CHAPTER 2.4.5.

BOVINE GENITAL CAMPYLOBACTERIOSIS

SUMMARY

***Definition 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*. The species is divided into two closely related subspecies:* C. fetus *subsp.* venerealis *and* C. fetus *subsp.* fetus*. By definition* C. fetus *subsp.* venerealis *is associated with BGC, causing fertility problems with considerable economic losses, particularly in endemic regions. Bovine infections with* C. fetus *subsp.* fetus *are associated with abortion and have a more sporadic occurrence.*

***Description of the disease**: BGC is a venereal disease that is characterised by infertility, early embryonic death, and abortion. The disease is caused by* C. fetus *subsp.* venerealis*, a bacterium with pronounced tropism for the genital system of cattle. Transmission of the causal agent takes place mainly during natural mating, and the presence of* C. fetus *subsp.* venerealis *in the semen of bulls creates the risk of spread of the disease through artificial insemination.*

***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, seagull-shapes and spirals, and can be cultured at 37°C for at least 3 days in a microaerobic atmosphere. Confirmation of the isolate and discrimination between the subspecies of* C. fetus *can be performed by biochemical or molecular methods. Immunofluorescence may also be used to identify the organism, but it will not differentiate between different subspecies.*

***Serological tests:** Enzyme-linked immunosorbent* (*ELISA*) *can be used for testing herd immunity, but is not suitable for diagnosis of the infection in individual animals. This test can not differentiate between infections caused by the two subspecies.*

***Requirements for vaccines and diagnostic biologicals:** A vaccine may be prepared from* C. fetus *subsp.* venerealis *and/or* C. fetus *subsp.* fetus *that shares antigens with* C. fetus *subsp.* venerealis*. This vaccine is inactivated with formalin, and may be administered in an oil-emulsion adjuvant.*

A. INTRODUCTION

1. 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*. It can be isolated from the genital tract of cattle (e.g. preputial smegma, vaginal mucus) or internal organs of aborted fetuses.

Campylobacter fetus is divided into the two closely related subspecies: *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* (28). An intermediate biovar of *C. fetus* subsp. *venerealis* has been described. Whether this variant has specific clinical features is unclear. By definition *C. fetus* subsp. *venerealis* is associated with BGC, causing fertility problems with considerable economic losses particularly in endemic regions. *Campylobacter fetus* subsp. *fetus* can be recovered from the intestinal tract of cattle and other animal species (6). *Campylobacter fetus* subsp. *fetus* can be isolated from aborted bovine fetuses showing its clinical relevance in cattle. However, *C. fetus* subsp. *fetus* is associated with sporadic cases of abortion in bovine whereas *C. fetus* subsp. *venerealis* is associated with endemic abortion and fertility problems in certain areas.

Although *C. fetus* is primarily recognised as a veterinary pathogen, *C. fetus* subsp. *fetus* is occasionally diagnosed as an opportunistic emerging pathogen in humans. Infections usually occur in pregnant or immuno-compromised individuals and are often systemic with a variety of neurological and vascular complications (21).

2. Taxonomy

In 1991 a revision of the taxonomy and nomenclature of the genus *Campylobacter* was proposed. According to the Bergey's Manual, the genus *Campylobacter* comprises sixteen species and six subspecies. More recently, two additional species have been proposed. Two subspecies of *C. fetus* have been recognised. Although the clinical signs of two subspecies overlap, they were originally defined by the differences in clinical presentation (19, 28). The two subspecies can be differentiated in the laboratory by one biochemical trait: glycine tolerance. Subspecies *venerealis* is considered as glycine sensitive and subspecies *fetus* as glycine tolerant. *Campylobacter fetus* subsp. *venerealis* biovar *intermedius* strains have been described (18), yet their taxonomic position needs to be clarified. On the basis of protein-banding patterns using polyacrylamide gel electrophoresis (PAGE) of whole cell proteins, no discrimination can be made between the two *C. fetus* subspecies (27). Studies of DNA–DNA hybridisation have failed to reveal any major difference between the *venerealis* and *fetus* subspecies (10). However, several molecular methods have been shown to be able to differentiate the two subspecies, including polymerase chain reaction (PCR) (12, 22, 25, 30), PFGE (pulsed-field gel electrophoresis) (17), multilocus sequence typing (MLST) (23) and amplified fragment length polymorphism (AFLP) (29) (see also Section B.1.h).

B. DIAGNOSTIC TECHNIQUES

1. Isolation and identification of the agent (the prescribed test for international trade)

a) Collection of samples

i) *Male: preputial smegma and semen*

In bulls, smegma may be obtained by different methods: scraping (20), aspiration (3), and washing (4). Smegma is commonly collected by scraping and can be used for isolation of the bacteria, or is rinsed into a tube with approximately 5 ml of phosphate buffered saline (PBS) with 1% of formalin for immunofluorescence (IFAT) diagnosis. 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 sown directly onto culture medium or transport and enrichment medium.

ii) *Female:* (*cervico*) *vaginal mucus* (*CVM*)

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

For aspiration, the vulva region is cleaned with a tissue paper, 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 (3). Gentle suctioning is applied while moving the pipette gently backwards and forwards. The pipette is removed, and the collected mucus is sown directly onto culture medium or transport and enrichment medium.

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 onto culture medium or transport and enrichment medium.

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

iii) *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 inoculated directly in transport and enrichment medium, or into PBS with 1% formalin for IFA testing.

b) Transport of samples

The use of a transport medium is essential if the samples are not processed in the laboratory within the same day after collection. For dispatch to the laboratory, if the samples are not in transport medium, the samples must be placed in an insulated container (within the temperature range 4–10°C), and protected from light.

Various transport and enrichment media are available, such as Clark's, Lander's, SBL, Foley's and Clark's, Weybridge's, Cary-Blair's (7, 11, 15).

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

c) Treatment of samples

On arrival at the laboratory, samples should be inoculated directly onto culture medium, or processed further if required.

i) *Genital tract samples*

Preputial washings may be centrifuged (3500 ***g***) to concentrate the sample. The final sample (reduced to 250 µl) may be inoculated onto the culture medium (directly and/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 cysteine solution (aqueous solution of cysteine hydrochloride at 0.25 g/100 ml, pH 7.2, sterilised by membrane filtration). After 15–20 minutes, the diluted and liquefied mucus can then be inoculated onto isolation medium.

ii) *Aborted fetuses, placentas*

Fetal stomach contents are inoculated directly onto 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.

d) Isolation of *Campylobacter fetus*

i) *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* due to antimicrobials (e.g. cephalosporins) that may inhibit C. *fetus* growth (24). 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.

ii) *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 (26). 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.

e) Identification of *Campylobacter* species

i) *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.

II) *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.

iii) *Biochemical tests:* see Table 1.

iv) *Atmosphere: Campylobacter* does not grow under aerobic conditions.

f) 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 can not differentiate between different subspecies.

i) *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. Heterologous sera are pooled. In a recent study, a conjugate prepared from chicken IgY was described as an alternative to rabbit antibodies. Monoclonal antibodies that can be used for immunodiagnostic detection of *C. fetus* have been described (2).

ii) *Preparation of conjugates*

Conjugates are prepared as described by Harlow *et al*. (9). 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.

iii) *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.

iv) *Immunofluorescence test* (14)

The sample (20 µl) is applied in duplicate to microscopic 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. Subsequently, the slides are 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 an epifluorescent 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 are used as negative control. Samples that show fluorescent bacteria presenting the typical morphology of *C. fetus* is considered positive.

g) Biochemical identification of *Campylobacter fetus* subspecies

Tests described in Table 1 must be done on pure cultures.

***Table 1**. Differential characteristics of* Campylobacter *species potentially isolated from the bovine genital tract and aborted fetuses (according to Bergey's Manual 2nd edition, 2005)*

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

(a) = Although *C. fetus* does not belong to the thermophilic *Campylobacter*s, a considerable number of strains of this species grows at 42°C; (b) = On triple sugar iron agar medium; (c) *C.jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is negative; (d) *C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is variable; (e) according to Bergey's Manual strains are sensitive, however resistant strains have frequently been reported; (+) = 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.d ii); V = variable results; S = sensitive; R = resistant.

i) *Growth at 25°C and 42°C*

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

ii) *Oxidase and catalase*

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

iii) *Growth in the presence of sodium chloride*

A cell-suspension is inoculated onto 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.d.ii). Control strains are tested in parallel.

iv) *Growth in the presence of 1% glycine*

A cell-suspension (~McFarland no. 1) is inoculated onto a glycine medium (15 ml of blood-based medium + 1.65 ml of 10% aqueous solution of filter sterilised glycine), and onto the same medium without glycine. Incubation is performed under the specified atmospheric conditions (see Section B.1.d.ii). Two control strains (of subspecies *venerealis* and *fetus*) 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 (18).

v) *Hydrogen sulphide (H_2S) production in TSI medium*

This hydrogen sulphide (H_2S) test is done on triple sugar iron agar (TSI) under the specified growth conditions (see Section B.1.d.ii). The medium contains peptone (20 g/litre), meat extract (2.5 g/litre), yeast extract (3 g/litre), sodium chloride (5 g/litre), ferric citrate (0.5 g/litre), sodium thiosulphate ($Na_2S_2O_3$) (0.5 g/litre), lactose (10 g/litre), sucrose (10 g/litre), glucose (1 g/litre), phenol red (0.024 g/litre), agar (11 g/litre), and distilled water (to 1 litre). This medium is sterilised after distribution into tubes by autoclaving at 115°C for 15 minutes and are solidified to obtain a slope. A cell-suspension (~McFarland no. 1) is inoculated onto the slope and into the medium by a loop. A colour change from red to black indicates H_2S production. Control strains are tested in parallel.

vi) *Hydrogen sulphide production (H_2S) in cysteine medium* (not listed in the Table 1)

The H_2S test is done in a *Brucella* broth medium containing 0.02% cysteine. H_2S production is detected by a lead-acetate strip that is attached inside the top of the tube. A cell suspension (~McFarland no. 1) is inoculated into the medium. Blackening of the lead acetate strip is considered as a positive reaction. Control strains are tested in parallel.

vii) *Sensitivity to cephalothin and nalidixic acid*

Sensitivity to cephalothin (CN) and nalidixic acid (NA) is tested by the disks containing CN (30 µg) or NA (30 µg).

For the test, 72-hour cultures are suspended in PBS at a concentration of 10^9 bacteria/ml. The culture medium is dried before the culture is deposited on the surface. Using the suspension, 100 µl are spread onto the basic blood medium. The sensitivity disks are then placed on top. These plates are incubated at 37°C in the specified atmosphere (see Section B.1.d.ii), and examined after 48 hours and 72 hours. A zone of inhibition of at least 3 mm around a disk indicates that the strain is sensitive to this antibiotic. All *C. fetus* subsp. *fetus* strains and most of the *C. fetus* subsp. *venerealis* strains are resistant to NA (16). All *C. fetus* are sensitive to CN (16).

h) Molecular identification of *Campylobacter fetus* subspecies

Several molecular methods for the identification of *C. fetus* subspecies have been described, including 16S sequencing (8, 17), PFGE (17), AFLP (29), and MLST (23). However, most of these methods are time consuming and/or require expensive apparatus and knowledge. Routine diagnostic laboratories would be served best by a simple PCR. Several PCRs have been claimed to be subspecies specific including those developed by Hum *et al*. (12), Wang *et al*. (30), and more recently by Tu *et al*. (22) and Van Bergen *et al*. (25).

The multiplex PCR described by Hum *et al*. (12) is currently the most cited PCR. It 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. Thus, performance of this multiplex PCR allows differentiation of the two subspecies (*C. fetus* = one amplification product vs *C. fetus* subsp. *venerealis* = two amplification products). *Campylobacter fetus* subsp. *venerealis* biovar intermedius strains have not been evaluated in Hum's study, but isolates identified as belonging to biovar intermedius with AFLP, classify in the PCR of Hum as either *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* (23). Comparison of this PCR against AFLP and MLST (23) and against the glycine test (31) confirms that PCR can give false positive and negative reactions.

The PCR described by Wang *et al*. (30) reveals only a *C. fetus* subsp. *fetus*-specific product. These results were obtained only for a very limited number of strains. Recent evaluations of its value for subspecies differentiation using larger sets of strains yielded both false positive and negative reactions (25).

The random amplification of polymorphic DNA (RAPD)-PCRs described by Tu *et al*. (22) are published only recently, and are apparently evaluated with a very limited number of *C. fetus* subsp. *venerealis* strains. Their value should be evaluated more extensively with a larger group of strains.

The recently described PCR by Van Bergen *et al*. (25) showed full consistency with the *C. fetus* subsp. *venerealis* as defined by AFLP and is therefore considered as the best PCR for detection method of *C. fetus* subsp. *venerealis* currently available. However, *C. fetus* subsp. *venerealis* biovar intermedius as defined by AFLP is not identified by this PCR.

2. Serological tests/antibody detection

An ELISA is available to detect 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 (13).

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.

a) 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 $OD_{610\,nm}$ = 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.

- **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:

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

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

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 (5). Experimental *C. fetus* subsp. *fetus* vaccine confers immunity against *C. fetus* subsp. *venerealis* because both strains share common antigens (1), 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

a) 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.

b) 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.d.ii). 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.

c) 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

a) Sterility

Tests for sterility and freedom from contamination of biological material may be found in Chapter 1.1.9.

b) 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.

c) 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

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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NB: There is an OIE Reference Laboratory for bovine genital campylobacteriosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).