they differ from the classical cholera and halophilic groups in certain fundamental respects.[72 75]

The genus Campylobacter is now defined as comprising bacteria with the following characteristics [78]:

(i) gram-negative

(ii) slender,curved rods

(iii) non-sporing

(iv) motile by means of polar flagellae,usually one but there may be one at both ends of the cell

(v) microaerophilic with a respiratory mechanism

(vi) non-fermentative, i.e. does not produce acid in a carbohydrate medium

(vii) have a G+C ratio of between 29 and 36 mol %
for their DNA component.

In contrast, the members of the genus *Vibrio* ferment glucose and possess DNA with a G+C ratio of between 40 and 53 mol %.[78]

The Campylobacter have now been included in the family *Spirillaceae* because of morphological and physiological similarities with the genus *Spirillum*. In addition, striking analogies between the anatomical features of members of the genus *Campylobacter* and the genus *Spirillum* have been observed under the electron microscope.[61] These similar features are : an outer wavy membrane, a complex cytoplasmic membrane, and individual flagellar basal granules.

Since the original description of *Vibrio fetus*, other species and subspecies of microaerophilic curved bacteria have been reported, which have now been included in the genus *Campylobacter*. In recent years, much controversy has arisen over the system of nomenclature used for members of this genus. However, names appearing in the Approved Lists of Bacterial Names are now the only names which are nomenclaturally valid as at the 1st January, 1980.[70]

Table I shows the approved names and some of the previous names used prior to 1st January, 1980.

TABLE I : CLASSIFICATION OF CAMPYLOBACTER SPECIES

APPROVED NAMES [70]	SMIBERT [71]	VERON & CHATELAIN[78]	PREVIOUS CLASSIFICATION AS VIBRIO	SEROLOGICAL CLASSIFICATION[4]
C.fetus subsp.*fetus*	*C.fetus* subsp. *intestinalis*	*C.fetus* subsp.*fetus*	*V.fetus* subsp.*intestinalis*	A-2, B
C.fetus subsp.*venerealis*	*C.fetus* subsp. *fetus*	*C.fetus* subsp.*venerealis*	*V.fetus* subsp.*venerealis*	A-1, A-sub 1
C.coli	*C.fetus* subsp. *jejuni*	*C.coli*	*V.coli* and "related vibrios" [35] [36]	C
C.jejuni	*C.fetus* subsp. *jejuni*	*C.jejuni*	*V.jejuni*	
C.sputorum subsp. *sputorum*	*C.sputorum* subsp. *sputorum*	*C.sputorum* subsp.*sputorum*	*V.sputorum*	
C.sputorum subsp. *bubulus*	*C.sputorum* subsp. *bubulus*	*C.sputorum* subsp.*bubulus*	*V.bubulus*	

a) *C.fetus* subsp. *venerealis*

Both *venerealis* and biotype *intermedius* are associated with Campylobacteriosis (Vibriosis) in cattle, with the former being the more prevalent biotype. The disease is characterised by repeated irregular returns to service with many interservice intervals of 28 to 35 days. This infertility will continue for three to five months with only 20 to 40% becoming pregnant after two services. A small percentage (5 to 10%) will abort after approximately five months of pregnancy.[10]

The natural habitat is within the reproductive tract; in bulls, the organisms are confined to the preputial cavity, whereas in females, they are found within the lumen of the genital tract.[9] Neither biotype will multiply in the intestinal tract of man or animals.[72]

b) *C.fetus* subsp. *fetus*

This organism causes sporadic abortions in sheep and cattle. It is found primarily as a commensal organism in the intestinal tract of cattle, sheep, swine, birds and humans.[78] Isolations from placentas, lungs and stomach contents of aborted foetuses from both sheep and cattle and from blood, intestinal contents, faeces, and bile of infected cattle, sheep and humans have also been made. [72]

Because the organism seems adapted to the intestinal environment, it is unable to survive for long periods in the reproductive tracts of non-pregnant animals but blood-borne organisms can invade the gravid uterus and cause abortions. [72 78]

c) *C.coli*

This organism is a normal inhabitant of the intestines of swine, poultry, and (occasionally) man, but is absent in sheep and cattle. It can cause dysentery in swine, hepatitis in birds, and bloody diarrhoea in man. [78]

d) *C.jejuni*

This organism frequently occurs in the intestinal tracts of cattle and sheep, in which it can cause mild dysentery.[78]

e) *C.sputorum*

Subspecies *sputorum* is an occasionally pathogenic human parasite; it has been recovered from the oral cavity in gingivitis and from sputum in bronchitis.[72]

Subspecies *bubulus* appears to be non-pathogenic. It has been isolated from vaginal and preputial secretions, semen, foetuses, and foetal membranes as well as from faeces from sheep, horses, and cattle.[78]

DIFFERENTIATION

Although *C.fetus* has been known to be associated with diseases in cattle and sheep for many years, recognition has been largely based on its morphology and oxygen requirements. However, since many similarities exist among *Campylobacter* species, differentiation is now based on biochemical and growth tolerance tests.[78] The major tests used are :

(i) hydrogen sulphide production in medium containing cysteine (sensitive H_2S test) and in triple sugar iron medium (insensitive H_2S test)

(ii) catalase activity

(iii) salt tolerance test

(iv) glycine tolerance test

(v) heat tolerance test

Table II shows the differentiation of *Campylobacter* species based on these tests.

TABLE II : DIFFERENTIATION OF CAMPYLOBACTER SPECIES

SPECIES AND SUBSPECIES	CAT	H_2S(S)	H_2S(I)	GLY	NaCl	25°C	42°C
C.fetus subsp.*fetus*	+	+	-	+	-	+	-
C.fetus subsp.*venerealis*	+	-	-	-	-	+	-
C.fetus subsp.*venerealis* biotype *intermedius*	+	+	-	-	-	+	-
C.coli	+	+	±	+	-	-	+
C.jejuni	+	+	-	+	-	-	+
C.sputorum subsp.*sputorum*	-	+	+	+	-	-	-
C.sputorum subsp.*bubulus*	-	+	+	+	+	±	-

Abbreviations : CAT = Catalase activity
H_2S(S)= H_2S production in sensitive medium
H_2S(I)= H_2S production in insensitive medium
GLY = 1% Glycine
NaCl = 3.5% salt
\+ = positive or growth
\- = negative or no growth
± = variable result

MORPHOLOGY

Campylobacter are curved, spiral rods, 1.5 - 3.5μm long and 0.2 - 0.4μm wide. They are gram-negative, non-sporing microaerobes motile by means of a single polar flagellum at one or both ends of the cell. The rods may have one or more spirals and they may appear S-shaped and "gull-winged" when two cells form short chains. The ends of the cells are usually pointed. *C.fetus* may appear ribbon-shaped being comprised of a chain of single curved cells. In old cultures, cells may become coccoid ("ring forms"); these appear to be a degenerative form because cultures composed mainly of the coccoid forms are nonviable.[72]

Motility is by means of a long, polar flagellum two to three times the length of the cells; there may be one at both ends of the cell. When dark ground microscopy is used to examine liquid cultures, the typical unique corkscrew motility is observed. In older cultures, motility is often reduced and coccoid forms with one or more flagellae often occur.[72]

GROWTH CHARACTERISTICS

a) Solid Media

Considerable variations in colony type occur on solid media. The basic types are described as smooth, cut-glass, rough and mucoid. Colony size also varies greatly with both small (0.5mm diameter) and large (3mm diameter) colonies occurring on the same plate. The smooth colony type is the one most frequently recovered on primary isolation from animals. They usually have a diameter of 0.5mm, are round, slightly raised, colourless, and slightly translucent. Colony variation proceeds from smooth to cut-glass to rough. Cut-glass

colonies are about 1mm in diameter, round, raised, translucent, and granular with reflecting facets. Rough colonies are less commonly found, similar in size to smooth colonies except that they are granular and more opaque. Mucoid colonies are similar in size but viscid.[71]

After 48 hours incubation on blood agar, colonies attain a diameter of at least 1mm; and are round, raised, and translucent with a regular edge and a smooth glistening surface. Sometimes on initial isolation, colonies occur as a thin veil of translucent confluent growth, especially on very moist blood agar. Colour varies from translucent to opaque grey to buff.[71]

b) Semisolid Medium

In semisolid medium (0.14-0.16% agar), growth occurs as a disc-shaped zone 2-3mm below the surface. After 48 hours, growth becomes abundant in the upper portion. No growth occurs in the centre or at the bottom of the medium.[72]

CAMPYLOBACTERIOSIS IN CATTLE

In cattle, the disease is characterised by infertility, irregular returns to service, and occasional abortions. It is associated with genital tract infection by both *C.fetus* subsp. *venerealis* and *C.fetus* subsp. *venerealis* biotype *intermedius* of which the former is the more common. Antigenically, the biotypes are closely related and their differentiation is based on differing biochemical properties.

DISTRIBUTION

The causal association between genital tract infection with this organism and an infertility syndrome in cattle was first established in 1947.[58] Since then, the disease has been recognised internationally as an important cause of reduced breeding efficiency in cattle. However, more recently, the disease has become less important in dairy cattle in countries where control measures based on the widespread use of artificial insemination, rigorous testing, culling, and treatment of bulls for this disease, and the addition of effective antibiotics to semen, have been adopted. Less success in controlling the disease in beef cattle has been achieved due to the impracticality of widespread use of artificial insemination. Control measures consist of annual vaccination programmes, culling of bulls, and antibiotic therapy.

In New Zealand, it is generally accepted that the prevalence of the disease is low, more so in dairy than in beef herds. However, no effort to determine the prevalence or to identify the geographical distribution has been undertaken.

Indeed few reports of a definitive diagnosis of this disease in New Zealand can be found in the literature.

EPIDEMIOLOGY AND TRANSMISSION

The natural habitat of *C. fetus* subsp. *venerealis* is within the bovine reproductive tract. It is unable to survive and multiply in the

intestinal tract. In bulls, the organism is confined to the preputial cavity, particularly the mucosa of the glans penis, prepuce and distal portion of the urethra.[10] In cows and heifers, the sites of infection are within the lumen of the vagina, cervix, uterus and oviducts. [9]

Under natural conditions, transmission of infection occurs during coitus. Direct transmission between females is unlikely[38] and attempts to infect female cattle by contamination of the vulva have failed.[42] [51] However, transmission between bulls is possible during mounting especially when large numbers of bulls are kept together.[79] Furthermore, where bulls are regularly housed, contaminated bedding can act as a source of infection.[56] [65] Bulls may also be infected through the use of contaminated semen collection equipment. The only other way that transmission is known to occur is by intrauterine innoculation during the artificial insemination procedure, when semen collected from infected bulls is used.[9]

PATTERN OF HERD INFERTILITY

The classic manifestations of Campylobacteriosis are repeat breeding, delayed conception, and eventual development of convalescent immunity. This situation will only occur when most of the female population is susceptible and all are exposed to infection at first breeding. It is a relatively rare occurrence in beef herds but when it does occur, infertility is severe. Where breeding seasons are restricted to 60 days or less, conception rates will be only 20-30%.[8] Often the initial introduction of the disease into a beef herd does not result in a

dramatic reduction in breeding efficiency. The most common sign of infection in a beef herd is a prolonged calving season with a high percentage of cows calving in the latter part of the calving period.

Typically, Campylobacter infected herd under extensive management in the western United States is characterised by normal reproductive rates in heifers, lowest calf crop in those with first and second calves, and higher pregnancy rates in the older age groups.[32] The abortion frequency is usually less than 1% and the herd pregnancy rate about 85%. The variable pattern of infertility in infected beef herds can usually be explained by combinations of different circumstances. Normal reproductive rates in heifers are due to the practice of mating heifers as a separate group to young and often newly-purchased bulls. Under these circumstances there are no carrier animals and no sources of infection. The poorest reproductive performance occurs in first and second calvers, which have joined the main mixed age groups. They are highly susceptible and are exposed to infection from carrier bulls and cows. In the older age group, there is an increasing proportion of animals with some level of convalescent immunity that have a higher pregnancy rate. In chronically infected herds, these cows will not necessarily be exposed to infection each year. Thus there may be many combinations of carrier,susceptible, and immune cattle in the herd with the result that marked variation in the pattern of infertility is shown.

As far as the male is concerned, most bulls, five years old and younger, will not carry the infection from one breeding season to the next.[8] Individual bulls, if not infected, will become active in transmitting

the disease only after they have bred a carrier cow. Widespread dissemination only occurs when most of the bulls are carriers at the beginning of the breeding season.

INFECTION IN BULLS

Infection with *C.fetus* subsp. *venerealis* in the bull does not produce any associated histological changes nor any changes in semen characteristics. In effect, the bull acts as a true carrier.[9]

The susceptibility of younger bulls to natural or experimental infection is very low whereas bulls over five years old are highly susceptible and retain the organisms for long periods.[79] This increased susceptibility is attributed to an increase in number and size of crypts in the epithelium of the prepuce and penis. [64] Whilst the organism may be found on the epithelial surface throughout the entire preputial cavity, greatest numbers are found in the fornix of the prepuce and on the penis. The greatest concentration of organisms are found in the lumina of the epithelial crypts, suggesting that these structures provide ideal environmental conditions for their survival.[64]

There is considerable variability in the ability of infected bulls to transmit infection. Although younger bulls may not become infected and harbour organisms for long periods, they are still able to infect susceptible female cattle during the time of their transient infection by mechanical venereal transmission. When rested or simply washed out, many become clear of the infection but may subsequently become reinfected.[32] In older bulls, the number of viable organisms that can be isolated

varies considerably between bulls (<100 to >200,000 per ml). Furthermore the numbers of organisms may decline substantially if bulls are serving several cows every day.[9]

It is not surprising therefore that the ability of different bulls to transmit infection to cows varies greatly. Newsam[48] found that 38%, 53%, 61% and 76% of heifers served once by four different bulls respectively became infected.

INFECTION IN FEMALE CATTLE

Cows and heifers that have had no previous contact with *C.fetus* subsp.*venerealis* are usually highly susceptible. However, it is not unusual for a small percentage not to become infected in spite of being challenged with large doses of viable organisms.[9] The susceptibility of female cattle does not appear to alter with increasing age.

Following transmission, organisms can be found in the vagina, cervix, uterus and oviducts. Infection of the uterus and oviducts may persist for about two months, but thereafter infection is progressively eliminated so that by the third month, it is usually confined to the cervix and vagina.[54]

Clinically, the disease is manifested as an infertility syndrome characterised by irregular returns to service with many interservice intervals of 28 to 35 days.[10] A high percentage of infected females return to service for three to five months and only 25 to 40% may be pregnant after two services. The average time from first service to pregnancy often exceeds 60 days. Although most will eventually

conceive, 5 to 10% will abort after about five months gestation and the organisms can be recovered readily from fresh aborted foetuses.[9] It is more difficult to isolate the organism from heifers after they become pregnant[14] and up to 10% may still be infected after calving; most are free of infection by three months post-partum. Non-pregnant females tend to remain infected for six months or more[77] and 50% may still carry a vaginal infection after ten months.[59] These carrier animals can therefore act as a source of infection in the next breeding season.

The histopathology of Campylobacteriosis in the non-pregnant animal has been described.[54] The presence of the organism in infected animals is not always associated with histological alterations; however, a frequent finding is a mild endometritis. Less frequently, mild inflammatory changes can be observed in the cervix and oviducts. The main lesions are lymphocytic and plasmacytic reactions of the endometrium, more often focal than diffuse in distribution. These lesions are not pathognomonic for the disease and it is arguable if they are sufficient on their own to produce the characteristic infertility syndrome.[54] The lesions are observable by two to three weeks post infection and are still present at 8-12 weeks. Complete recovery generally occurs at four to five months. Although the endometritis is mild, its duration is similar to the period of infertility. The infertility has been attributed to a combination of the mild endometritis and a direct effect of the organism on the conceptus.[77]

Cows that have recovered from the disease possess partial immunity against reinfection and a well developed resistance to the

pathogenic effects of the organism.[77] Although 30-70% may become reinfected when mated with infected bulls, their fertility is only marginally impaired.[9] Immunity declines and has largely vanished by three to four years after the initial infection in most cases.[30]

DIAGNOSIS OF CAMPYLOBACTERIOSIS

Diagnosis of Bovine Campylobacteriosis is based on herd history and on the results of cultural tests from preputial scrapings from bulls and vaginal mucus and aborted foetuses from cows.[10] Early attempts to recover the organism from contaminated material were often not very successful. The major obstacles were the susceptibility of the organism to normal atmospheric levels of oxygen (20%), its fastidious growth requirements, and overgrowth by contaminants. Consequently, diagnoses described in the early literature were largely based on the microscopic recognition of organisms with spirillum morphology.

The susceptibility of the organism to normal atmospheric oxygen levels and the beneficial effects of microaerophilic conditions for culturing the organism have been reported by Plastridge[57] and Doyle.[21] The organism was found to grow optimally in the presence of an atmosphere of 5% oxygen, 10% carbon dioxide, and 85% nitrogen.[33] The oxygen concentration that gives best growth is 6%.[72] The organism will not grow anaerobically.[71]

Up until 1958, little was known of the basic nutritional requirements of *C.fetus*. It was reported to be unable to grow anaerobically and to obtain energy by oxidation of compounds of the tricarboxylic acid cycle or compounds which are easily introduced into the cycle.[33] The

microaerophilic nature of *C.fetus* subsp. *venerealis* as well as its requirements for carbon dioxide have increased the difficulty of its recovery from animal and human sources.

1. BOVINE PREPUTIAL SAMPLES

a) Culture

Despite the realisation of the importance of the bull in transmitting the disease, early diagnostic attempts were confined mainly to culture of vaginal mucus samples from female cattle. This situation resulted because of the difficulty encountered in isolating *C.fetus* subsp. *venerealis* organisms from the bovine prepuce due to the presence of more rapidly growing contaminants.[23] Another complicating factor is a variability in the number of viable organisms in preputial samples.

Two approaches were pursued in an effort to reduce the overgrowth by contaminants. The first approach was the use of inhibitors and the second was selective filtration.

Various inhibitors were incorporated into the culture media to reduce overgrowth and selectively improve the growth of *C.fetus* subsp. *venerealis*. However most either inhibited *C.fetus* or were ineffective in controlling contaminants. As early as 1945, crystal violet and brilliant green were reported to permit the growth of *C.fetus*.[63] It was not until 1956, that a reasonably successful medium incorporating bacitracin, polymyxin, actidione, ox bile and ethyl violet was introduced.[37] Shepler *et al*.[69] reported that the most effective medium was a combination of bacitracin (15 units/ml), polymyxin (1 unit/ml),

and novobiocin (5µg/ml) added to a basal medium of brain-heart infusion agar with 10% defibrinated ox blood. This medium eliminated the spread of *Proteus sp.* and other microorganisms but *Pseudomonas* was not always suppressed. Further improvement was achieved by the addition of cycloheximide (20µg/ml) to prevent the fungal growth, which occurred occasionally when culturing preputial samples.[22]

The selective filtration of preputial samples through millipore type filters, prior to seeding, was reported to be of value in reducing overgrowth by contaminants.[60] It has also been reported to be useful in culturing semen samples especially where heavy contamination has occurred. The combined use of filtration and culture on selective media is considered of great value in the diagnosis of *C. fetus* infection in bulls.[22 69] However, because the technique markedly reduces the number of organisms innoculated onto the culture medium, it has limited application, especially for samples collected from bulls which are excreting small numbers of the organism.[22]

Although both of these techniques contribute to the successful recovery of organisms from infected bulls, the sampling technique used must also enable the detection of bulls harbouring small numbers of the organism.

Sample collection is therefore an important aspect of diagnosis of infection in the bull. The technique of preputial washing[47] using 16ml of peptone broth for preputial lavage was compared with that of preputial scraping using a bevelled Bartlett's pipette[3] and flushing the resultant material into 4ml of peptone broth.[23] The scraping

technique was considered superior to the washing technique for detecting the organisms since *C.fetus* was isolated in greater numbers and from more samples using the former technique.[23] This may be attributed to the inherently smaller dilution factor and to direct sampling of areas most likely to yield the organism.

Because of the sensitivity of *C.fetus* subsp. *venerealis* to normal atmospheric oxygen levels and overgrowth by preputial saprophytes, it is necessary to culture preputial samples within six to eight hours of collection. This requirement imposes severe limitations on this approach to isolation, especially where bulls are long distances from diagnostic facilities. To overcome this, a transport medium was developed.[16] It was based on a modified Stuart's medium with the addition of 250mg of cysteine hydrochloride per litre. This was dispensed in 20ml aliquots and the air in contact with the medium replaced with nitrogen. Preputial samples were innoculated into the medium and stored at 4-6^{o}C for 48 hours prior to culture on solid selective medium.[22] Results showed that the authors were equally successful in isolating *C.fetus* subsp. *venerealis* from samples in the transport medium compared with those from freshly collected samples. However, the problem of poor isolation from bulls with small numbers of viable organisms was not overcome.

In 1974, an enrichment medium which selectively increased the numbers of *C.fetus* organisms prior to culture on the solid selective medium, was developed.[17] It contains 300µg/ml of 5-fluorouracil, 100 units/ml of polymyxin B sulphate, 50µg/ml of brilliant green, 3µg/ml of nalidixic acid, and 100µg/ml of cycloheximide in bovine serum. This is dispensed

in 10 ml aliquots and after boiling, the solidified medium is broken up. Air in contact with the medium is replaced with a mixture of 5% O_2, 5% CO_2 and 90% N_2. Prior to use, the medium is stored for one week at 4^oC, during which time it turns deep green and becomes more selective against many microorganisms. Yellowing of the medium during the storage period indicates an incorrect gas mixture and the medium's selectivity does not develop. This enrichment medium, when correctly prepared, can be stored for up to three months at 4^oC.

Preputial samples collected by pipette[3] are washed into 4ml of sterile physiological saline, protected from direct sunlight, and allowed to stand, to permit epithelial cells and gross debris to settle. Within eight hours, 1 ml of supernatant is innoculated into the medium using a syringe and 18 gauge needle and the container shaken. The sealed containers are transported at ambient temperature (18^oC - 37^oC) and incubated at 37^oC on arrival at the laboratory.

After four days, 2-3 ml of saline are added, mixed, and all available liquid removed. This is then filtered through an 0.65µ Millipore filter and 0.1ml is distributed over the surface of petri dishes containing solid selective medium.[22] These plates are then incubated for three to six days at 37^oC in an atmosphere of 5% O_2 and 95% N_2. Although it is possible for many other microorganisms to grow slowly in the enrichment medium, their growth is usually sparse and following subculture most of the contaminants cannot grow under microaerophilic conditions on the solid selective medium.[17]

This enrichment medium and the procedure outlined above have been used

on numerous occasions on bulls in the field at the CSIRO Laboratories, Parkville, Victoria, Australia since 1974.[11] Although *Pseudomonas sp.*, *Proteus sp.*, and *C.sputorum* subsp. *bubulus* are inhibited and are uncommon plate contaminants, certain aerobic organisms that resemble *C.fetus* may grow in the enrichment and on the solid selective medium.[17] These have to be differentiated from both biotypes of *C.fetus* subsp. *venerealis*.

Following the culturing of 120 samples from 28 bulls, there was no contamination of solid selective medium in 80 of these samples (66.7%) and only 14 samples (11.7%) were contaminated in excess of 20% of the total surface area of two plates. *C.fetus* subsp. *venerealis* was isolated from 57 of 63 samples (90.5%) from nine infected bulls. Both biotypes were isolated equally as easily with this technique.[17]

A quantitative assessment of this enrichment medium to test its effectiveness as a transport medium has been carried out.[80] Abundant yields of *C.fetus* subsp. *venerealis* were obtained with an innoculum size of 10^4 of this organism even after keeping the medium in air at 22-23^{o}C for four days prior to incubation at 37^{o}C under microaerophilic conditions. With a smaller innoculum (100 organisms), isolation can still be achieved after being kept in air at 22-23^{o}C for at least two days. Winter and Caveney [80] concluded that this formulation comprised a convenient and effective transport medium for field use.

Where the numbers of viable *C.fetus* organisms isolated from preputial secretions vary considerably between bulls, from less than 100 to more

than 200,000 organisms per ml, a reliable selective enrichment system is essential for detecting the presence of disease in a herd by cultural examination. Diagnostic efficiency in samples containing few *C.fetus* cells and large numbers of resistant contaminating organisms is improved by using an enrichment medium which selectively increases the numbers of *C.fetus* organisms prior to culture on a solid selective medium.

Even with the improvement in isolation techniques as described above, accurate diagnosis of the carrier state in the bull still remains a difficult and demanding procedure. A negative diagnosis may not accurately reflect a non-carrier status. For herd diagnosis, it has been suggested that great reliance can be placed on a single examination of bulls from a suspect herd. In practice, it is advisable to collect samples from all available bulls, four years and older, where these total ten or less. In larger herds, a representative larger number should be sampled.[9] Where single bulls are being tested, at least four consecutive negative results should be recorded before the animal can be safely considered free of the disease.[22]

b) Immunofluorescence

Since it was first reported that fluorescein labelled antibodies could be used for the identification of homologous antigens in tissue preparations,[19] the procedure has been used successfully in the diagnosis of many bacterial and viral diseases. In 1956, Moody *et al.*[45] described the ease with which bacteria could be identified in smears using the fluorescent antibody technique (FAT). Thomason *et al.*[76] were able to detect as few as 200 bacteria per ml even though 10^7 times

as many contaminating organisms were present in the sample.

The possibility of applying this technique to the diagnosis of Campylobacteriosis in the bovine was first investigated in 1963. Mellick *et al.*[44] used a fluorescent antibody conjugate prepared by labelling the gamma globulin fraction from a pool of rabbit antisera for one strain of *C.fetus* subsp. *venerealis* with fluorescein isothiocyanate. Nonspecific fluorescence was minimised by separation of the conjugate by ion-exchange chromatography. Although there were cross reactions with *C.fetus* subsp. *fetus* and other strains of *C.fetus* subsp. *venerealis*, none was observed with *C.sputorum* subsp. *bubulus* or 17 other species of bacteria tested. In their comparison of fluorescent antibody tests on preputial samples and cultural examinations on antibiotic media of semen samples from a group of 24 bulls, complete agreement between the results was obtained. They concluded that the FAT provided a highly accurate and sensitive method for the detection of *C.fetus* carrier bulls.

Other workers[11 22 56 62 65 82] have used similar methods for immunofluorescence and compared it with cultural examinations on solid selective media with success.

Dufty[22] used similar procedures to those of Mellick *et al.*[44] except that a Sephadex G-200 column was used for final purification of the conjugate instead of ion-exchange chromatography. Of the 100 samples from five infected bulls, 94 were positive to FAT. It was more sensitive than either direct culture on solid selective medium (78%) or the use of millipore filtration prior to culture on solid selective medium (81%). Clark and Dufty[11] tested immunofluorescence in conjunction

with culture after transportation of samples collected in a selective enrichment medium.[17] In this trial, 89 samples from five infected bulls were examined. *C.fetus* subsp. *fetus* was demonstrated in 84 (94.4%) by FAT, and in 77 (86.5%) by culture. The results recorded by other workers [56] [63] [65] [82] are shown in Table III.

Table III : COMPARISON OF IMMUNOFLUORESCENCE AND CULTURE FOR DIAGNOSING CAMPYLOBACTERIOSIS IN THE BULL.

	Winter [82] *et al.*	Phillpot[56]	Schutte[65]	Ruckerbauer[62] *et al.*
FAT +, Cult.+	44 (16.3%)	8 (29.6%)	125 (81.7%)	12 (17.9%)
FAT +, Cult.-	12 (4.5%)	6 (22.3%)	12 (7.8%)	6 (9.0%)
FAT -, Cult.+	6 (2.2%)	1 (3.7%)	13 (8.4%)	2 (3.0%)
FAT -, Cult.-	202 (75.0%)	12 (44.4%)	3 (2.0%)	47 (70.1%)
Doubtful	5 (1.9%)	-	-	-
Total	269	27	153	67

The concensus of opinion is that FAT provides a rapid, reliable, and highly sensitive diagnostic tool for the diagnosis of Campylobacteriosis in the bull. However, diagnostic efficiency is improved when a combination of FAT and culture is employed. Although a single positive FAT result is conclusive, a single negative FAT result is not and additional tests are required before making a final diagnosis. It has been suggested that a minimum of four consecutive negative FAT results is required before a bull can be safely regarded as free from disease.[22]

Other advantages of the FAT are :

(i) it overcomes the problem of having to carry out biochemical tests to distinguish between *C.fetus* subsp. *venerealis* and *C.sputorum* supsp. *bubulus* since there is no cross reaction. Although *C.fetus* subsp.*fetus* cross reacts with antisera produced against *C.fetus* subsp. *venerealis*, this organism is an uncommon contaminant of preputial samples.[44]

(ii) it is able to detect low numbers of *C.fetus* subsp. *venerealis* organisms in highly contaminated samples. As low as 50-100 organisms/ml can yield a positive FAT result.[55]

(iii) it is possible that it may overcome the problem of delays of greater than eight hours between collection and testing. Although this is the view of Dufty;[22] Phillpot;[56] and Winter *et al.*[82] it is not supported by Ruckerbauer *et al.*[62] They stated that neither culture nor FAT was of value if samples were not examined within 24 hours. Failure to detect organisms by FAT in fresh samples was due to the low number of organisms present (less than 50) and in frozen samples, the disruption of their cell walls by freezing and thawing. This problem can now be overcome by using a suitable transport medium.

(iv) in conjunction with cultural examinations, all positive cases can be identified following collection of two preputial samples.[65]

The disadvantages and limitations of the FAT are :

(i) there are greater difficulties in preparing smears and in interpretation of findings, which require skilled and experienced technicians, when compared to cultural examinations.

(ii) where the numbers of organisms are low, false negative diagnoses are likely to occur.

(iii) the problem of autofluorescence and debris in smears may be a complicating factor especially where there are few organisms.

(iv) the variability in the intensity of fluorescence depending on the strain of *C.fetus* subsp. *venerealis* involved may contribute to false negative diagnoses. Schutte[65] reported that *C.fetus* organisms from one bull, which proved positive to culture, showed low intensity of fluorescence. Winter *et al.*[82] also reported doubtful FAT results due to the presence of a few dimly fluorescent forms, usually atypical in nature.

(v) the possibility of false positive FAT diagnoses cannot be ruled out, even when carried out in combination with cultural examination. In most instances where FAT results have been positive with corresponding negative cultural results, these have been explained by failure of cultural examinations. These failures have been due to a high degree of bacterial contamination, small numbers of viable organisms in the preputial samples, reduction in numbers following millipore filtration, or delays in processing samples and the consequent loss of viability of the organisms.

c) Heifer Test-Mating

Diagnosis of Campylobacteriosis in the individual bull has been achieved by virgin heifer test mating.[1] The bull is allowed to breed test heifers after negative cervicovaginal mucus cultures have

been obtained. Subsequently, cervicovaginal mucus is collected for cultural examinations. Where test mating is used with A.I. bulls not used for natural breeding, preputial scrapings or semen combined with nutrient broth washings of the artificial vagina are instilled in the cervices of the test heifers.

Adler[1] claims that one heifer is sufficient for test mating and that a definitive diagnosis can be made within three weeks. Based on the fact that only 77% of females become infected following intravaginal application of *C.fetus* subsp. *venerealis* culture and 75% after infected preputial material is used, Newsam[48] casts doubt on the reliability of the single heifer test.

2. BOVINE VAGINAL MUCUS SAMPLES.

a) Culture

The collection of vaginal mucus for the isolation and identification of *C.fetus* subsp. *venerealis* is a valuable diagnostic procedure in the examination of non-pregnant cows and heifers. Because of the susceptibility of the organism to normal atmospheric levels of oxygen, samples must either be cultured within six to eight hours of collection, or special precautions for transportation must be taken. Satisfactory methods for transportation are either forwarding samples in sealed plastic insemination pipettes stored at -79^{o}C in dry ice[67] or innoculation of a transport enrichment medium.[17 29]

The main sites of infection are the cervix and anterior vagina.[9] Techniques for the collection of samples of cervical and vaginal mucus for bacteriological examination have been described.[2 28 31]

Isolation of *C.fetus* subsp. *venerealis* has been more readily achieved from vaginal mucus samples from infected cattle at oestrus than at other stages of the oestrous cycle. *C.fetus* subsp. *venerealis* was isolated from 83% of samples collected at oestrus compared to 26.5% of samples collected at other stages of the cycle.[38] A solid selective medium for culturing vaginal mucus samples was developed in 1969.[14] Clark *et al.*[14] collected 565 vaginal mucus samples from experimentally infected heifers (using the method of Anon[2]) and cultured these on a solid selective medium. They reported a slight degree of contamination in 54% of samples, moderate contamination in 32% of samples, and heavy contamination in 14% of samples, but were able to isolate *C.fetus* subsp. *venerealis* from 78% of samples collected from two days before to two days after oestrus and from 57% of samples collected at other stages of the oestrous cycle. They also noted a marked decline in the rate of isolation of the organism from vaginal mucus samples accompanying pregnancy in infected heifers.

The bacteriological examination of vaginal or cervical mucus collected from non-pregnant cows and heifers has been recommended as a herd test for Campylobacteriosis in the western States of the U.S.A. It can also serve as a valuable adjunct to the examination of bulls in establishing a herd diagnosis.[31]

b) Vaginal Mucus Agglutination (VMA) Test

Specific agglutinins are formed in the vagina as a result of genital infection with *C.fetus* subsp. *venerealis* and their demonstration by an immunological test can be used in the diagnosis of infection. The vaginal mucus agglutination test is not very sensitive, being capable

of demonstrating agglutinins in only approximately 50% of samples collected from infected females.[9] However, it can be used successfully as a herd test to diagnose past or present infection.[50]

The VMA test can usually detect antibody in vaginal mucus by 30 to 70 days after initial infection but reactions may be delayed until three to four months.[9] While some cows may remain positive for years, others become negative within two months with about 50% of reactors becoming negative within six months. Positive VMA test reactions can be expected in 10 to 50% of samples collected from pregnant or non-pregnant infected female cattle that were mated one and a half to six months previously. In the average infected herd, at least one positive sample should be obtained if ten infected females are sampled correctly; however it is advisable to collect 20 samples.[10] Furthermore, it is recommended that samples be collected during the dioestrous phase because of an increase in false negative results when vaginal mucus is collected at oestrus.[34] This has been attributed to a dilution effect due to the greater volume of mucus present at oestrus.[9]

A number of factors can contribute to false positive results with the VMA test. The presence of blood will do so because of the presence of cross reacting antibody in serum directed against *C. fetus* subsp. *fetus*.[9] Minor inflammatory lesions have also been shown to contribute to false positive reactions.[15] The incidence of false positive reactions may also be higher if vaginal mucus is collected by tampon compared to collection by pipette.[14]

c) Haemagglutination Tests

Two indirect haemagglutination tests have been developed for the diagnosis of Bovine Campylobacteriosis. The first utilises normal sheep erythrocytes sensitised with a heat-stable fraction of *C.fetus* subsp. *venerealis* as antigen.[52] [75] It is equal to the VMA test in sensitivity and ease of performance. The second utilises sheep erythrocytes which have been treated with tannic acid and sensitised with a phenol soluble fraction of *C.fetus* subsp. *venerealis* as antigen.[53] It is more sensitive than the VMA test and enables antibody to be demonstrated sooner and with greater frequency after initial infection. False positive reactions to both tests occur in about 1% of samples of vaginal mucus collected from non-infected cattle. This may be due to the presence of antibody directed against *C.fetus* subsp. *fetus*.[10]

CONTROL AND TREATMENT

The best way of achieving complete elimination of Bovine Campylobacteriosis from an infected herd is by disposing of all herd bulls and implementing the exclusive use of artificial insemination, using semen from non-infected bulls, for two years.[10] In cases of recent infection, treatment of non-pregnant females with intra-uterine infusions of penicillin and streptomycin, can be of value. However, such a treatment regime is not justified after three to four months post-exposure as immunity builds up rapidly and the non-pregnant animals can be expected to conceive soon.[49]

This type of control measure is obviously more suited to dairy herds but would be impractical on a large population of beef herds. In such instances, the most practical method of control is vaccination of

female cattle.

Vaccination of females by subcutaneous injections with bacterins prepared with a high cell content suspended in an oil adjuvant will give adequate protection against the infertility effects of the disease.[8] The immunity produced by an adjuvant bacterin is equal to convalescent immunity acquired after natural infection.[8 30] A single injection in older cows and heifers (15 to 18 months of age and older) will give immunity for at least two years after vaccination. However, younger heifers (12 months old) require two injections for the same degree of protection.[12]

In large herds under extensive management, annual or bi-annual vaccination of adult cows and annual vaccination of yearling heifers, one to two months prior to mating, would be the best procedure to adopt for control of the disease.[10] In conjunction with this, other ancilliary control measures can be taken to enhance the value of a control programme. Antibiotic treatment of bulls by recommended methods is known to be highly effective in eliminating infection from the preputial sac.[40 65 68] Although treated bulls may subsequently become reinfected by serving an infected cow, the opportunity for doing so is reduced in vaccinated herds. Where possible, disposal of all bulls, four years of age or older, and the use of young bulls only for service, would be beneficial in a control programme. The lower susceptibility of young bulls[79] should limit the spread of the disease and ensure better conception .

With the recognition of the therapeutic and prophylactic effects of

vaccination of bulls, this approach may offer a more practical and effective method of controlling the disease than that of vaccination of females. In 1968, Clark *et al.*[13] reported on the elimination of *C.fetus* subsp. *venerealis* organisms in four carrier bulls after two subcutaneous injections, five weeks apart. Each injection contained 46 mg dry weight of cells in a mineral oil adjuvant. Subsequent attempts to reinfect these bulls at six months after vaccination by infusion of a culture of *C.fetus* subsp *venerealis* into the preputial cavity did not result in re-establishment of infection. These results have been substantiated by other workers using larger numbers of bulls.[5 18] Bouters *et al.*[5] reported that thirty of forty-one bulls (70%), positive to *C.fetus* subsp. *venerealis* prior to vaccination, were found free of the organism after one injection and the remainder (30%) were free of infection after the second injection. They also found that none of 288 uninfected bulls, serving in an infected area, became infected following a single injection and conclude that vaccination of bulls is a safe and effective procedure for treatment and prevention of *C.fetus* subsp. *venerealis* infection in bulls. Clark *et al.*[18] reported that none of sixteen vaccinated bulls became infected after five attempts to artificially infect them at six-monthly intervals. Thirteen of the seventeen control bulls, which were not vaccinated, became infected.

Before vaccination of bulls can be considered a safe alternative to vaccination of female cattle as a method of controlling Bovine Campylobacteriosis, certain important points need to be clarified; whether there is a need for annual vaccination for continuous protection, whether vaccination against *C.fetus* subsp. *venerealis* will also protect against genital infection by biotype *intermedius*, and whether vaccinated bulls can transmit infection mechanically.[18]

II. MATERIALS AND METHODS

ORGANISM

The original organism, *Campylobacter fetus* subsp. *venerealis*, isolate D78 was obtained from the C.S.I.R.O., Parkville, Victoria. Following preliminary trials at Massey University, in which two bulls and two cows were challenged with the organism, it was recovered from vaginal mucus samples from one cow pre-slaughter and from the preputial cavity of one bull post-slaughter. The organism was sub-cultured, lyophilised, and stored at Massey University for reference purposes as FD15.

The lyophilised organism, FD15, was reconstituted and seeded onto blood agar plates, which were incubated at 37°C in an atmosphere of 90% N_2 : 5% CO_2 : 5% O_2. A series of biochemical tests were carried out to establish its identification prior to using it to challenge seven heifers and two bulls.

Three to four day old growth of the organism was harvested in phosphate buffered saline (PBS) pH7.2 and standardised to Brown Opacity Tube (BOT) number 2 or 3. For heifers, 2 ml of this suspension were introduced into the uterine cavity or into the cervical canal using an artificial insemination (A.I.) pipette. For bulls, 3 ml of the suspension were introduced deep into the preputial cavity using an A.I. pipette. The preputial orifice was then closed off and the infective material massaged vigorously in the preputial cavity.

ANIMALS AND FACILITIES

Because of the highly infectious nature of the disease, animals were

pastured in isolated paddocks at Massey University under strict supervision. At the completion of the trials or following failure to recover the organisms by culture four weeks after exposure, animals were sent directly to the abattoirs.

Seven 15 to 18 month old virgin Friesian and Friesian cross heifers were available for the experimental work. One vaginal mucus sample was collected and cultured from each heifer prior to challenge with the organism. There was no history of previous exposure to the disease.

Two Jersey bulls, 74473 and 75400, six and five years of age respectively were challenged with the organism. Both were culled from the New Zealand Dairy Board's Artificial Breeding Centre at Awahuri. There was no previous exposure to the organism and previous cultural examinations of preputial samples from these bulls were negative. Two other Jersey bulls, 76420 and 76422, both four years of age, were used as controls. They were made available from the same Artificial Breeding (A.B.) Centre and had no previous exposure to the organism as well as negative results to cultural examination of preputial material.

In addition to these bulls, twenty-two preputial samples from twenty-one bulls of various ages and breeds at the A.B.Centre were collected for routine examinations. These were cultured and examined by immunofluorescence as control samples. They were also cultured independently by the Animal Health Laboratory, Ministry of Agriculture and Fisheries, Palmerston North.

SAMPLE COLLECTION

Sampling of bulls and heifers commenced one week following infection and continued either once or twice weekly for not less than three months. Where animals remained negative to culture four weeks post-infection, a second infective dose of similar concentration (BOT No.2-3) was used. Failure to recover the organism by culture within four weeks following the second attempt at infection resulted in culling and immediate slaughter.

a) Female

Cervicovaginal mucus was collected by aspiration using the equipment shown in Plate 1. The heifer was restrained in a head-bale and crush, and the perineal area was cleaned using dry paper towels. The A.I. pipette with a six inch piece of rubber tube attached to one end was placed within a sterile glass tube so that the free end was protected from contamination within the glass tube. The vulval lips were spread apart, and the glass tube introduced into the vagina. Once in place in the anterior vagina, the A.I. pipette was then extruded and placed into the external os of the cervix and suction applied. Mucus was sometimes collected from the anterior vagina. A column of 15-20 mm of mucus in the A.I. pipette was considered sufficient for culture. The quantity and nature of the mucus varied from small and tenacious during the luteal phase of the oestrous cycle, to large and thin during the follicular phase. Prior to withdrawal from the vagina, the A.I. pipette was pulled back within the glass tube. The cervicovaginal mucus was then flushed into 5ml of saline or thioglycollate medium in a labelled universal bottle, protected from direct sunlight, and despatched immediately to the laboratory for culture.

b) Bulls

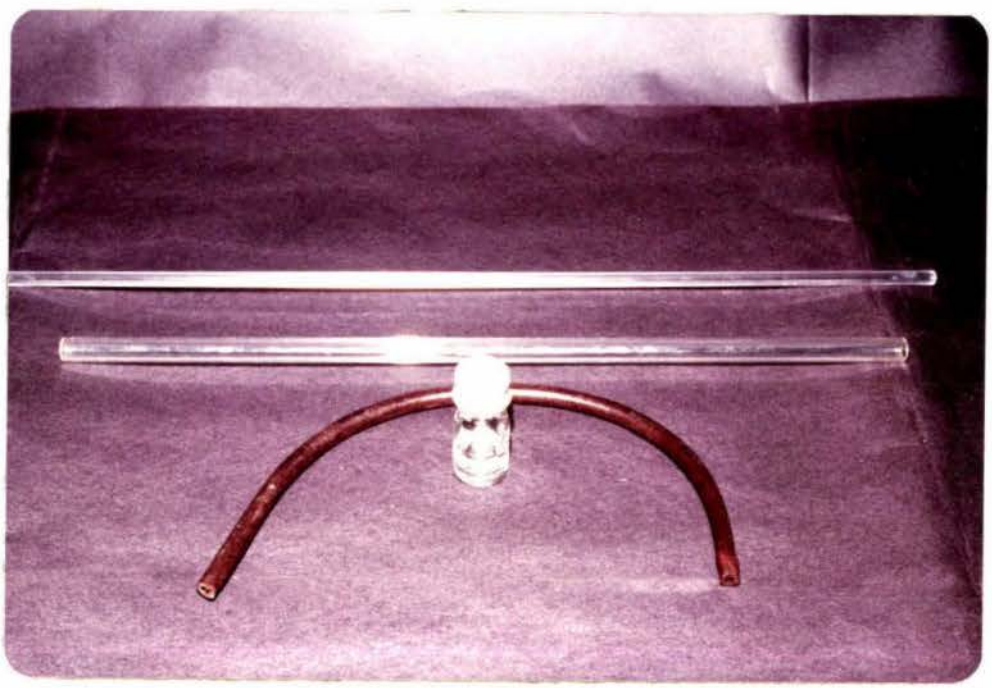
Preputial samples were collected using a Bartlett pipette (see Plate 2) to which a 120ml rubber bulb was attached for aspiration. The bull was restrained in a crush and head-bale with the hindleg on the side of the operator secured with a leg-rope. It was not found necessary to trim the hairs around the preputial orifice or to clean up the area before sample collection unless there was heavy soiling and matting of the hairs. The preputial orifice was everted and the bevelled-end of the Bartlett pipette introduced into the preputial cavity and placed in the area of the fornix. The bend in the pipette enabled the operator to position the pipette with the bevel against the preputial epithelium. The fornix was scraped firmly but not excessively vigorously and suction was applied by depressing and releasing the rubber bulb. The pipette was then worked anteriorly over the dorsal surface of the penis, scraping and applying suction over this area as well. Prior to removal of the pipette, the rubber bulb was either depressed partially and held in that position or removed completely from the pipette. This procedure was adopted to prevent aspiration of grit and contaminating materials from around the orifice whilst removing the pipette. The preputial scrapings and smegma were then flushed into 4ml of saline in a labelled universal bottle, protected from direct sunlight, and sent to the laboratory for culture and FAT.

Prior to use, the Bartlet pipette was disinfected by immersion in a hypochlorite solution (3% solution of "White Magic"[a]) for at least 24 hours, thoroughly rinsed with tap water, and dried in a 27°C incubator prior to use.

[a]Smith and Nephew NZ Limited,Petone,Wellington.

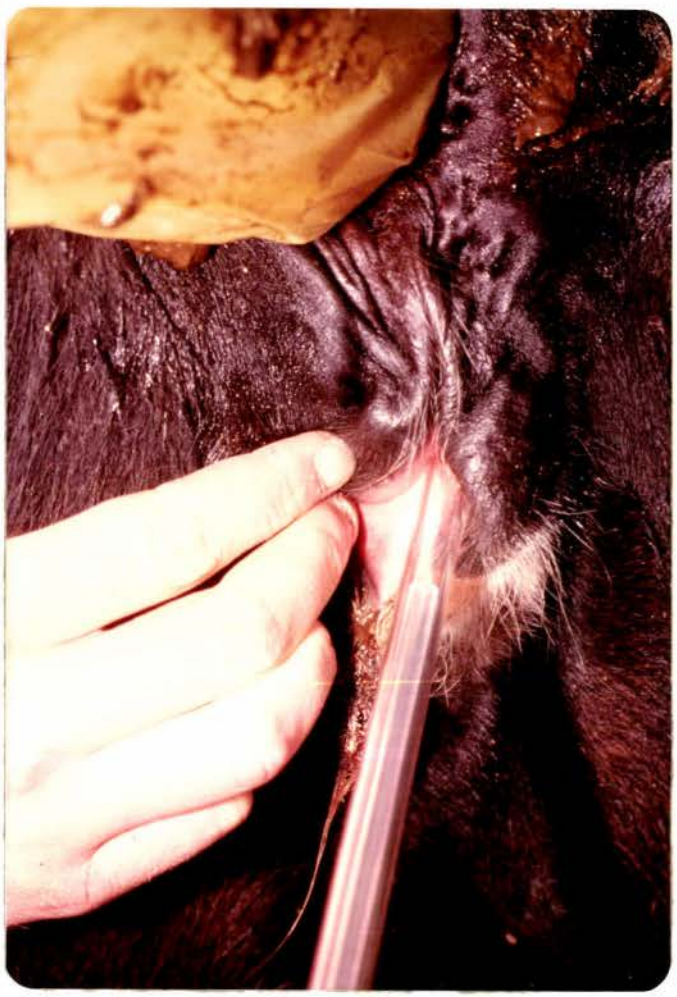
PLATE 1 : CERVICAL COLLECTION OF MUCUS SAMPLES

a) Equipment



b) Restraint of heifer

c) Introduction of equipment into vulva

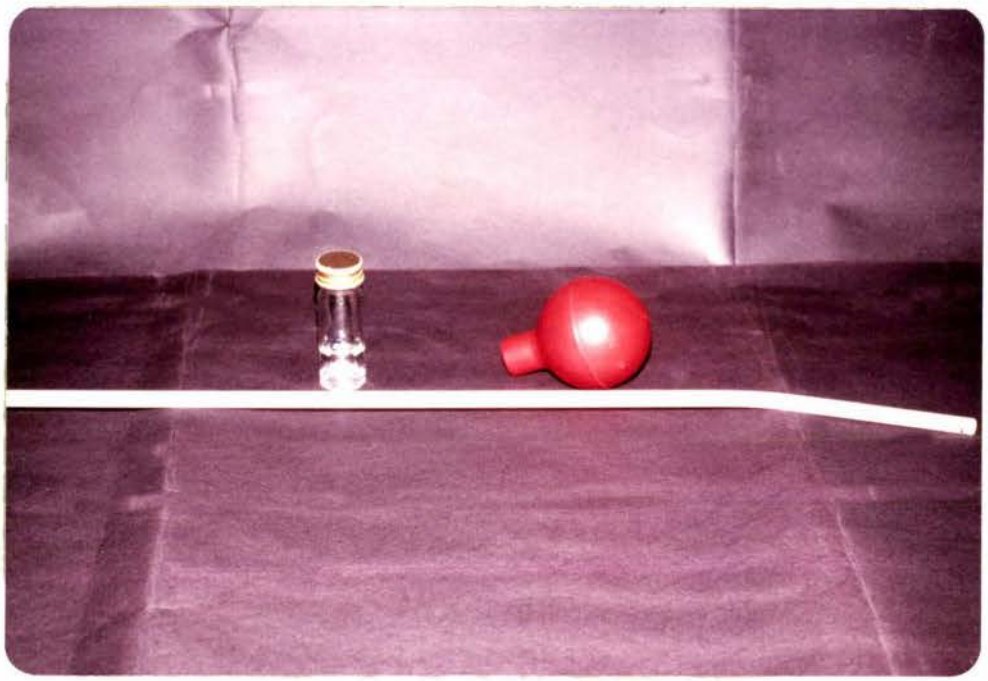


d) Volume of mucus in pipette

e) Mucus sample

PLATE 2 : COLLECTION OF PREPUTIAL SAMPLES

a) Equipment



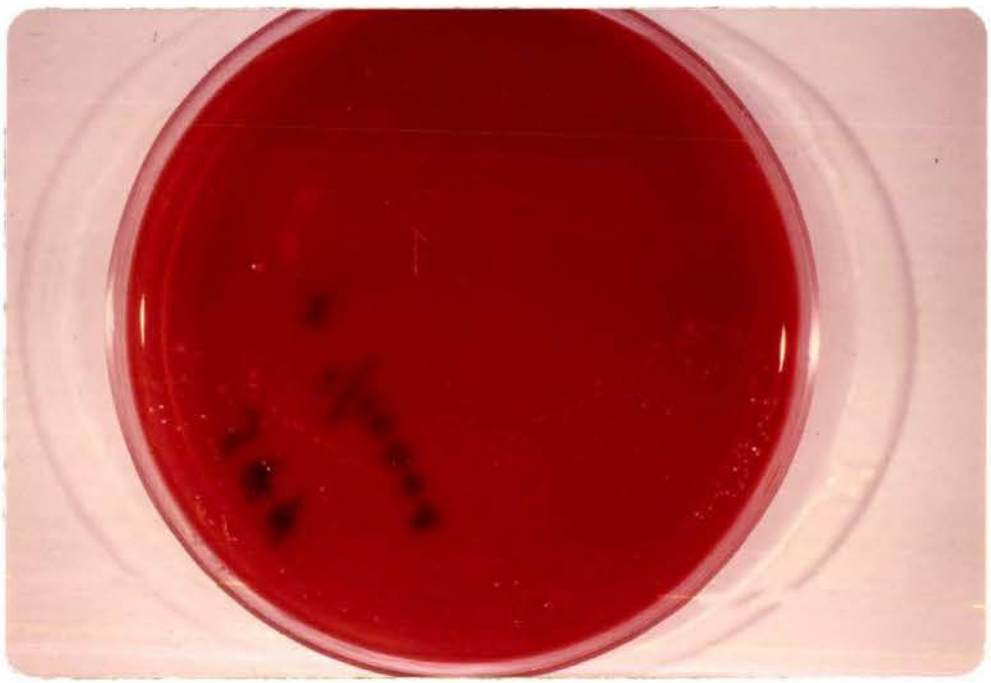
b) Restraint of bull

c) Pipette into preputial cavity

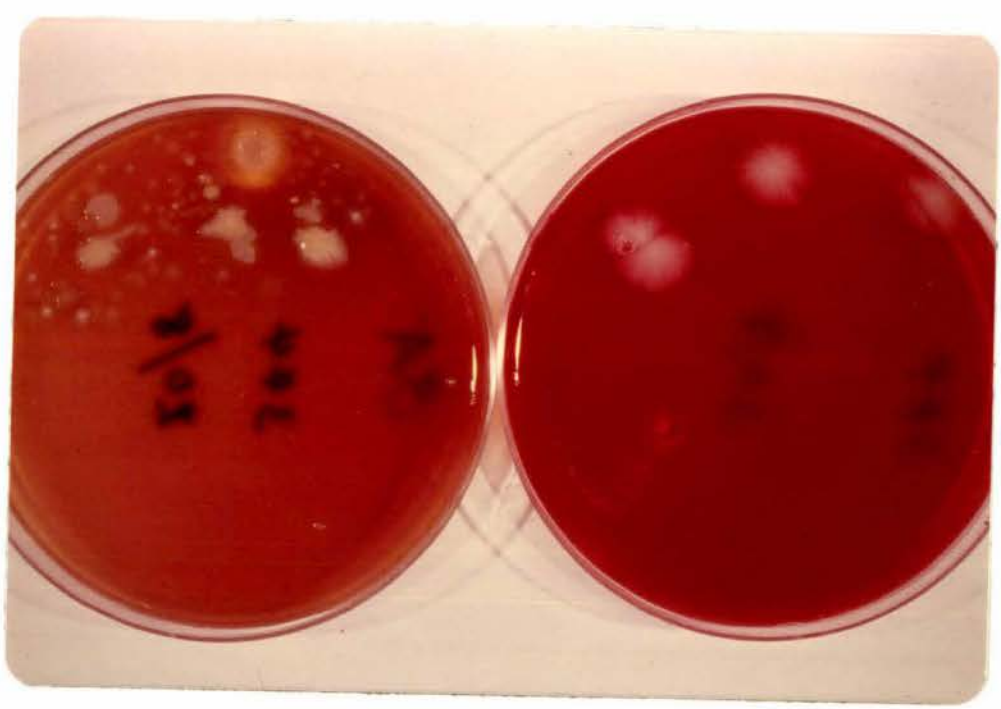
d) Preputial sample

PLATE 3 : BACTERIOLOGY

a) *C.fetus* subsp. *venerealis* colonies on solid selective medium



b) Comparison of degree of contamination on blood agar and solid selective medium



SOLID SELECTIVE MEDIUM

The solid selective medium used for culture of *C.fetus* subsp. *venerealis* has been described by Dufty.[22] The nutrient agar base consists of 1% Bacto-peptone, 0.5% NaCl, 0.4% powdered Lab-Lemco beef extract, and 1.5% powdered agar dissolved in distilled water. The pH was adjusted to 7.6 and the solution autoclaved. To this was added 10% sterile defibrinated ox blood, 15 units/ml of bacitracin, 1 unit/ml of polymyxin B sulphate, 5 µg/ml of sodium novobiocin, and 20µg/ml of cycloheximide. The mixture was dispensed in plastic petri dishes and stored at $4^{o}C$ for a maximum of two weeks. Prior to use, the plates were dried for ½ to 1 hour at $37^{o}C$.

CERVICOVAGINAL MUCUS SAMPLES

Cervicovaginal mucus samples were plated out within two hours of collection. The mucus was spread out on duplicate plates with sterile bacteriological swabs and then spread out using a sterile loop. With each batch of plates, a control plate inoculated with original organism was included. These were incubated at $37^{o}C$ in McIntosh-Fildes jars in an atmosphere of 90% N_2 : 5% O_2 : 5% CO_2 for one week. A preliminary check on growth of the organism was made at three days and the final check at seven days. Colonies which initially could not be confirmed as *C.fetus* subsp.*venerealis* were subcultured onto two blood agar plates, one of which was incubated at $37^{o}C$ in normal atmospheric conditions, the other in the microaerophilic conditions of 90% Na : 5% O_2 : 5% CO_2. In addition, a smear was made, Gram stained, and examined for the presence of the organism. The subcultured plates were checked for growth at three and seven days. Growth in microaerophilic conditions without corresponding growth in aerophilic conditions was taken as positive circumstantial evidence that the organism was *C.fetus* subsp. *venerealis*. A series of biochemical tests, as outlined

later (see Biochemical Tests) was carried out to further establish the identity of the organism.

PREPUTIAL SAMPLES

Because of the susceptibility of *C.fetus* subsp. *venerealis* to normal atmospheric levels of oxygen, preputial samples were processed immediately on arrival at the laboratory. In all instances, this was within two hours of collection. The samples were first centrifuged at 2500 rpm for ten minutes and the residue discarded. The supernatant was then thoroughly agitated and divided in half, one for culture and the other for FAT.

a) Culture

Two to three drops of the supernatant (approximately 0.1ml) were sown on to the solid selective medium in duplicate and spread out with a sterile wire loop. The rest of the supernatant was filtered through a 0.65 μ Millipore filter using a syringe and Swinny Hypodermic Adaptor. Two to three drops of the filtered supernatant were sown onto duplicate plates and spread with a sterile wire loop. With each batch of plates a control plate of the original organism was included and all were incubated at 37°C in an atmosphere of 90% N_2 : 5% O_2 : 5% CO_2. Plates were examined for growth at three and seven days and where suspicious colonies were observed they were treated in similar fashion to that outlined for vaginal mucus samples.

b) Fluorescent Antibody Test

(i) Preparation of Antisera

The original organism was grown on blood agar plates incubated at 37°C

in an atmosphere of 90% N_2 : 5% O_2 : 5% CO_2 for three days. The resulting growth was then harvested in saline and standardised to B.O.T. No. 6. This was used as the source of antigen with which two rabbits were challenged by intravenous innoculation using the ear vein. The following regime was used to expose the rabbits to the antigen :- 0.5ml on Tuesday, 1.0 ml on Friday, 1.5 ml on Tuesday, 2.0 ml on Friday and 2.0 ml on Tuesday. The rabbits were test bled on the following Monday and the titre of antibody production assessed quantitatively by tube agglutination test. If titres exceeded 1:6400, the rabbits were bled on Tuesday; if they had been below this level, then a further 2ml of antigen would have been innoculated on Tuesday and bleeding would have been delayed until the following Friday. However, in both cases, titres exceeded 1:6400 when the rabbits were test bled.

(ii) Tube Agglutination Test

For the agglutination test, the original serum was diluted 1/50 with saline (1.0 ml serum in 49.0 ml saline). A series of tubes with 0.5 ml of saline were set up and 0.5ml of 1/50 diluted serum was added to the first tube to give a 1/100 dilution. Twofold dilutions were then achieved by adding successive 0.5ml aliquots to 0.5ml saline resulting in dilutions of 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800 and 1/25600 respectively. An 0.5 ml aliquot of the antigen was then added to each tube and the mixture incubated at $37^{o}C$ in a water bath for up to 18 hours. Recording was done after allowing the tubes to stand at ambient temperature for two to three hours.

(iii) Precipitation of γ-Globulin Fraction

To a known volume of the original serum, an equal volume of 70% ammonium sulphate solution was added under continuous stirring and left standing for three hours. The γ-globulin precipitate was collected by centrifugation at 14000 G for 15-20 minutes at $4^{o}C$. The precipitate was then redissolved to half the original volume with phosphate buffered saline (PBS) pH 7.2. (This procedure can be repeated if a purer γ-globulin fraction is desired.) The γ-globulin fraction was dialysed against two changes of PBS pH 7.2 over 24 hours. If any precipitate formed during dialysis, it was centrifuged off and discarded. The γ-globulin fraction was then placed on a DEAE Sephadex A50 medium column, equilibrated with PBS pH7.2, and eluted to purify the fraction further. The protein content was then determined by spectrophotometry at 280nm and the concentration adjusted to 1.0 - 1.5 g protein per 100 ml by addition of PBS.

(iv) Conjugation with Fluorescein Isothiocyanate (FITC)

A solution of 1mg FITC per ml 0.15M $Na_2 HPO_4.2H_2O$ pH 9.0 was prepared and added to the γ-globulin fraction at the rate of 10ml per gram of protein while stirring at room temperature. Free FITC was removed by gel filtration on a Sephadex G25 coarse column equilibrated and eluted with PBS pH 7.2. The conjugate was then adsorbed with pig's liver powder to reduce non-specific staining.

(v) Specificity of Conjugate

Smears of the original organism were prepared, stained with serial dilutions of the conjugate and examined for brightness of fluorescence. The optimal dilution was taken as one half the dilution which produces

maximum fluorescent intensity. The batch of conjugate was diluted accordingly and tested against homologous and heterologous strains. The homologous strains included several isolates of *Campylobacter fetus* subsp. *venerealis*, *C.fetus* subsp. *fetus*,and *C.jejuni* which were lyophilised and stored by the Microbiology Department at Massey University. The heterologous strains comprised an array of bacteria including *Pseudomonas aeruginosa*, *Proteus sp.*, *Corynebacterium pyrogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus sp.* The conjugate was then dispensed in 2ml aliquots and stored in a freezer at -10°C until required.

(vi) Processing Preputial Samples

The supernatant, which remained after culture, was centrifuged at 4500 rpm for 40 minutes and the sediment resuspended in 0.2ml of PBS pH7.2. Two smears were made by using a Pasteur pipette to place two drops and to spread these in a circular manner onto clean slides. The smears were air dried and then fixed in 95% ethyl alcohol for ten minutes. They were rinsed in distilled water and air dried. Each smear was then flooded with the conjugate and placed in a moist chamber at 37°C for half an hour. Excess conjugate was washed off with PBS pH7.2 and the smear washed thoroughly in PBS pH7.2 for 30 minutes. The smear was then blotted dry, mounted in glycerol buffered at pH8.5, and examined by ultraviolet microscopy. With each batch of smears, a control slide of the original organism was always included.

Smears were first examined on the periphery because the circular motion applied in spreading the smear tended to push the organism towards the periphery. Failure to locate *C.fetus* subsp.*venerealis* organisms on the

periphery was then followed by scanning of the entire smear until organisms were observed, or for a maximum of 15 minutes per slide. Where smears needed to be re-examined or where they could not be examined immediately after staining, the edges of the cover-slip were sealed with nail varnish and the slides stored at $4^{o}C$ covered by tinfoil.

BIOCHEMICAL TESTS

Isolated colonies of the organism being tested were cultured in a fluid thioglycolate medium [a] for three days at $37^{o}C$. One ml of this culture was used as an innoculum for all metabolic tests.

(a) Hydrogen sulphide production

For the sensitive test, Brain-heart Infusion Broth[b] with 0.1% agar and 0.2% cysteine was used as the test medium. Hydrogen sulphide production is detected by a blackening of a strip of lead acetate impregnated filter paper after 24 hours incubation and then after five days incubation.

For the insensitive test, a triple sugar iron medium was innoculated by the stab method and after five days incubation, hydrogen sulphide production recorded. Blackening of the medium was seen if the organism was strongly H_2S positive.

(b) Catalase Test

One ml of culture was innoculated into fresh thioglycolate medium. After three days incubation, 10ml of 3% hydrogen perioxide was added to the

[a] = Baltimore Biologics Laboratory,Div.of Becton & Dickinson,Cockeysville,M.D. U.S.A.
[b] = DIFCO,Detroit, Mich. U.S.A.

culture tube. A special stopper with a capillary tube was used to cover the tube, which was then inverted. Gas production was recorded as the amount of fluid displaced by gas in the capillary tube.

(c) Heat Tolerance Tests

One ml of culture was innoculated into each of two tubes of thioglycolate medium; one was incubated at 25°C and the other at 42°C. Growth was recorded after five days incubation.

(d) Glycine Tolerance Tests

One ml of culture was innoculated into thioglycolate medium with 1% glycine. After five days incubation, the medium was examined for growth of the organism.

(e) Salt Tolerance Test

Thioglycolate medium with 4.5% salt (NaCl) was innoculated with 1ml of the test culture and incubated at 37°C for five days. It was then examined for growth of the organism.

III RESULTS

HEIFERS

Eight 15 to 18 month old virgin Friesian and Friesian-cross heifers were available for challenge with *C.fetus* subsp. *venerealis* (FD15). One heifer was culled because of her poor condition and small inactive ovaries. The remaining seven heifers were challenged with 2ml of a suspension of the organism in phosphate huffered saline (PBS) pH7.2, which was standardised to Brown Opacity Tube (BOT) No. 2 or 3.

Only two (29%) of the seven heifers became infected as determined by consistent recovery of *C.fetus* subsp. *venerealis* from vaginal mucus samples collected twice weekly beginning one week after exposure to the organism. In spite of a second challenge, using a similar concentration of organisms, four weeks after the first, the other five heifers remained negative to cultural examination of vaginal mucus samples.

A total of 144 samples were collected and cultured from the seven heifers. Seventy-four of these were from the two infected heifers; the remaining 70 were from the five non-infected heifers. The latter have been excluded from further analysis based on the assumption that they did not become infected.

Forty-eight (65%) of the 74 samples cultured from heifers number D07 and D23 were positive, with the first recovery of the organism taking place on day 13 and 21 post-exposure respectively. Both heifers remained positive for 152 days. After this period, eight consecutive

samples from D07 (day 155 to 200) were negative and post-slaughter swabs (day 210 after exposure) from the anterior vagina, cervix, and uterus of this animal were also negative on culture (see Table IV).

In contrast, heifer D23 remained infected up to slaughter (day 162 after challenge) as shown by recovery of the organism from swabs of the anterior vagina and cervix. A swab taken from the uterine body was negative on culture.

Twenty-six (60.5%) of the 43 samples from heifer D07 were positive. In view of the fact that eight consecutive vaginal mucus samples from this heifer were negative, including the post-slaughter samples, it was assumed that she overcame her infection. By discounting the final eight samples from analysis, recovery by culture was 74.3% (26 of 35 samples). Twenty-two (71%) of the 31 samples from heifer D23 were positive on culture. Results for both animals combined were 72.7% (48 of 66 samples) for culture. These results are shown in Table V.

TABLE IV : DURATION OF RECOVERY OF *C. fetus* subsp. *venerealis* FROM INFECTED HEIFERS

	D07	D23
Exp. to 1st + (days)	13	21
Exp. to last +(days)	152[a]	152
Exp. to slaughter (days)	210[b]	162[c]

Exp = Exposure
\+ = Recovery of organism
a = 8 samples from day 155 to 200 after exposure negative
b = Post-slaughter samples negative
c = Post-slaughter samples from anterior vagina and cervix positive.

TABLE V : CULTURAL EXAMINATION OF VAGINAL MUCUS SAMPLES FROM INFECTED HEIFERS

	D07	D07[a]	D23	Total	Total[b]
No. samples	43	35	31	74	66
No. + samples	26	26	22	48	48
% + samples	60.5	74.3	71	65	72.4
No. - samples	17	9	9	26	18

a and b = Eight consecutive negative samples from D07 (days 155 to 200 after exposure) discounted from analysis.
\+ = Recovery of *C.fetus* subsp.*venerealis*
\- = No recovery of *C.fetus* subsp.*venerealis*

Seven samples (9.5%) suffered from gross contamination in the form of a fungal overgrowth, which covered the entire surfaces of both duplicate plates of the solid selective medium. This occurred in spite of the fungicide, cycloheximide, being incorporated in the medium. Varying degrees of contamination (nil to moderate) occurred on other samples but the contamination did not interfere with identification and sub-culturing of colonies of *C.fetus* subsp. *venerealis*. During the early stages of the experiment, culture work was carried out using blood agar plates as well as the selective medium. It soon became evident that the latter had great advantage in reducing contamination whilst still allowing good growth of the organism. The use of blood agar plates was then discontinued.

In one instance, the quantity of vaginal mucus collected was less than 12mm in the plastic pipette used for collection. This sample showed no growth on culture.

Post-slaughter samples from the anterior vagina, cervix, and uterine body of four heifers, D07, D23, D05 and D236 were cultured. Positive cultures were obtained from the anterior vagina and cervix of only one of these, heifer D23.

BULLS

Thirty-two preputial samples were collected from the two bulls which were challenged with *C.fetus* subsp. *venerealis* (sixteen from each bull). Sampling began one week after exposure and continued at weekly intervals for sixteen weeks.

As controls, five preputial samples were collected from each of two bulls in a similar manner. A further twenty-two preputial samples from twenty-one bulls at a nearby Artificial Breeding Centre were examined by culture and immunofluorescence. All the control samples were negative to both examinations.

Twenty-six of the 32 samples (81.25%) collected from the two bulls challenged with the organism were positive on FAT examination and 22 (68.75%) on cultural examination. One FAT result was classed as questionable due to the presence of a few faintly fluorescent organisms. The corresponding cultural result was negative. Two cultural examinations were classed as questionable because their agar plates had collapsed under negative pressure in the McIntosh -Fildes jar. The corresponding FAT examinations were both positive. Results are shown in Table VI.

Of the remaining five negative FAT results, one cultural result was

positive and the other four were negative. Three of these occurred consecutively in weeks 15, 16 and 17 following exposure in bull number 75400. The number of organisms recovered from culture and seen on smears by immunofluorescence from this bull steadily decreased over the experimental period. Preputial samples for weeks 12 and 13 only showed growth of one *C.fetus* subsp. *venerealis* colony on one of the duplicate plates of solid selective medium.

Three samples which were negative on culture were positive on FAT examination. Two of these were heavily contaminated, making it impossible to determine whether *C.fetus* subsp. *venerealis* colonies were present. Varying degrees of contamination occurred on other plates but this did not interfere with the recovery and identification of the organism.

The use of a millipore filter, prior to innoculation of the solid selective medium, reduced the degree of contamination compared to direct culture. However, in one instance, when only one colony was recovered by direct culture, no growth of the organism was recorded following millipore filtration.

TABLE VI: CULTURAL AND IMMUNOFLUORESCENCE EXAMINATION OF PREPUTIAL SAMPLES FROM INFECTED BULLS

Bull	Culture Result	Fat Results +	-	S	Total
75400	+	8	1	0	9
	-	1	4	1	6
	S	1	0	0	1
	Total	10	5	1	16
74473	+	13	0	0	13
	-	2	0	0	2
	S	1	0	0	1
	Total	16	0	0	16
Grand Total		26	5	1	32

+ = positive or growth, - = negative or no-growth
S = suspicious or questionable result.

IV DISCUSSION

HEIFERS

The objectives of this experiment were :

(a) to determine whether heifers could be artificially infected with *C.fetus* subsp. *venerealis* (FD15).

(b) where infection was achieved, to determine the period, during which the infecting organism could be recovered, and

(c) to determine the efficiency of recovery of organisms using cultural examination with the solid selective medium as described by Dufty.[22]

The fact that only two of the seven heifers challenged with the organism becamse infected, indicates a low infectivity rate. Based on their previous history and a negative culture of vaginal mucus samples prior to challenge, it is unlikely that these animals had been previously exposed to *C.fetus* subsp. *venerealis* and had developed any resistance to the organism. It is more likely that the strain used was of low virulence. A previous attempt to infect two bulls and two cows at Massey University with this same strain (FD15) resulted in limited success. Although the organism was recovered from the preputial sac after slaughter, preputial samples collected before slaughter failed to yield any organism. Organisms were recovered from vaginal mucus samples of one cow prior to slaughter. Varying degrees of virulence of different strains of *C.fetus* subsp. *venerealis* have been reported,[49] [51] and it has also been suggested that strains may vary in their ability to cause infertility

in individual heifers.[77] Whether the subculturing and lyophilisation of FD15 had in any way affected its virulence remains unknown.

The challenge dose was estimated to contain 10^9 *C.fetus* subsp.*venerealis* organisms per ml. Based on previous reports [48] [49] [51] this should have been sufficient to infect susceptible heifers.

The period over which organisms could be recovered from the two infected heifers, D07 and D23, was 152 days and at least 162 days respectively. Previous reports indicate a return to normal fertility in infected heifers of three to five months.[9] [10] Non-pregnant females tend to remain infected for six months or more[77] and as many as 50% may still be infected in the vagina ten months after challenge.[57]

The results of the cultural examinations from cervicovaginal mucus samples collected at more or less random stages of the oestrous cycle in this experiment (see Table V) were slightly better than the 60% success rate recorded by Clark *et al.*[14] These authors found that the isolation rate from samples collected during the oestrous phase of the cycle was 78% and from the dioestrous phase 57%. The difference was attributed to the increased flow of mucus during oestrus, which carried more organisms into the vagina thereby increasing the chances of isolation. Lawson and Mackinnon[38] had earlier recorded an 83% success rate from samples collected at oestrus compared with 27% from samples collected at other stages of the cycle. Hoerlein and Kramer,[31] using a technique for collecting cervical mucus similar to that of the author, suggest that different stages of the oestrous cycle make little difference in the bacteriological examination of

cervical mucus. They achieved an isolation rate of 53.7% using a culture medium incorporating brilliant green and mycostatin.

The slightly higher recover rates obtained by the author could have been associated with the method of collection where material was actually aspirated from the caudal cervical canal as well as from the anterior vagina.

Only 9.5% of the samples resulted in plate overgrowth on the solid selective medium that led to plates being totally discarded; the degree of contamination of other samples was insufficient to cause any real problem in isolation. This result compares favourably with the 14% heavy, 32% moderate, and 54% slight contamination recorded by Clark *et al.*[14] and suggests that the method of sampling adopted was very satisfactory.

BULLS

The objectives of this trial were to attempt to artificially infect two bulls and to compare the efficiency of cultural and immunofluorescence examinations as methods of diagnosing disease due to *C.fetus* subsp. *venerealis*.

The organism was shown to be present in preputial samples collected from both bulls by both techniques. However, in the younger bull, 7540 0, the number of organisms seen by immunofluorescence and the number of colonies of *C.fetus* subsp. *venerealis* recovered on culture decreased over a period of 13 weeks following exposure. For the last three weeks prior to slaughter (weeks 15,16 and 17 post-exposure)

preputial samples from this bull were negative to both methods. Using the criteria established by other workers,[65] [82] this bull would have been classed as negative. It would appear therefore that although infection was established in 75400, it was only transient.

Although it has been reported that bulls, five years of age and older, are more prone to becoming infected,[64] Schutte[65] reported on failure to artificially infect a five year old bull in spite of three attempts. One eight year old bull remained negative after two attempts at artificial infection using a high concentration of *C.fetus* subsp. *venerealis* organisms suspended in broth. This bull was subsequently infected using sheathwashings from a known carrier bull.

Several factors may have contributed to the failure to establish a long standing infection in bull 75400. Firstly, the preputial environment may not have been favourable for establishing infection because of the lack of mucosal crypts of sufficient depth. This has been advanced as one reason for young bulls being more resistant to infection than older bulls.[64] Secondly, weekly preputial sampling would also have reduced the number of organisms present in the prepuce. Thirdly, the bull was not used for mating at any time during the experimental period. Sexual rest and/or preputial washing in young bulls has been reported as enabling them to overcome infection.[32]

In contrast, a long-standing infection was established in bull number 74473. Organisms were present in moderate numbers and positive immuno-fluorescence results were obtained with all sixteen samples that were

collected. One cultural examination was questionable and one of the two negative results obtained with this bull was due to heavy contamination of the culture plates.

Eighty-one percent of samples were positive to FAT examination and 69% to culture for the two experimental bulls; 84% were positive to both tests. If it is accepted that bull 75400 did become negative (based on three consecutive negative results to both methods), then the efficiency of FAT examination increased to 90% and that of culture to 76%; both together yielded a success rate of 93%.

Compared to the results of other workers [11] [22] [65] the efficiency of positives from known infected animals is slightly lower. Dufty[22] for example, reported 94% FAT positive, 78% positive on direct culture, and 81% positive on culture after millipore filtration for 100 preputial samples collected from five infected bulls; Clark and Dufty [11] demonstrated the presence of *C. fetus* subsp. *venerealis* in 94% of samples by FAT and 86.5% by culture for 89 samples from five infected bulls; and Schutte[65] recorded 90% FAT positive samples and 90% culture positive samples for 153 samples from 44 bulls. Results for other workers are shown in Table III (see Literature Review).

A contributing factor to the lower efficiency of both methods in this trial may have been associated with the small number of organisms recovered from bull 75400, and the possibility that a long-standing infection was not established in this bull. It is noteworthy that Schutte[65] recorded 76% FAT positive results and 81% culture positive results for 37 preputial samples collected from 14 bulls which did not respond to antibiotic treatment - as many as 12.5% false negative

diagnoses were recorded in the early post-treatment period. These he attributed to the smaller number of organisms present and the further reduction following millipore filtration prior to culture. Large amounts of cellular debris plus autofluorescence may have also masked the smaller number of organisms when employing the FAT method.[65]

Twenty-two samples yielded positive isolates on direct culture and 21 following millipore filtration. The difference was accounted for by one direct culture where only one colony on one plate was recovered. The degree of contamination following millipore filtration was less than that using direct culture, an advantage that has been observed by other workers.[22 56 60 69] However, the technique suffers from the disadvantage of removing *C.fetus* subsp. *venerealis* organisms as well as contaminants and Dufty [22] has estimated that over 90% of the organism can be removed by filtration. This could be a major problem when testing bulls which only have few organisms present in the preputial cavity.[65]

One of the major problems noted when using immunofluorescence was the presence of cellular debris and autofluorescence when the smears were examined using ultra-violet light microscopy. Where organisms occurred in moderate to large numbers there was usually no difficulty in detecting them, but where small numbers were present, identification became much more difficult.

In the initial stages of the experiment, smears were made prior to millipore filtration, after millipore filtration, and after final centrifugation. Because of the dilution factor, the numbers of *C.fetus* subsp.*venerealis* seen were smaller in the first two smears

but less cellular debris was present. By contrast more organisms, but accompanied by more debris, were observed on smears made from the residue after final centrifugation. In spite of these problems, this latter technique was used throughout the entire experiment.

On one particular smear, a few dimly fluorescent organisms were observed, a result that was recorded as questionable. This phenomenon of less intense fluorescence has been observed by others.[56 65 82]

Philpott[56] reported on the variations in intensity of fluorescence shown by different isolates. He found *C.fetus* subsp. *venerealis* biotype *intermedius* showed reduced fluorescence and postulated that loss of superficial antigenic material either *in vivo* or alternatively whilst suspended in the saline wash could have contributed to this. He went on to suggest that a given conjugate may not detect all strains of *C.fetus* subsp. *venerealis* but that a conjugate of serotype 1 fluoresced with reference strains and with all recent isolates associated with infertility that had been used in his experimental work.

In terms of diagnosing disease caused by this organism in the field, it is generally accepted that although a single positive result with either test is conclusive, single negative results cannot be considered indicative of non-infection. Dufty[22] suggested that four consecutive FAT examinations of preputial samples with negative results are required before considering a bull free of infection. Winter *et al.*[82] pointed out the complementary nature of both examinations and suggested their combined use. They appear to be less conservative than Dufty and stated that a single negative result with both tests on one sample was indicative of a non-carrier status; the percentage of carriers

missed using this approach would, in their view, be negligible. Schutte[65] suggests two consecutive negative results with both tests as indicative of a non-infected status. In the light of the results obtained with the experiment reported in this thesis, Schutte's criterion would be more acceptable. The main limitation with both techniques is the difficulty in obtaining positive diagnoses when only small numbers of organisms are present in the preputial cavities of infected bulls.

V CONCLUSIONS

Where Bovine Campylobacteriosis is suspected, herd diagnosis should be based on cultural examinations of vaginal mucus samples from non-pregnant females and cultural and immunofluorescence examination of preputial samples from bulls. Based on the results of this experiment and the findings of others, the following guidelines are suggested in order to increase diagnostic efficiency.

(i) Where possible, cervical and/or vaginal mucus should be collected from a minimum of ten non-pregnant females and preputial samples from all bulls used on the suspect property. Where the number of bulls is greater than twenty, samples should be collected from all bulls, five years and older, up to a maximum of ten, as there is a greater chance of long-standing infections in this age group.[9]

(ii) Preputial samples should be collected by aspiration of secretions from the fornix and surrounding preputial mucosa and the dorsal surface of the penis using the Bartlett pipette and rubber bulb.[23]

(iii) Cervical and/or vaginal mucus should be collected using the method and equipment of Hoerlein and Kramer.[31] Where the quantity of mucus collected equals or exceeds 40mm in the artificial insemination pipette, it may be split with one half submitted for cultural examination and the other for vaginal mucus agglutination (VMA) test.

(iv) Where there are anticipated delays of greater than six hours between collection and processing for cultural examination, an enrichment medium[17] should be used for transportation.

(v) Cultural examination should be carried out on duplicate plates of solid selective medium incubated at 37°C in an atmosphere of 90% N_2 : 5%CO_2 : 5%O_2. Cervical/vaginal mucus should be plated out directly; preputial samples should be plated out directly as well as after millipore filtration.

(v) For preputial samples, immunofluorescence examination should be carried out on the rest of the sample, which is centrifuged at 2700 X g for one hour. A single smear is made up by resuspending the residue in distilled water.[22] The smear is stained with a suitable conjugate and examined by ultra-violet microscopy.

(vii) Provisions to resample the animals should be made in case of doubtful results.

This procedure is suitable for herd diagnosis of the disease. For individual bulls, although a single positive result with either FAT or cultural examination can be considered indicative of a carrier status, it would require a minimum of two consecutive weekly samples being negative to both tests to consider an individual bull free of the organism. Using this criterion to declare nine bulls free of the

organism, Schutte[65] failed to recover organisms from virgin heifers which were test-mated to these bulls. For individual females, a single positive culture test is indicative of infection. However, based on the heifer test-mating procedure, it may require as many as three consecutive weekly samples being negative on culture to establish that an animal is not infected.

In view of the efficiency of vaccination as a therapeutic and prophylactic measure in the bull,[5 13 18] and as a protective measure in the females[8] it is the author's view that the ability to declare individual animals free of the disease based on immunofluorescence and cultural examinations, either singly or combined, is not only a difficult but also an unnecessary and unwarranted task. Of greater importance, is the ability to diagnose the disease in a herd based on these two tests. It must be noted that with FAT examination of preputial samples, a positive result can be obtained within a day. This is of importance where remedial measures have to be effected immediately.

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