

## BOVINE GENITAL CAMPYLOBACTERIOSIS

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### SUMMARY

**Description of the disease:** Bovine genital campylobacteriosis (BGC) is a venereal disease also known as bovine venereal campylobacteriosis (BVC). The causal agent of this sexually transmissible disease is *Campylobacter fetus* subsp. *venerealis*, one of three subspecies of *C. fetus*. BGC causes infertility, early embryonic death, and abortion with considerable economic losses. Bovine infections with *C. fetus* subsp. *fetus* are associated with abortion and have a more sporadic occurrence.

*Campylobacter fetus* subsp. *venerealis* has a pronounced tropism for the male and female genital systems of cattle. Bacterial transmission takes place mainly during natural mating. The disease may also spread through artificial insemination with semen from infected bulls.

**Identification of the agent:** Samples taken from bulls, cows or aborted fetuses can be analysed for the presence of the causal organism. The organism is a thin Gram-negative curved rod that may form S-shapes, flying seagull silhouette shapes and spirals, and can be cultured on selective media at 37°C after at least 2 days in a microaerobic atmosphere. Confirmation of the organism and discrimination between the *C. fetus* subspecies can be achieved using biochemical or molecular methods although in the latter case an assay with a sufficient specificity is required. Immunofluorescence may also be used to identify the organism, but it will not differentiate between different subspecies.

A monoclonal antibody-based capture enzyme-linked immunosorbent assay (ELISA) may also be used to detect *C. fetus* from Clarke's transport enrichment medium. Like immunofluorescence, the ELISA cannot differentiate adequately between the two *C. fetus* subspecies and is limited to a screening method with traditional cultural methods to confirm identification of any positive ELISA result. However, compared with immunofluorescence, it has the advantage of higher sensitivity and specificity for *C. fetus* identification and much higher throughput for both negative and positive results where larger volume testing is conducted.

**Serological tests:** ELISA can be used for evaluating herd immunity, but is not suitable for diagnosis of the infection in individual animals. This test cannot differentiate between infections caused by the two subspecies *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*.

**Requirements for vaccines:** A vaccine may be prepared from *C. fetus* subsp. *venerealis* or *C. fetus* subsp. *fetus* as the two subspecies appear to share common protective antigens. This vaccine is inactivated with formalin, and may be administered in an oil-emulsion adjuvant.

### A. INTRODUCTION

#### 1. Description and impact of the disease

Bovine genital campylobacteriosis (BGC, also known as bovine venereal campylobacteriosis [BVC]) is a venereal disease characterised by infertility, early embryonic death, and abortion in cattle. The causal agent of this sexually transmissible disease is *Campylobacter fetus* subsp. *venerealis* (Cfv). It can be isolated from the genital tract of cattle (e.g. preputial smegma, vaginal mucus) or internal organs of aborted fetuses and causes fertility problems with considerable economic losses. Cff can be recovered from the intestinal tract of cattle and other animal species (Garcia *et al.*, 1983); it can be isolated from aborted bovine fetuses indicating that it has clinical relevance

in cattle. Cff is associated with sporadic cases of abortion in bovine whereas Cfv is associated with endemic abortion and fertility problems in certain areas.

Although *C. fetus* is primarily recognised as a pathogen in animals, Cff is occasionally diagnosed as an opportunistic pathogen in humans. Infections usually occur in pregnant or immuno-compromised individuals and are often systemic with a variety of complications depending on the site of infection (Wagenaar *et al.*, 2014). Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

## 2. Taxonomy

*Campylobacter fetus* is one of the 27 currently recognised species within the genus *Campylobacter* (<http://www.bacterio.cict.fr/c/campylobacter.html>). Three subspecies of *C. fetus* have been recognised: *C. fetus* subspecies *testudinum* (Cft), *C. fetus* subsp. *fetus* (Cff) and *C. fetus* subsp. *venerealis* (Cfv), of which Cft is reptile-associated and both Cff and Cfv are mammal-associated. Although the clinical signs related to infections by Cfv and Cff overlap, they were originally defined by the differences in clinical presentation (Florent *et al.*, 1959; Veron & Chatelain, 1973). Cfv and Cff can be differentiated by the 1% glycine tolerance test and H<sub>2</sub>S production in cysteine-rich medium; Cfv is not able to grow in the presence of 1% glycine and not able to produce H<sub>2</sub>S from cysteine-rich medium, whereas Cff is 1% glycine tolerant and H<sub>2</sub>S production positive (Florent *et al.*, 1959; Veron & Chatelain, 1973). Cfv includes a variant designated Cfv biovar *intermedius* (Florent *et al.*, 1959; Veron & Chatelain, 1973). Neither DNA–DNA hybridisation (Harvey & Greenwood, 1983) nor protein-banding patterns using polyacrylamide gel electrophoresis (PAGE) of whole cell proteins (Vandamme *et al.*, 1990) revealed any major difference between Cfv and Cff. MALDI-TOF (matrix assisted laser desorption ionisation–time of flight) mass spectrometry can be used to identify *C. fetus* at species level (Bessede *et al.*, 2011), but this assay is not able to differentiate between Cff and Cfv. Of all described molecular tests, only polymerase chain reaction (PCR) assays targeting the *nahE* gene, both conventional and real-time, are able to identify *C. fetus* species reliably (Abril *et al.*, 2007; van der Graaf *et al.*, 2013), as well as multilocus sequence typing (MLST) and amplified fragment length polymorphism (AFLP) (van Bergen *et al.*, 2005a; Wagenaar *et al.*, 2001). Several molecular methods have been described claiming to differentiate between Cff and Cfv, including PCR (Abril *et al.*, 2007; Hum *et al.*, 1997; Tu *et al.*, 2005; van Bergen *et al.*, 2005c; Wang *et al.*, 2002), pulsed-field gel electrophoresis (PFGE) (On & Harrington, 2001), MLST (van Bergen *et al.*, 2005a) and AFLP (Wagenaar *et al.*, 2001) (see also Section B.1.9), but none of these molecular tests is able to identify the *C. fetus* isolates reliably to subspecies level (Iraola *et al.*, 2015; van der Graaf *et al.*, 2013). The PCR assay described by McGoldrick *et al.* (2013) for *C. fetus venerealis* identification had a sensitivity of 98.7% and specificity of 99.8%. Whole genome sequencing can be used to differentiate the mammal-associated *C. fetus* strains based on their core genomes (van der Graaf *et al.*, 2014), but this method is not in full concordance with the phenotypic subspecies identification of *C. fetus* strains.

## B. DIAGNOSTIC TECHNIQUES

**Table 1.** Test methods available for diagnosis of bovine genital campylobacteriosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
<b>Agent identification<sup>1</sup></b>						
Culture (including phenotypic characterisation)	+++	+++	+++	+++	+++	–
IFAT	++	++	++	++	++	–

<sup>1</sup> A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
MAb-based ELISA	++	++	++	++	++	–
MALDI-TOF	+	+	+	+	+	–
MLST	+	+	+	+	+	–
<i>C. fetus</i> species PCR ( <i>nahE</i> )	++	++	++	++	++	–
Detection of immune response						
Antibody ELISA	–	–	–	–	–	+

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method but may need further validation; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = purpose not applicable.

IFAT = indirect fluorescent antibody test; MAb = monoclonal antibody; ELISA = enzyme-linked immunosorbent assay; MalDI-tof = matrix assisted laser desorption ionisation–time of flight; MLST = multilocus sequence typing; PCR = polymerase chain reaction.

Culturing and identification of the organism is a suitable test for certifying individual animal prior to movement. However, the routine culture methods can be time consuming. The validated sensitive and specific monoclonal antibody (MAb)-based capture enzyme-linked immunosorbent assay (ELISA) screening procedure (Brooks *et al.*, 2004; Devenish *et al.*, 2005) can accurately detect *C. fetus* subsp. from incubated Clarke's transport enrichment medium (TEM). Only positive ELISA results are cultured for confirmation of the specific subspecies using standard isolation and identification methods. This procedure has the advantage of more rapid turnaround times for reporting, and ease, compared with traditional cultural methods.

## 1. Isolation and identification of the agent

### 1.1. Collection of specimens

#### 1.1.1. Male: preputial smegma and semen

In bulls, smegma may be obtained by different methods: scraping (Tedesco *et al.*, 1977), aspiration (Campero *et al.*, 2003), and washing (Clarke & Dufty, 1978). Smegma is commonly collected by scraping and can be used for isolation of the organism, or is rinsed into a tube with approximately 5 ml phosphate buffered saline (PBS) with 1% formalin for diagnosis by immunofluorescence. Smegma can also be collected from the artificial vagina after semen collection, by washing the artificial vagina with 20–30 ml of PBS.

For preputial washing, 20–30 ml of PBS is introduced into the preputial sac. After vigorous massage for 15–20 seconds, the infused liquid is collected.

Semen is collected under conditions that are as aseptic as possible. Semen samples must be diluted with PBS and are inoculated directly on to culture medium or into transport and enrichment medium.

#### 1.1.2. Female: cervico vaginal mucus

Samples may be obtained by aspiration or washing the vaginal cavity.

For aspiration, the vulva region is cleaned, and an artificial insemination (AI) pipette or Cassou pipette (blue sheath type) is inserted into the vaginal cavity so that the anterior reaches the cervix (Campero *et al.*, 2003). Gentle suction is applied while moving the pipette gently backwards and forwards. The pipette is removed, and the collected mucus is inoculated directly on to culture medium or into transport and enrichment medium.

Cervico-vaginal mucus (CVM) may also be collected by washing the vaginal cavity: 20–30 ml of PBS is infused into the cavity through a syringe attached to an AI pipette. The fluid is sucked out and re-infused four to five times before being collected and spread directly on to culture medium or added to transport and enrichment medium. Washing fluid in the vaginal cavity may also be collected by a tampon or gauze held inside the vagina for 5–10 minutes after PBS infusion. Samples of CVM obtained by suction may be diluted with PBS, or sown directly on to culture medium or transport and enrichment medium.

CVM is transferred into approximately 5 ml of PBS with 1% of formalin for IFAT.

### 1.1.3. Aborted fetuses, placentas

The placenta as well as the liver, lungs and stomach contents of the fetus provide the best samples for isolation of the causative bacteria. Samples are collected aseptically and inoculated into transport and enrichment medium, or into PBS with 1% formalin for IFA testing.

## 1.2. Transport of specimens

The use of a transport medium is essential if the specimens are not processed in the laboratory within the same day after collection. For dispatch to the laboratory specimens must be placed in an insulated container (within the temperature range 4–8°C) protected from light and shipped to reach the laboratory as soon as possible, preferably on the same day as sampling.

Various transport and enrichment media are available, such as Clark's, Lander's, SBL, Foley's and Clark's, Weybridge's, Cary-Blair's (Garcia *et al.*, 1984; Hum *et al.*, 1994; Monke *et al.*, 2002).

Some of the transport and enrichment media mentioned above contain cycloheximide. Because of its potential toxicity, amphotericin B can be used as an alternative.

## 1.3. Treatment of specimens

On arrival at the laboratory, specimens should be inoculated directly on to culture medium, or, if required immediately, processed further.

### 1.3.1. Genital tract samples

Preputial washings may be centrifuged (3500 **g**) to concentrate the sample. The final sample (reduced to 250 µl) may be inoculated on to the culture medium (directly or using the filter method).

If the CVM is not very viscous it can be inoculated directly or diluted with an equal volume of PBS. When the CVM is very viscous, it may be necessary to liquefy it by adding an equal volume of L-cysteine solution (100 mM *N*-Acetyl-L-cysteine, CAS number 616-91-1<sup>2</sup>, dissolved in PBS and sterilised by membrane filtration). After 15–20 minutes, the diluted and liquefied mucus can then be inoculated on to isolation medium.

For the filter method; sterile cellulose acetate membranes of 0.45–0.65 µm pore size are placed on the surface of non-selective agar supplemented with 5% blood. A suspension of the sample is made in in PBS or saline, and 10–15 drops of this suspension are placed on top of the membrane and allowed to filter passively at 37°C (microaerobic conditions are not required). After 30–40 minutes, the filter membranes are removed and the culture plates incubated for 1–2 days at 37°C under microaerobic conditions (Steele *et al.*, 1984).

### 1.3.2. Aborted fetuses, placentas

Fetal stomach contents are inoculated directly on to culture medium. Internal organs or pieces of organs are flamed to disinfect the surface, and are subsequently homogenised. The homogenate is inoculated on to culture medium.

After washing placental membranes with PBS to eliminate the majority of the surface contamination, the chorionic villi are scraped and the scrapings are transferred to culture medium.

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2 This is the unique number for this chemical assigned by Chemical Abstracts Service (CAS).

## 1.4. Isolation of *Campylobacter fetus*

### 1.4.1. Culture media for isolation

Many media are currently in use for the bacteriological diagnosis of BGC. It should be noted that several media used for the isolation of *Campylobacter* spp. are not suitable for the isolation of *C. fetus* because of antimicrobials (e.g. cephalosporins) that may inhibit *C. fetus* growth (van Bergen *et al.*, 2005b). Most culture media contain cycloheximide. Because of its potential toxicity, this antifungal agent can be replaced by amphotericin B. The recommended selective medium for isolation of *C. fetus* is Skirrow's. Skirrow's medium is a blood-based medium with 5–7% (lysed) defibrinated blood and contains the selective agents: polymyxin B sulphate (2.5 IU/ml), trimethoprim (5 µg/ml), vancomycin (10 µg/ml), and cycloheximide (50 µg/ml).

Alternatively, a non-selective blood-based (5–7% blood) medium in combination with filtration (0.65 µm) can be used; however, it may be less sensitive when compared with a selective medium.

Quality control of each batch of media should be performed using control strains.

### 1.4.2. Incubation conditions

Plates are incubated at 37°C and under microaerobic atmosphere of 5–10% oxygen, 5–10% carbon dioxide and preferably 5–9% hydrogen for optimal growth (Vandamme, 2000). Appropriate microaerobic conditions may be produced by a variety of methods. In some laboratories the suitable atmosphere is created by a gas replacement in a jar. Gas generator kits are also available from commercial sources. Variable atmosphere incubators can also be used.

Conditions of culture and incubation are systematically verified by using control strains of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Such controls should be set up for each isolation attempt.

## 1.5. Identification of *Campylobacter* species

### 1.5.1. Colony morphology

Colonies of *C. fetus* usually appear on culture media after 2–5 days. To prevent overgrowth of specific colonies by contaminants, it is recommended that the media be evaluated daily and suspicious colonies be subcultured. After 3–5 days of incubation, colonies measure 1–3 mm in diameter. They are slightly grey-pink, round, convex, smooth and shiny, with a regular edge.

### 1.5.2. Macroscopic morphology

*Campylobacter* is motile, a property that may disappear during sub-culturing. *Campylobacter* often takes the form of a thin, curved bacillus, 0.3–0.4 µm wide and 0.5–8.0 µm long. Short forms (comma-shaped), medium forms (S-shaped), and long forms (helical with several spirals) may be observed simultaneously in the living state. Old cultures may contain coccoid bacteria.

### 1.5.3. Biochemical tests

See Table 2.

### 1.5.4. Atmosphere

*Campylobacter* does not grow under aerobic conditions.

## 1.6. Immunological identification of *Campylobacter fetus*

The IFAT can be applied to identify the organism directly from samples or to confirm the identification of a strain after isolation. It cannot differentiate between different subspecies.

### 1.6.1. Preparation of immune sera

*Campylobacter* strains, preferably standard strains from recognised culture collections (*C. fetus* subsp. *venerealis* or *C. fetus* subsp. *fetus*), are grown on blood-based medium at 37°C under microaerobic conditions for 3 days. The organisms are harvested into PBS, and washed twice by centrifugation. Rabbits aged 3 months are inoculated intramuscularly with 2 ml of

$10^{11}$  organisms/ml of a *C. fetus* subspecies resuspended in PBS and Freund's incomplete adjuvant. Inocula are administered at four sites, 0.5 ml at each site. The animals are bled before inoculation and at weekly intervals thereafter. When the serum titres reach high levels, as estimated by the immunofluorescence test or agglutination test, 0.1–1.0 ml of  $10^{10}$  viable organisms/ml are injected intravenously. The rabbits are bled for serum 7 days later. The rabbit sera are pooled. In a recent study, a conjugate prepared from chicken IgY was described as an alternative to rabbit antibodies. MAbs that can be used for immunodiagnostic detection of *C. fetus* have been described (Brooks *et al.*, 2002).

### 1.6.2. Preparation of conjugates

Conjugates are prepared as described by Harlow & Lane (1988). The working dilution of the conjugate is determined by checkerboard titration against smears of a *C. fetus* culture using positive and negative control dilutions, and selecting twice the lowest concentration that produces brilliant fluorescence with *C. fetus* bacteria.

### 1.6.3. Sample preparation

The genital fluid (fetal abomasal content, preputial smegma or CVM) samples are rinsed into approximately 5 ml PBS 1% formalin. Two centrifugation steps are carried out. First, samples are centrifuged at 600 **g** for 10 minutes at 4°C to remove debris. Subsequently, the supernatant is centrifuged at 8000 **g** for 30 minutes at 4°C. The pellet is dissolved in ~100 µl remaining supernatant.

### 1.6.4. Immunofluorescence test (Mellick *et al.*, 1965)

The sample (20 µl) is applied in duplicate to microscope slides. The material is air-dried and fixed in acetone at –20°C for 30 minutes or ethanol at 18–25°C for 30 minutes. Glass slides will be air-dried and the fluorescein isothiocyanate isomer (FITC)-conjugated antiserum is added at the appropriate dilution. Staining is carried out in a humid chamber at 37°C for 30 minutes in dark condition. The slides are then washed three times for 10 minutes in PBS. The slides are mounted in buffered glycerol (90% glycerol: 10% PBS). The cover-slips are sealed to prevent drying, and the slides are examined under ultraviolet light in a fluorescent microscope. Positive and negative control slides will be used each time the test is done. *Campylobacter fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* reference strains are used as positive controls, and another *Campylobacter* species is used as negative control. Samples that show fluorescent bacteria presenting the typical morphology of *C. fetus* are considered positive.

## 1.7. Biochemical identification of *Campylobacter fetus* subspecies

Tests described in Table 2 must be done on pure cultures.

**Table 2.** Differential characteristics of *Campylobacter* species potentially isolated from the bovine genital tract and aborted fetuses (according to Bergey's Manual 2<sup>nd</sup> edition, 2005)

	25°C	42°C	Oxidase	Catalase	NaCl 3.5%	Glycine 1%	H <sub>2</sub> S cysteine
<i>C. fetus</i> subsp. <i>venerealis</i>	V	√ <sup>(a)</sup>	+	V	–	–	–
<i>C. fetus</i> subsp. <i>fetus</i>	V	√ <sup>(a)</sup>	+	+	–	+	+
<i>C. jejuni</i>	–	√ <sup>(b)</sup>	+	√ <sup>(c)</sup>	–	V	+
<i>C. hyointestinalis</i>	–	+	+	+	–	V	n.d.
<i>C. sputorum</i>	–	+	+	V	+	+	n.d.

(a) = Although *C. fetus* does not belong to the thermophilic *Campylobacters*, a considerable number of strains of this species grows at 42°C;

(b) *C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is negative;

(c) *C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is variable; ;

(+) = positive reaction or growth and (–) = negative reaction or absence of growth of the strain on an appropriate medium under specified conditions (see Section B.1.4); V = variable results; n.d. = not determined.

### 1.7.1. Growth at 25°C and 42°C

A cell-suspension (~McFarland No. 1) is inoculated on to two blood-based medium-plates. Each plate is incubated under the specified atmospheric conditions (see Section B.1.4.2) at 25°C and 42°C. Control strains are tested in parallel.

### 1.7.2. Oxidase and catalase

Tests are performed according to a standard bacteriological protocol. Control strains are tested in parallel.

### 1.7.3. Growth in the presence of sodium chloride

A cell-suspension is inoculated on to blood medium containing 3.5% NaCl (15 ml of blood medium + 2.04 ml of 5 M sodium chloride solution), and on to plain blood medium. Incubation is performed under the specified atmospheric conditions (see Section B.1.4.2). Control strains are tested in parallel.

### 1.7.4. Growth in the presence of 1% glycine

A cell-suspension (~McFarland No. 1) is inoculated on to a glycine medium (15 ml of blood-based medium + 1.65 ml of 10% aqueous solution of filter sterilised glycine), and on to the same medium without glycine. Incubation is performed under the specified atmospheric conditions (see Section B.1.4.2). Two control strains (Cff and Cfv) are tested in parallel. As all strains are fastidious, small changes in media can be important, and lack of growth in the presence of glycine should be considered to be a presumptive test for *C. fetus* subsp. *venerealis*. The reproducibility of the assay is poor and intermediate strains have been described (Salama *et al.*, 1992 and Van Bergen *et al.*, 2005a). Furthermore, the reliability of the 1% glycine tolerance test can be influenced by the fact that glycine tolerance can be transduced by phages Chang & Ogg, 1971).

### 1.7.5. Hydrogen sulphide production in cysteine medium

The H<sub>2</sub>S test is done in a *Brucella* broth medium containing 0.02% cysteine. A cell suspension (~McFarland No. 1) is inoculated into the medium. H<sub>2</sub>S production is detected by a lead acetate strip that is attached inside the top of the tube during 72 hours. Blackening of the lead acetate strip is considered as a positive reaction. Control strains are tested in parallel.

## 1.8. Monoclonal antibody-based capture ELISA

The MAb-based capture ELISA procedure can be used to detect the presence of *C. fetus* species cultured in Clarke's TEM. The validated procedure and reagents are described by Brooks *et al.* (2004) and Devenish *et al.* (2005). Briefly, the samples (preputial washes, vaginal mucus, fetal fluids, placental tissues, liver tissue) are placed in Clarke's TEM and incubated for 4–5 days. Approximately 1.5 ml of the TEM fluids is withdrawn, heated and tested by ELISA. A rabbit polyclonal antiserum (to six different *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* serotype A and B strains) is used to capture antigen from the TEM fluid. The detection of any captured antigen is accomplished with further testing using four mouse MAbs specific to lipopolysaccharide (LPS) epitopes; *C. fetus* subsp. core LPS (1 MAb: M1825), serotype A-specific side chain LPS (2 MAbs: M1177 and M1194) and serotype B-specific side chain LPS (1 MAb: M1183). The test has been shown to be 100% sensitive and 99.5% specific for the detection of *C. fetus* species from the TEM fluids, and large numbers of samples can be tested at the same time.

### 1.8.1. Test procedure

- i) **Solid phase:** ELISA plate wells are coated with an optimal dilution of a rabbit polyclonal anti-*C. fetus* subsp. serum (in 0.06 M carbonate buffer, pH 9.6) and incubated for 18 hours at room temperature (RT). Coated ELISA plates can be sealed and stored at –20°C for 1 month. Prior to testing, ELISA plates, at RT, are washed four times using 0.01 M PBS containing 0.15 M NaCl and 0.05% Tween 20 (PBST).
- ii) **Controls:** There are three control antigens: 1) *C. fetus* subsp. *fetus* (ATCC 27374), a serotype B strong positive control that binds with the LPS core MAb (M1825) and the single serotype B specific MAb (M1183); 2) *C. fetus* subsp. *venerealis* (ATCC 19438), a weak serotype A positive control that binds to the LPS core MAb (M1825) and the two serotype A-specific MAbs (M1177 and M1194); and 3) *C. sputorum* biovar *sputorum*, a negative control that does not bind with any of the four MAbs. Lots of the three control antigens are grown, washed, concentrated and stored at –20°C.

- iii) *Testing samples:* After 4–5 days' incubation at 37°C of inoculated TEM samples, approximately 1.5 ml of fluid is withdrawn from the TEM vials. Optimally diluted control antigens and undiluted TEM fluid samples are heated at 100°C for 15 minutes and cooled to RT. Each heated test fluid sample is added in duplicate and each heated control antigen is inoculated in quadruple to appropriate wells in the solid-phase ELISA plate and incubated for 1 hour at RT.
- iv) *Detector:* To economise on reagents, screening of fluids is done initially with the core MAb M1825. Diluted MAb M1825 (optimally in PBST) is added to each well of the ELISA plates and the plates are incubated for 1 hour at RT. Fluids that are positive using MAb M1825 are further tested using all four MAbs.
- v) *Conjugate:* Horseradish-peroxidase goat anti-mouse immunoglobulin G (heavy and light chains) conjugate, optimally diluted in PBST, is added to all wells and plates are incubated for 1 hour at RT.
- vi) *Substrate:* 3,3',5,5'-tetramethylbenzidine-hydrogen peroxide substrate is added to wells and the plates are placed on an orbital shaker for 10 minutes at RT. Optical densities (OD) are immediately measured at 620 nm (OD<sub>620</sub>) using an ELISA reader.

All reagent lots, including capture antiserum, control antigens, mouse MAbs and conjugate are tested beforehand by checkerboard titration to determine optimal dilutions used in routine testing of sample fluids. Throughout the test procedure, 100 µl volumes are used and plates are washed four times between stages using PBST.

- vii) *Interpretation of the results:* To be ELISA positive, the fluid must be positive against not only the core MAb but also to at least one of the serotype-specific MAbs. In initial screening using MAb M1825, the mean OD<sub>620</sub> of the each test sample is divided by the mean OD<sub>620</sub> obtained from strong positive *C. fetus* subsp. *fetus* serotype B control antigen and multiplied by 100% to obtain a per cent positivity (%P). Any test fluid with a %P greater than or equal to 14% is considered positive and tested further with all four MAbs in the procedure. A repeat positive with M1825 and an OD<sub>620</sub> value greater than or equal to 0.2 with at least one of the other three serotype-specific MAb is a positive ELISA result for the detection of *C. fetus* ssp. in the original test fluid sample. All TEM vials corresponding to ELISA positive fluid samples are cultured as described in Section B.1.4 *Isolation of Campylobacter fetus* for confirmation and to determine the subspecies of the cultured isolate.

### 1.9. Molecular identification of *Campylobacter fetus* subspecies

Several molecular methods for the identification of *C. fetus* subspecies have been described, including 16S sequencing (Gorkiewicz *et al.*, 2003; On & Harrington, 2001), PFGE (On & Harrington, 2001), AFLP (Wagenaar *et al.*, 2001), and MLST (van Bergen *et al.*, 2005a). MLST was recommended for the differentiation of Cff and Cfv strains; a recent study however described a Cff strain that was isolated with the Cfv-associated MLST ST-4 genotype (Iraola *et al.*, 2015), showing that MLST is also not fully reliable for *C. fetus* subspecies differentiation. Whole genome sequencing can be used for a very reliable characterisation of *C. fetus* strains (van der Graaf *et al.*, 2014), but this method is expensive and currently not widely used in diagnostic laboratories.

Routine diagnostic laboratories would be served best by a PCR assay. Several PCRs have been claimed to be subspecies specific including those developed by Hum *et al.* (1997), Wang *et al.* (2002), Tu *et al.* (2005), van Bergen *et al.* (2005c) and Abril *et al.* (2007). However, a PCR assay that identifies *C. fetus* isolates reliably to subspecies level is not available (van der Graaf *et al.*, 2013) (Table 3).

**Table 3.** Sensitivity and specificity of *C. fetus* (sub)species identification of PCR assays

PCR assay	Identification	Target	Sensitivity <sup>a</sup>	Specificity <sup>a</sup>
Abril <i>et al.</i> , 2007	<i>C. fetus</i> Cfv	<i>nahE</i> <i>ISCfe1</i>	100% (143/143) 97% (58/60)	100% (12/12) 100% (95/95)
Van Bergen <i>et al.</i> , 2005c	Cfv	unknown	45% (27/60)	100% (95/95)
Hum <i>et al.</i> , 1997	<i>C. fetus</i> Cfv	<i>cstA</i> <i>parA</i>	100% (143/143) 58% (46/70)	100% (12/12) 83% (79/95)



PCR assay	Identification	Target	Sensitivity <sup>a</sup>	Specificity <sup>a</sup>
McMillen <i>et al.</i> , 2006	Cfv	<i>parA</i>	53% (32/60)	100% (95/95)
Wang <i>et al.</i> , 2002	Cff	<i>sapB2</i>	76% (63/83)	72% (52/72)
Van der Graaf <i>et al.</i> , 2013	<i>C. fetus</i>	<i>nahE</i>	100% (143/143)	100% (12/12)

<sup>a</sup>Represented as percentage (number of correct identified strains/total number of strains). Study included; 143 *C. fetus* strains, 60 Cfv strains, 83 Cff strains, 12 non-fetus *Campylobacter* strains, 95 non-Cfv strains and 72 non-Cff strains (van der Graaf *et al.*, 2013).

The multiplex PCR assay described by Abril *et al.* (2007) proved to be reliable for the correct identification of *C. fetus* species, with 100% sensitivity and 100% specificity, though the Cfv-specific target described for this PCR is 97% sensitive and cannot be used for a reliable differentiation of Cff and Cfv isolates (van der Graaf *et al.*, 2013). The *C. fetus* target of this PCR, gene *nahE*, can be used to identify *C. fetus* species and has also been developed as a real-time assay (van der Graaf *et al.*, 2013; McGoldrick *et al.*, 2013).

The PCR described by Van Bergen *et al.* (2005c) is able to detect Cfv strains as defined by AFLP, but the assay does not identify the *C. fetus* subsp. *venerealis* biovar *intermedius* as defined by. Therefore, this PCR is not suitable for diagnostic purposes (van der Graaf *et al.*, 2013).

The multiplex PCR described by Hum *et al.* (1997) enables the amplification of a *C. fetus*-specific DNA fragment (approximately 200 bp smaller than the 960 bp described in the original publication), as well as a *C. fetus* subsp. *venerealis*-specific fragment. Comparison of this PCR against AFLP and MLST (van Bergen *et al.*, 2005a) and against the glycine test (Willoughby *et al.*, 2005) confirms that PCR can give false positive and negative reactions (van der Graaf *et al.*, 2013). In a recent study, this PCR showed a positive result with a *C. hyointestinalis* strain isolated from a bull (Spence *et al.*, 2011). This observation renders the Cfv-specific target gene *parA*, and consequently all other PCR assays using this target, unsuitable for diagnostic purposes.

The PCR described by Wang *et al.* (2002) reveals only a *C. fetus* subsp. *fetus*-specific product. These results were obtained only for a very limited number of strains. Evaluations of its value for subspecies differentiation using larger sets of strains yielded both false positive and negative reactions (Van Bergen *et al.*, 2005c; Van der Graaf *et al.*, 2013).

The random amplification of polymorphic DNA (RAPD)-PCRs were described by Tu *et al.* (2005) but apparently have been evaluated with a very limited number of *C. fetus* subsp. *venerealis* strains. In a recent study, the sensitivity of this assay was shown to be very low (Van der Graaf *et al.*, 2013).

## 2. Serological tests – antibody detection

An ELISA is available for the detection of antigen-specific secretory IgA antibodies in the vaginal mucus following abortion due to *C. fetus* subsp. *venerealis*. These antibodies are long lasting, and their concentration remains constant in the vaginal mucus for several months (Hum *et al.*, 1991). The ELISA does not differentiate an antibody response to the different subspecies.

Initial sampling can be done after the early involution period (usually 1 week after abortion) when mucus becomes clear.

An ELISA for the detection of the serum humoral IgG response after vaccination is described.

### 2.1. Antigen preparation and coating

Cultures are transferred to PBS with 0.5% formalin for 1 hour, centrifuged at 17,000 *g*, washed twice with PBS, and then resuspended in 0.05 M carbonate buffer, pH 9.6. The final absorbance is adjusted to OD<sub>610 nm</sub> = 0.21. Flat-bottomed polystyrene microtitre plates coated with 10 µl of antigen are left overnight at 4°C, and then stored at –20°C. Before use, the plates are rinsed twice with distilled water and then tapped gently to remove moisture.

### 2.1.1. Test procedure

- i) Diluted vaginal mucus (100 µl) is added to each well, and the plate is incubated at 37°C for 2 hours. The plates are then washed as before, and 100 µl of rabbit anti-bovine IgA is added. After 2 hours incubation at 37°C, the plates are washed and 100 µl of goat anti-rabbit IgG conjugated to horseradish peroxidase is added to each well. After a further 2 hours incubation at 37°C, the plates are washed, and 100 µl of substrate is added (0.8 mg/µl 5 amino-salicylic acid; pH 6.0), immediately activated by the addition of 2% 1 M hydrogen peroxide). The plates are left at room temperature for 30 minutes and the reaction is stopped by the addition of 50 µl of 3 M sodium hydroxide. The absorbance is measured on an ELISA reader at 450 nm. Each sample is tested in duplicate, and positive and negative controls are included in each plate. The absorbance measurements yielded by the test sample are corrected for the absorbance measurement of positive and negative controls according to the formula:

$$\text{Result} = \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{negative control}}}{\text{Absorbance}_{\text{positive control}} - \text{Absorbance}_{\text{negative control}}} \times 100$$

- ii) The test is considered to be positive if the result is above 40. Vaccinated animals will not react to IgA ELISA as their vaginal mucus contains only IgG isotype antibodies.

## C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

**THIS SECTION WAS ADOPTED IN 2008, AND IS CURRENTLY BEING CONSIDERED FOR REVISION**

In some countries commercial vaccines are available for sheep and or cattle. Two groupings of antigens of *C. fetus* are recognised: the thermolabile 'H' flagellar antigens and the thermostable 'O' somatic antigens. In addition, a capsular 'K' antigen should be present. The K antigen is easily destroyed under *in vitro* conditions. The vaccine must incorporate these different antigens. Other vaccine preparations have also been described (Clarke *et al.*, 1972). Experimental *C. fetus* subsp. *fetus* vaccine confers immunity against *C. fetus* subsp. *venerealis* because both strains share common antigens (Bouters *et al.*, 1973), however, the addition of a second strain of *C. fetus* subsp. *venerealis* to the biological product is widely practised and strongly suggested. The presence of four to five heat-labile glycoprotein immunogens, shared by many *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* strains, is critical. The presence of such immunogens should be confirmed. The vaccine concentration (dry weight) should be around 40 mg protein per dose in order to have a good protection level.

In infected herds, all breeding animals (bulls, cows and heifers) will be vaccinated twice prior to the breeding season. In most of the cases, the vaccine reduces the length of the infection and carrier-cows can keep the infection from one season to the next. Bulls require two vaccine doses annually, because the vaccine may not always be effective in terminating established infections. The next year's bulls and replacement heifers are vaccinated, and from the third year, bulls are vaccinated annually.

In non-infected herds, only the bulls are vaccinated annually, and this will be done twice a year (two doses with 21 days interval; 2 weeks before the start of the breeding season).

### 1. Seed management

#### 1.1. Characteristics of the seed

The seed consists of a large, homogeneous batch of a culture of *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* that has been thoroughly characterised as to identity and purity, preserved in small aliquots.

#### 1.2. Method of culture

The initial growth of the seed is accomplished in semisolid medium. This consists of basal medium with the addition of 0.16% agar. Basal medium is composed of 2.8% *Brucella* broth, 0.5% yeast extract, 1.2% sodium succinate, and 0.001% calcium chloride. The initial culture is maintained for 3 days at

37°C under specified conditions (see Section B.1.4.2). The growth is transferred to additional tubes with semisolid medium and incubated for 48 hours. The resulting growth is used for vaccine production.

This culture should be stored at 4°C.

### 1.3. Validation as a vaccine

The seed must be free from contaminating organisms. The purity of the seed must be checked by a suitable culture method.

It is not practicable to test efficacy under laboratory conditions. It is determined in the field on the basis of epidemiological observations.

## 2. Method of manufacture

The working seed material is seeded into broth medium consisting of basal medium with the addition of 0.025% sodium thioglycollate. These cultures are incubated at 37°C for 24 hours while being shaken at a rate of 80 rpm. The fluids are harvested, and formaldehyde is added to a final concentration of 0.2% (0.74 g/litre).

The vaccine is mixed with an oil-emulsion adjuvant.

## 3. In-process control

The identity of the organism should be checked by culture and identification, as well as the absence of contaminating organisms.

## 4. Batch control

### 4.1. Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

### 4.2. Safety

The inactivation process must be complete and the method to insure inactivation should be validated before it can safely be used. Inactivation is checked by inoculating the equivalent of one dose on to the same medium under the same conditions as those used in the production process. This culture is incubated under the same conditions for 72 hours, after which there should be no evidence of bacterial growth. The final product must also be shown to be free from viable bacterial and fungal contaminants, using suitable culture methods.

Two guinea-pigs are inoculated with 2 ml of the product, either intramuscularly or subcutaneously. They must not have an adverse reaction attributable to the vaccine during a 7-day observation period following inoculation.

### 4.3. Potency

Potency of the vaccine may be measured by seroconversion in rabbits. Their serum titres are measured by immunofluorescence or by the tube agglutination test. Five rabbits, serologically negative at 1/100 serum dilution, are vaccinated twice subcutaneously with half the dose used in cattle, at an interval of 14 days. Serum from at least four of the five rabbits, collected 14 days after the second vaccination, must show at least a four-fold increase in titre.

## 5. Tests on the final product

### 5.1. Safety

See Section C.4.2.

## 5.2. Potency

See Section C.4.3.

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**NB:** There is an OIE Reference Laboratory for bovine genital campylobacteriosis (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: <http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/> ).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine genital campylobacteriosis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017