

Bovine Genital Campylobacteriosis

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Part 1. Diagnostic Overview

Summary

Bovine genital campylobacteriosis is a venereally transmitted bacterial disease caused by *Campylobacter fetus* subsp *venerealis*. The disease is characterised by temporary infertility of female cattle as a result of a subacute diffuse mucopurulent cervicitis, endometritis and salpingitis. Abortion occurs in a small percentage of infected cows, months after initial infection. Infected bulls show no clinical signs but many become carriers and subsequently infect females at service.¹ All breeds of cattle are susceptible. The disease is often suspected in herds in which infertility or abortions occur.

Diagnosis currently relies on the identification of the causal bacterium by culture of preputial washing, vaginal mucus or aborted material. An ELISA is also available to demonstrate the presence of specific antibodies in the vaginal mucus. Recently, a real-time PCR assay has also been reported for detection of *C fetus* subsp *venerealis* directly in clinical samples.²

Bovine genital campylobacteriosis is present in all Australian States and Territories. In New Zealand, the disease was last reported in 1993. Since then extensive surveillance has failed to identify the organism in NZ cattle herds indicating a low prevalence of infection.³

Bovine genital campylobacteriosis is included in the OIE category B list of diseases (second highest priority) and considered an important international trade barrier, with many countries requiring certification of negative disease status.⁴

1. Aetiology

C fetus subsp *venerealis* is a venereally transmitted obligate parasite of cattle, adapted to the genital mucosa lining the preputial and vaginal cavities. It is a gram-negative, non-spore forming bacterium, which is microaerophilic, fragile, and survives for only 6 hours under normal atmospheric conditions.

C fetus subsp *fetus* is similar to *C fetus* subsp *venerealis* but is ubiquitous, colonising other species as well as cattle, including sheep and birds. Although it has been known to cause sporadic abortions in cattle, it is not usually associated with infertility.⁵

Infection of humans by *C fetus* has been reported^{5,6} and is probably due to a general increased awareness of campylobacter infections and the application of more sophisticated diagnostic techniques.

Sub-speciation of *C fetus* via culture-based methods requires experienced interpretation of results, and outcomes may vary considerably between laboratories.^{5,7,8}

1. Taxonomy

There has been much confusion over the epidemiology of bovine genital campylobacteriosis as a result of previous taxonomic difficulties and nomenclature changes (Table 1). *C fetus* subsp *venerealis* is divided into two biotypes on the basis of minor antigenic and biochemical differences; *C fetus* subsp *venerealis* biotype *venerealis* and *C fetus* subsp. *venerealis* biotype *intermedius* (not listed in Table 1).

Table 1: Historical perspective of nomenclature used for *Campylobacter fetus*.

Smith & Taylor (1919) <i>Original Description</i>	<i>Vibrio fetus</i>	
Florent (1959) ¹¹	<i>Vibrio fetus</i> var <i>intestinalis</i> (glycine tolerant)	<i>Vibrio fetus</i> var <i>venerealis</i> (glycine intolerant)
Veron & Chatelain (1973) ¹²	<i>Campylobacter fetus</i> subsp <i>fetus</i>	<i>C fetus</i> subsp <i>venerealis</i>
Smibert (1974) ¹³	<i>C fetus</i> subsp <i>intestinalis</i>	<i>C fetus</i> subsp <i>fetus</i>
Skerman (1980) ¹⁴	<i>C fetus</i> subsp <i>fetus</i>	<i>C fetus</i> subsp <i>venerealis</i>

Strains of *C fetus* subsp *venerealis* and *C fetus* subsp *fetus* cannot be distinguished from one another by DNA homology experiments.⁹ Despite this finding, the maintenance of subspecies designations for *C fetus* is useful because of their significant epidemiologic differences.

Based on serotyping schemes, isolates of *C fetus* subsp *venerealis* belong to only one serotype, serotype A. Isolates of *C fetus* subsp *fetus* are more diverse and can be allocated to serotype A or serotype B.¹⁰

3. Clinical signs

Bovine campylobacteriosis is characterised by temporary infertility of female cattle, and, in a small percentage of infected animals, sporadic abortion.^{15,16} An important aspect of the disease is its subclinical nature in bulls, in which there are no overt clinical signs and a potential long-term carrier stage. In females, apart from late return to oestrus or irregular extended oestrus cycles, no obvious clinical signs can be detected. Shortly after infection, vaginal discharge occasionally develops as a result of subclinical endometritis, cervicitis and vaginitis, but these signs are not pathognomonic and can be overlooked. Most infected females show no clinical signs during the acute

stage of infection. Abortions are usually sporadic and generally occur in carriers around the third trimester of pregnancy, long after the initial infection.

Cows and heifers that have had no previous contact with *C fetus* subsp *venerealis* are highly susceptible to infection and susceptibility does not appear to change with increasing age.¹⁵ Cows that have recovered from the disease develop immunity against reinfection. The immune status of recovered cows declines with time and in most cases has largely vanished 3 to 4 years after the initial infection.^{16,17}

In outbreaks among newly-infected herds, most females return to service and only 25 to 50% become pregnant after two services. The average time from first service to establishment of pregnancy often exceeds 60 days.¹⁵ In seasonal calving herds, this results in most calves being born towards the end of the calving season. Although most infected females will eventually conceive, a small percentage may abort. There is a marked decline in the frequency with which *C fetus* subsp *venerealis* can be isolated from the vagina of infected heifers after they become pregnant.¹⁸ In spite of this, up to 10% may still be infected after calving, although most are free of infection by three months after parturition.

4. Transmission/Epidemiology

Transmission of *C fetus* subsp *venerealis* is strictly venereal. The infection is most commonly introduced to a herd by a persistently infected bull, and less frequently by a persistently infected female. After a bull becomes infected by serving a carrier female, it may remain persistently infected, disseminating the infection during breeding. Factors influencing the establishment of persistent infection probably relate to the genetic changes of the infecting strain, as it attempts to evade the immune system.^{19,20,21} This antigenic drift has not been well characterised, and bulls vary considerably in both their ability to harbour *C fetus* and the length of time they may remain as carriers.²² There is little published information on the frequency of the carrier stage in females, but it appears that persistent infection is less frequent in this sex. This is thought to be due to a vigorous IgG-dominated immune response to bacterial colonisation of the cervix and uterus.²³

Following the introduction of the infection into a herd, the extent of the subsequent infertility episode largely depends on management practices. Field observations in Australia and overseas consistently document pregnancy rates of 40-60% in newly infected herds.^{24,15,16} Since the average time from first service to establishment of pregnancy after infection often exceeds 60 days,¹⁵ the infertility is more severe in herds where restricted joining is practised. In susceptible herds, infertility in individual breeding units (a single sire mating with an infected bull) is always dramatic, compared with multiple sire mating practices, where infected and non-infected bulls are available. In herds in which the disease is endemic, pregnancy rates are generally higher and may appear to be satisfactory. This is mainly because fertility is not usually impaired in previously infected females due to acquired immunity. The degree of infertility will largely depend on the proportion and the number of susceptible cows or replacement virgin heifers being introduced to the endemically infected breeding unit.

5. Occurrence, Distribution and Control

Bovine genital campylobacteriosis is of worldwide distribution and commonly occurs in all States and Territories of Australia. The disease has not been reported in NZ since 1993, indicating a low level of disease distribution in this country.³

In infected herds reproductive wastage is substantial and represents a large financial loss for producers, particularly in the first year of infection, when gross margins may be reduced by as much as 66%. Once the disease becomes established in a herd, gross margins are usually 36% below those of uninfected herds. Infection often remains undetected and is responsible for continuing production losses in many Australian herds.²⁵

Bovine genital campylobacteriosis can be controlled by vaccination. Bivalent vaccines (see appendix H) containing *C fetus* subsp *venerealis* biotype *venerealis* and biotype *intermedius*, injected twice 4 weeks apart have a preventive effect and may also have a curative effect. Recommendations on vaccine usage are different in infected and uninfected herds.¹

5.1. Infected herds: All breeding animals are vaccinated in the herd following diagnosis. Additional antibiotic treatment of an infected bull is recommended at the time of the second vaccination, because vaccination alone may not effectively terminate established infections.²⁶ Treatment consists of a preputial infusion of 3.0 g amoxicillin suspension.

One year later, all bulls and replacement heifers are vaccinated, and bulls are subsequently vaccinated annually.

5.2 Uninfected herds: Annual preventive vaccination of bulls.

6. Diagnostic Tests and Specimens

Bovine campylobacteriosis is usually diagnosed on the basis of herd history and may be confirmed by laboratory investigations.

6.1 Isolation.

The unequivocal diagnosis of bovine genital campylobacteriosis requires the isolation and identification of *C fetus* subsp *venerealis*, and is the prescribed test for international trade.⁴

The challenges of isolating *C fetus* subsp *venerealis* are well recognised. The organism is fragile, slow-growing, and requires specific micro-aerophilic conditions for growth and survival. In preputial and semen samples, *C fetus* subsp *venerealis* must be isolated from among a wide variety of fast- and easy-growing contaminating micro-organisms, which are invariably present in large numbers. The sampling technique used in collecting preputial samples may also be important. Scraping the preputial and penile mucosa appears to be the most successful²⁷ in comparison with preputial washes and aspiration techniques.²⁸

Vaginal mucus tends to be less contaminated than preputial samples. However variations in the number of organisms present during the oestrus cycle, and the reduced number of bacteria occurring during pregnancy mean that culturing techniques are often not adequately sensitive to successfully isolate the organism from females.

C fetus subsp *venerealis* is most successfully isolated from aborted foetuses submitted to the laboratory, because the organism is not exposed to normal atmospheric conditions, and where samples can be collected without contamination and then directly cultured under microaerophilic conditions.

6.2 Antibody detection

An ELISA has been developed to detect antigen-specific secretory IgA antibodies in the vaginal mucus of cattle that are suspected of being previously infected with *C fetus* subsp *venerealis*.^{29,25} With a reported specificity of 98.5%, the IgA ELISA appears to be the current test of choice for the retrospective diagnosis of bovine campylobacteriosis.

6.3 Molecular detection

Molecular identification and differentiation of *C fetus* subsp *venerealis* and *C fetus* subsp *fetus* is achieved using a multiplex PCR assay.^{8,30} This assay has been widely validated and used for the identification and subspeciation of culture isolates.

Recently, a real-time PCR assay has also been reported.² It is awaiting validation and approval by the New Test Development Working Group within the Subcommittee of Animal Health Laboratory Standards.

Part 2. Test Methods

1. Bacteriology

1. Collection of Specimens for Laboratory Diagnosis

C fetus subsp *venerealis* inhabits the mucosa of the prepuce and vagina. Therefore, in decreasing order of sensitivity, preputial smegma scrapings, preputial washings, vaginal lavage, semen and vaginal swabs are all suitable for culture.

1.2 Bulls

1.2.1 Collection of preputial smegma

Equipment needed.

- (i) Plastic bevelled pipette. If more than one bull is tested, use new equipment or disinfect pipette/syringe with a standard disinfectant solution and then rinse with water between bulls.
- (ii) Rubber bulb
- (iii) Universal containers

- (iv) Campylobacter enrichment transport medium (CETM) (10 mL in 30 mL Universal containers) (See Section 1.4.1).

Introduce the pipette to the full length of the preputial cavity. Squeezing and releasing the bulb, scrape along the dorsal surface of the penis and the surrounding preputial mucosa for half to one minute. Control the placement of the penis with a hand externally on the wall of the prepuce. Keep the opening in the bevelled end of the pipette directed downwards onto the penis. The angle of the 'handle' of the pipette should be downwards, away from the animal's body. Withdraw the pipette with the bulb compressed to avoid picking up too much debris from around the preputial opening. Flush out the scrapings with 4 mL of phosphate buffered saline (PBS) into the universal container, stand to allow impurities to settle and inoculate the CETM with 1 mL of the supernatant.

1.2.2 Collection of preputial washings

Equipment needed

- (i) Sterile insemination pipette connected by plastic or rubber tube to a 20 mL sterile disposable syringe
- (ii) Universal container with 20 mL of PBS.
- (iii) Universal container with 10 mL of CETM

Introduce the pipette to the full length of the preputial cavity and hold the prepuce orifice firmly with one hand around the pipette to prevent PBS from leaking from the cavity. Inject 20 mL of PBS into the prepuce and wash thoroughly by forcing the saline up and down along the penis for several times. Withdraw the pipette close to the orifice where the washing fluid has accumulated and withdraw the fluid into the syringe. Transfer the washings into the universal container and stand to allow impurities to settle, before inoculating CETM with 1 mL of the supernatant.

This technique is better tolerated by bulls than the smegma collection method, consequently less physical restraint is required.

1.2.3 Collection of semen

Semen is collected by one of the standard techniques, using artificial vagina, electro-ejaculator or massage of the accessory sex glands. The CETM is inoculated with 1 mL of semen.

1.3 Cows and Heifers

Since persistent infection occurs in some female cattle a representative number (10%) of infertile animals should be sampled. However, the sensitivity of culturing vaginal mucus is quite poor.

1.3.1 Collection of vaginal mucus by lavage of each animal

Equipment needed

(i) A length of 45 cm of fairly rigid polythene tubing (6 mm external and 4 mm internal diameter) is closed at one end by gentle heating in a flame. In the 3 cm of tubing adjacent to the closed end about 16 small holes are made using a hot pin or needle. The holes should be made at various parts of the circumference so that when liquid is forced through the tubing jets will spray from the holes to adequately cover a 360° arc.

(i) 20 mL disposable syringe

(ii) Sterile PBS.

The open end of the sterile tube is connected by a plastic or rubber tube to the syringe. PBS is drawn into the tube and syringe so that air is excluded. The blocked end of the tube is inserted into the vagina as far as the cervix and the saline solution is expelled forcefully into the vagina. The liquid is sucked back into the syringe and the process repeated four to six times. It may be necessary to move the tube backwards and forwards along the floor of the vagina while applying suction with the syringe in order to locate the fluid. The contents of the syringe and tube are transferred into a sterile container of appropriate capacity.

1.3.1 Collection of vaginal mucus by swab of each animal

Equipment needed

(i) 1.5 mL sterile PBS

(ii) Sterile swab (15 cm or longer)

After cleaning the perineum, the swab is introduced into the vagina up to the cervix. Urine contamination is avoided by opening the vaginal cleft and placing the swab behind the external urethral opening. At this point the swab is turned and slightly pulled back and forth a few times to ensure full saturation. The mucus from the swab is then transferred to the PBS by thorough rinsing.

1.3.2 Aborted foetus and placenta

Foetal stomach content is collected aseptically from a freshly aborted foetus using a sterile syringe. Other tissues (lung, liver, spleen and placenta) can also be collected for direct culture.

When placenta is available for examination an additional sample for microscopic examination is collected. Since *C fetus* subsp *fetus* can be present in the faeces of healthy animals, microscopic examination may be useful in determining the significance of *C fetus* subsp *fetus* if isolated.

1.4 Forwarding samples to the laboratory

If specimens can be delivered to a laboratory within six hours, CETM is unnecessary. These specimens should not be exposed to sunlight or temperatures below 4°C or above 30°C. If delivery takes longer than six hours, then the use of CETM is necessary for the submission of foetal stomach contents, preputial washings and vaginal mucus samples.

The organism may remain viable for one or two days in chilled tissues and in the placenta.

1.4.1 Transport media

A range of CETMs have been developed for *C fetus*. These include Clark's, Lander's, Weybridge's and Cary-Blair's media.^{32,33,34}

1.4.1.1 Modified Lander's Medium (MLM)

Lander's medium (see Part 3) has been reported to be the preferred transport medium.^{32,34} The recommended polymyxin B concentration for this medium is 10 IU/mL. However, Platridge and Koths³⁴ (1961) have demonstrated that some isolates of *C fetus* can be sensitive to polymyxin B at a concentration of 5 IU/mL. It has also been demonstrated that 5-fluoro-uracil, which is a constituent of Lander's medium, is inhibitory for *C fetus* subsp *venerealis* (Hornitzky and Berg, unpublished). Thus 5-fluor-uracil has been omitted and the concentration of polymyxin B sulphate has been reduced to 2.5 IU per mL (see Part 3).

Using a sterile disposable syringe and needle, withdraw 1 mL from the top of the sample and inoculate the MLM.

The inoculated containers should be clearly marked. Information regarding the nature of the sample, the identification of the animal, and the time and date of collection should be included. Forward samples to the laboratory within two days. The optimal temperature range for inoculated containers during transport is 18-37°C. Samples should not experience temperatures outside this range.

1.4.2 Laboratory procedures

1.4.2.1 Samples received in transport medium

MLM medium is incubated at 37°C for three days. There is no need to incubate the broth medium under microaerophilic conditions. The MLM medium is then subcultured onto a selective agar plate and incubated under microaerophilic conditions (5% oxygen, 5% carbon dioxide and 90% nitrogen) at 37°C for 3 to 9 days.³⁵ The preferred selective medium for isolation of *C fetus* is Skirrow's medium. *C fetus* does not grow under aerobic conditions.

1.4.2.2 Fresh Samples

Preputial smegma, preputial washings, semen or vaginal mucus received within six hours of collection may be inoculated into CETM and onto blood agar plates and selective agar plates.

Foetal tissues may be sampled aseptically and inoculated onto blood agar and selective agar plates. Foetal stomach contents should be examined by dark ground or phase

contrast microscopy and smears made and stained using Gram stain and modified Ziehl-Neelson method.

1.4.2.3 Culture of *C fetus*

A diagnosis of bovine genital campylobacteriosis is made by the isolation of *C fetus* subsp *venerealis* by culture. Although Skirrow’s medium is preferred, a non-selective blood-based (5-7% blood) medium can be used. When used in combination with filtration through 0.65 µm filter, most contaminants are eliminated. However, filtered samples may be less sensitive when compared with unfiltered samples cultured on selective medium.

1.4.3 Identification procedures

After incubation for 2-5 days suspect *C fetus* colonies are 1-3 mm in diameter. They are slightly grey-pink, round, convex, smooth and shiny, with a regular edge. The organism is a gram negative, curved rod. Short forms (comma-shaped), medium forms (S-shaped) and long forms (spiral rod with several turns) may be observed simultaneously. Old cultures may contain *C fetus* as coccoid bacteria..

Differentiation from other *Campylobacter* spp may be achieved by standard biotyping methods (Table 2).

Table 2: Differential characteristics of *Campylobacter* spp potentially isolated from the bovine tract and aborted fetuses ^{4,37}

Bacterium	25° C	42° C	Oxidase	Catalase	NaCl 3.5%	Glycine 1%	H ₂ S
<i>C fetus</i> subsp. <i>venerealis</i>	V	-	+	V	-	-	-
<i>C fetus</i> subsp <i>fetus</i>	+	V	+	+	-	+	-
<i>C jejuni</i>	-	V	+	V	-	V	-
<i>C hyointestinalis</i>	-	+	+	+	-	V	V
<i>C sputorum</i>	-	+	+	V	+	+	+

V = variable

Alternatively, PCR may be used to identify and subspeciate *C fetus* (see Section 2).

2. Molecular Biology

2.1 Principal of procedures

Isolates of *C fetus* subsp *venerealis* and *C fetus* subsp *fetus* can be detected and differentiated by a multiplex PCR assay.^{8,30} This multiplex assay has been extensively validated for the differentiation of isolates, and has demonstrated a good correlation to phenotypic tests.^{38,39,40,31,41} Primers MG3F and MG4R amplify 764 bp of the chromosomally-encoded gene for a carbon starvation protein that is present in both subspecies of *C fetus*. Subspeciation is achieved using primers VenSF and VenSR,

which amplify 142 bp (GenBank entry AY750964) of a *parA* homologue encoded only by *C. fetus* subsp *venerealis* (Table 3).

Table 3: Multiplex PCR primers⁸

Primer	Sequence (5' → 3')	Product size
VenSF	CTGTAGCAGTTGCGATATTGCCATT	142 bp
VenSR	GCTTTTGAGATAACAATAAGCAGCTT	
MG3F	GGTAGCCGCAGCTGCTAAGAT	764 bp
MG4R	TAGCTACAATAACGACAACT	

Based on the multiplex assay's *C. fetus* subsp *venerealis parA* amplicon⁸, a 5' nuclease real-time PCR assay was subsequently developed for detection of this subspecies. The real-time assay amplifies an 86 bp region of the *parA* gene only from *C. fetus* subsp *venerealis*. This assay was developed for the direct detection of *C. fetus* subsp *venerealis* in preputial washes, asserting greater sensitivity than culture techniques.

Other molecular assays include a *C. fetus* subsp *fetus*-specific PCR, which targets the *sapB2* gene⁴², and a *C. fetus* subsp *venerealis*-specific assay, which uses primer set Cf C05 to amplify a sequence identified from ALFP analyses.⁴³

2.2 Laboratory procedures – multiplex PCR

Crude DNA extracts for PCR analysis can be obtained from bacterial colonies using a simple boil lysis method (for example, a suspension in sterile H₂O is incubated at 95-100°C for 5-10 min), or more purified DNA may also be used.

The multiplex⁸ PCR master mix is prepared fresh and kept on ice (Table 4).

Table 4: PCR master mix

Reagent	µL / reaction	Final concentration in 20 µL reaction
PCR dH ₂ O	10.06	
Qiagen 10× buffer (15 mM MgCl ₂)	2	1×
dNTPs (10 mM)	0.4	0.2 mM
VenSF (10 µM)	0.4	0.2 µM
VenSR (10 µM)	0.4	0.2 µM
MG3F (10 µM)	2	1 µM
MG4R (10 µM)	2	1 µM
Qiagen Taq polymerase (5 U/µL)	0.14	0.7 U
TOTAL (master mix)	19 µL	
Template DNA	1	

The Hum MG3F/MG4R primers are used at 5x the concentration of the VenSF/VenSR primers to compensate for the reduced amplification efficiency of the larger PCR

product. The multiplex assay primers tend to form dimers if the master mix is not kept well-chilled on ice at all times until cycling (Table 5) is commenced.

Table 5: Cycling conditions

Step	Temperature	Time	Number of cycles
Denaturation	94°C	3 min	1
Denaturation	94°C	40 s	36
Annealing	54°C	1 min	
Extension	72°C	40 s	
Extension	72°C	5 min	1

PCR products are analysed by agarose gel electrophoresis. Some primer dimer (<100 bp) may occur in the negative template control and negative samples without affecting the validity of the assay. Non-specific amplification products may occur, particularly when *Campylobacter sputorum* biovar *fecalis* DNA is present in the samples. Non-specific products are acceptable for samples, but not controls.

Test acceptance criteria:

1. Two bands of 764 bp and 142 bp in the *C fetus* subsp. *venerealis* positive control.
2. One band of 764 bp in the *C fetus* subsp. *fetus* positive control (if this is run).
3. No amplification products visible in the NTC (small amounts of primer dimer may be observed).

DNA extracts from both *C fetus* subsp. *fetus* and *C fetus* subsp. *venerealis* will amplify a 764 bp MG3F/MG4R product. Only *C fetus* subsp. *venerealis* will amplify a 142 bp VenSF/VenSR product. Some non-specific amplification products may occur.

2.3 Molecular challenges

2.3.1 Multiplex assay⁸

The assay is the most widely validated and applied assay for *C fetus* subsp. *venerealis*; however, it does have some problems and peculiarities. The MG3F/MG4R amplicon has been mis-reported as 960 bp in several publications, but has since been confirmed by sequence analysis to be 764 bp (GenBank accessions AY158813 and AY158814), which is consistent with its appearance in the published gel images.

Three of the four primers used in this multiplex assay are not perfectly homologous to all known *C fetus* sequences; of the three *C fetus* subsp. *venerealis* *parA* partial gene sequences available in GenBank (AY750964, AY903214 and EU443150), two have mismatches to the multiplex primer sequences⁸ (VenSF: 2/26 nucleotides; VenSR: 1/26 nucleotides). The MG3F/MG4R primers have no mismatches to the sequences available in GenBank (AY158813 and AY158814).

Although originally described as plasmid-encoded²⁹ and predicted to be involved in plasmid partitioning on the basis of sequence homology, it has since been observed that isolated plasmid DNA failed to generate a VenSF/VenSR PCR product, suggesting that this target is actually chromosomally encoded.⁴¹

Non-specific multiplex PCR products may be observed if the sample contains high numbers of non-target organisms. A consistent pattern of cross-reactivity is observed when *Campylobacter sputorum* biovar *fecalis* isolates are assayed using this multiplex PCR; however, these non-specific amplified products differ in size from the critical 764 and 142 bp bands and do not interfere with interpretation of the *C fetus* result. *C sputorum* is a commensal and opportunistic pathogen that has been described in bovines, ovines, and humans.⁴³

In the United Kingdom, a number of field isolates of *C fetus* subsp *venerealis*, isolated primarily from vaginal mucus and aborted fetuses, failed to amplify the 142 bp product, and were therefore mis-typed as *C fetus* subsp *fetus*.⁴¹

2.3.2 Alternative molecular assays

Several alternative molecular assays have been developed and many have been evaluated against the multiplex assay⁸ described above. Most of these studies describe unusual isolates for which PCR and phenotypic testing gave conflicting results, for example, glycine tolerant strains that are identified as *C fetus* subsp *venerealis* by PCR.

A *C fetus* subsp *fetus*-specific PCR that targets the *sapB2* gene has been described, however this assay has since been reported to be unreliable.^{39,42}

Another *C fetus* subsp *venerealis*-specific assay uses primer set Cf C05 to amplify a sequence that was identified by ALFP analyses.⁴³ Comparison of this and the multiplex assay⁸ showed some discrepancies for subspeciation of *C fetus*, but in each of these cases, the Cf C05⁴³ PCR results were consistent with subsequent AFLP typing.

3. Serology

3.1 Principle of IgA ELISA

Specific IgA antibodies appear in the vaginal mucus three to five weeks after infection, are long lasting and can be measured several months after infection.

The IgA ELISA is best used for herd infertility investigation, but it is also suitable for the diagnosis of abortion caused by *C fetus* subsp *venerealis* in individual animals.¹

3.2 Scope and application

The test can be used after infertility or abortion to detect antibodies against *C fetus* subsp *venerealis*. Antibody concentration may vary in the vaginal mucus at different stage of

the oestrus cycle and for infertility investigation the ELISA is best used as a herd test with representative number of problem animals being sampled. Antibody concentrations are less variable after abortion so the ELISA can be used to investigate individual abortions from one week to three months after the event.

The ELISA is not suitable to test preputial washing collected from bulls. Vaccination against the disease will not interfere with the IgA ELISA because only IgM and IgG is present in the vaginal mucus of vaccinated cattle.

3.3 Collection of Vaginal Mucus

Sampling kit (cotton swab, PBST) can be obtained from the testing laboratory. Standard sample of vaginal mucus is collected by a cotton swab as described in section 1.2.2. The head of the swab is then cut and put into 4.5 mL of sterile phosphate buffered saline containing 0.05% Tween 20 (PBST). Samples should be kept chilled after collection and send chilled to the testing laboratory. Time is not critical but samples should reach the laboratory within two or three days.

3.4 Laboratory Procedures

3.4.1 Preparation of Vaginal Mucus

The samples are agitated for 1 h, centrifuged at room temperature at 2500 rpm for 5 min and stored at 4°C until examined. The approximate dilution factor of mucus in PBS is 1:45.

3.4.2 Antigen Preparation and Coating

Campylobacter fetus subsp *venerealis* is grown on Columbia blood agar in microaerophilic conditions at 37°C for 3 days. The plates are checked for purity, and colonies are transferred to 0.5% formol saline for 1 h, centrifuged, washed twice with phosphate-buffered saline (pH 7.5) and then resuspended in carbonate buffer (0.05M pH 9.6). The final optical density (OD) is adjusted to 0.21 at 610 nm. Flat-bottomed polystyrene microtitre plates coated with 100 µL of antigen are left overnight at 4°C and then stored at -20°C. Before use, the plates are washed 4 times with PBST and tapped gently to remove moisture.

3.4.3 Conjugate titration

Each time a new batch of antigen is used, the anti bovine IgA, and the conjugate must be titrated. Optimal concentration of reagents will vary depending on the type and manufacture of microtitre plates and may also vary between batches of the same plates.

3.4.4 ELISA

Diluted vaginal mucus (100 µL) is added to each well and incubated at 37°C for 1 h. The plates are then washed and 100 µL of rabbit anti-bovine IgA (see appendix G) added. After 1 h incubation the plates are washed and 100 µL of goat anti-rabbit IgG conjugated to horseradish peroxidase (see appendix G) is added to each well. After a further 1 h incubation the plates are washed and 100 µL of substrate athylbenz thiazoline-6-sulfonic

acid, commonly known as ABTS, 0.0055 g/10 mL of citrate buffer pH 4.2 is added and immediately activated by 3 µL of 30% H₂O₂. Plates are shaken for 10-30 min in a plate shaker at room temperature. The optical density (OD) is measured on a plate reader at 414nm. The main target OD of the high positive control should be 1.2+/- 0.2 and that of negative control should be less than 0.2. Expected time to reach the target OD varies from 10-30 min depending on the batches of antigen and conjugates used. At 30 min the reaction is stopped by 50 µL of 3M NaOH. The OD is measured on a Titertec Multiscan or an equivalent reader at 450 nm and expressed as ELISA values (EV). The OD measured is expressed as ELISA unit (EU).

Each sample is tested in duplicate with high, low positive and negative controls are included in each plate. The OD measurements yielded by the test sample are corrected for the OD measurement of positive and negative controls according to the formula:

$$\text{EU} = \frac{\text{OD of sample} - \text{OD of negative control}}{\text{OD of positive control} - \text{OD of negative control}} \times 100$$

3.4.5 Interpretation of Results

Samples that are less than 23 EU are reported negative;
samples that are between 23 to 33 EU are inconclusive; and
samples that are greater than 33 EU are considered positive reactors.

ELISA units between 23 and 33 EU should be viewed with suspicion especially if several animals react in the range. In these cases further investigation by sampling 10 different animals in the herd could further clarify disease status.

In a herd of 100 cattle bovine genital campylobacteriosis is unlikely to be present if all 10 samples are negative. Finding 1 or 2 positive reactors would be consistent with infection but the infection in these cases may not be widespread in the herd. Finding 3 or more reactors may indicate widespread infection in the herd.

A positive test result of a sample collected after an abortion strongly indicates campylobacter infection.

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Part 3. Reagents and Test Kits

1. Modified Lander's Transport Enrichment medium

Composition per litre.

Mueller-Hinton Broth	1 L
Bacteriologic charcoal	5 g
Lysed horse blood	70 mL

Antibiotic supplement

Vancomycin	40 µg/mL
Polymyxin B sulphate	2.5 IU/mL
Cycloheximide	100 µg/mL
Trimethoprim	20 µg/mL)

FBP supplement

Sodium pyruvate	0.25 g/mL
Sodium metabisulphite	0.25 g/mL
Ferrous sulphate	0.25 g/mL

Preparation

Prepare Mueller-Hinton Broth according to manufacturer's instructions with the charcoal added. Autoclave at 121°C for 15 min and allow to cool before adding the lysed horse blood, antimicrobial agents and FBP supplement (sodium pyruvate, sodium metabisulphite, ferrous sulphate) aseptically.

The medium is dispensed in 10 mL volumes in 30 mL Universal containers, taking care to ensure even distribution of the charcoal. The medium is stored at 4°C and is stable for at least 3 weeks.

Commercially made alternatives may be used.

2. Skirrow's Campylobacter Agar

Composition per litre.

<i>Campylobacter</i> agar base	990 mL
Antibiotic supplement	10 mL

Campylobacter agar base.

Proteose peptone	15.0 g
Agar	12.0 g
NaCl	5.0 g
Yeast extract	5.0 g
Liver digest	2.5 g

Preparation of base

Add components to base distilled/deionised water and bring volume to 900 mL. Gently heat and bring to boiling. Mix thoroughly. Autoclave 121°C for 15 min. Cool to 50°C

Antibiotic supplement

Vancomycin	10 µg/mL
Trimethoprim	5 µg/mL
Polymixin B	2.5 IU/mL

Preparation of medium

Prepare 990 mL of *Campylobacter* agar base. Autoclave and cool to 45-50°C. Aseptically add 10 mL of the antibiotic supplement. Mix thoroughly. Pour into sterile Petri dishes.

Commercially made alternatives may be used.