AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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Bovine Campylobacteriosis

Bacteriology and Antibody Detection

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Contents

1. Introduction 3
2. Taxonomy 3
3. Diagnostic techniques 3
   3.1. Identification of the agent 3
   3.2. Antibody detection 5
4. Prevention and control 6
   4.1. Infected herds 6
   4.2. Non-infected herds 6
5. Acknowledgments 6
6. Addendum 6
7. References 6
8. Appendixes 6
   8.1. Appendix 1 — Cleaning and sterilisation of equipment 6
   8.2. Appendix 2 — Clarke's transport enrichment medium (Australian TEM) 7
   8.3. Appendix 3 — Lander's transport enrichment medium (Weybridge TEM) 7
   8.4. Appendix 4 — Clarke's selective agar 7
   8.5. Appendix 5 — Modified Skirrow's agar 7
   8.6. Appendix 6 — Modified Ziehl–Neelsen stain 7
   8.7. Appendix 7 — Immunoglobulins 7
   8.8. Appendix 8 — Vaccines 8

Bovine Campylobacteriosis

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Revised by B. L. Clark (1985) and reprinted by CSIRO for the Australian Agricultural Council (1987) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 22, *Bovine Campylobacteriosis*.

1. Introduction

Bovine venereal campylobacteriosis (BVC) is 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.

Infected bulls show no clinical signs but become carriers and infect females at service. The other subspecies, *Campylobacter fetus* subsp. *fetus* is known to cause sporadic abortion in cattle, but is not usually associated with infertility. This subspecies has also been isolated from the intestinal and reproductive tracts of healthy animals.

2. Taxonomy

There has been much confusion over the epidemiology of BVC because of the previous taxonomic difficulties and nomenclature changes (Table 1).

Because of a minor antigenic and biochemical difference *C. fetus* subsp. *venerealis* is divided into two biotypes, namely *C. fetus* subsp. *venerealis* biotype venerealis and *C. fetus* subsp. *venerealis* biotype intermedius.

Recent studies indicate that strains of *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* can not be distinguished from one another by DNA homology experiments. Despite this finding the maintenance of subspecies designation for *C. fetus* is desirable because of the epidemiologic differences between the two subspecies.

Infertility and sporadic abortion caused by *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* have been reported. However, due to its genital tropism *C. fetus* subsp. *venerealis* is far more important. Similarly, preventive measures can only be taken to control BVC caused by this subspecies.

All isolates of *Campylobacter fetus* subsp. *venerealis* belong to serotype A. Isolates of *Campylobacter fetus* subsp. *fetus* can be serotype A or serotype B.

Table 1. Nomenclatures used for *Campylobacter fetus*

Author	*Nomenclature*	
Original description Smith and Taylor (1919)	*Vibrio fetus*	
Florent (1959)	*Vibrio fetus* var *intestinalis* (glycine tolerant)	*Vibrio fetus* var *venerealis* (glycine intolerant)
Veron and Chatelain (1973)	*Campylobacter fetus* subsp. *fetus*	*C. fetus* subsp. *venerealis*
Smibert (1974)	*C. fetus* subsp. *intestinalis*	*C. fetus* subsp. *fetus*
Skerman *et al.*	*C. fetus*	*C. fetus*

3. Diagnostic Techniques

3.1. Identification of the Agent

The only unequivocal means to diagnose BVC is to isolate the organism from infected bulls, cows and heifers or aborted foetuses.

The bull is the animal of choice when attempting to isolate *C. fetus* subsp. *venerealis*.

3.1.1. Collection of Specimens for Laboratory Diagnosis

3.1.1.1. Bulls

The organism inhabits the mucosa of the glans penis, prepuce and the distal portion of the urethra. Therefore, preputial smegma, preputial washings and semen samples are all suitable for culture.

3.1.1.1.1. Collection of preputial smegma.

(a) *Equipment needed.*

(i) Plastic pipettes — Made from perspex, poly-styrene or polypropylene (10 mm external and 6 mm internal diameter), 60 cm in length, straight except for a bend at the end that is held by the operator during collection. The other end that contacts preputial surfaces is bevelled.

(ii) Rubber bulbs — Firm rubber bulbs to put on pipettes, capacity 85 mL.

(iii) Sterile screw-capped universal containers — 28 mL or 18 mL capacity each containing 4 mL of phosphate buffered saline pH 7.2 (PBS).

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 smegma with 4 mL of PBS into the universal container and stand to allow impurities to settle.

This technique is preferred when investigations are extended to include possible *Tritrichomonas foetus* infection.

3.1.1.1.2. Collection of preputial washing.

(a) *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.

Introduce the pipette to the full length of the preputial cavity and hold the preputial orifice firmly with one hand around the pipette to prevent PBS from escaping.

Inject 20 mL of PBS into the prepuce and wash thoroughly with one hand 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 to the universal container and stand to allow impurities to settle.

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

3.1.1.1.3. Collection of semen. Semen is collected by one of the standard techniques, using either artificial vagina, electro-ejaculator or massage of the accessory sex glands.

Culture of semen samples are not as reliable as culture of samples collected from the prepuce.

3.1.1.2. Cows and heifers

Since persistent infection exist in some female cattle this group of animals can be sampled when bulls are no longer available for sampling. A representative number (10%) of infertile animals should be sampled.

3.1.1.2.1. Collection of vaginal mucus by swab.

(a) *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.

3.1.1.2.2. Collection of vaginal mucus by lavage.

(a) *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.

(ii) 20 mL disposable syringe, 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.

3.1.1.3. Aborted foetus and placenta

Foetal stomach contents are 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 only is available for examination an additional sample for histology is collected. Since *C. fetus* subsp. *fetus* can be present in the faeces of healthy animals, histological examination may be useful in determining the significance if *C. fetus* subsp. *fetus* is isolated.

3.1.2. Forwarding Samples to the Laboratory

If specimens can be delivered to a laboratory within six hours of collection, the only precautions that need to be observed are avoidance of sunlight and of temperatures below 4°C or above 30°C during transport.

If longer delays are involved, the use of selective transport enrichment media (TEM) is necessary for the submission of foetal stomach contents, preputial and vaginal mucus samples. The organism may remain viable for one or two days in chilled foetal tissues and in the placenta.

3.1.2.1. Inoculation of transport enrichment media

3.1.2.1.1. Clark's — Australian TEM (see 8.2.) Using a sterile disposable syringe andneedle, withdraw 1 mL from the top of the sample and inject this through the lid of the bottle. Shake the bottle well to mix the material with the medium. During inoculation, avoid injecting air into the bottle and do not unscrew or remove the lid.

3.1.2.1.2. Lander's – Weybridge TEM (see 8.3.) Using a sterile disposable syringe and needle, withdraw 1 mL from the top of the sample and inoculate the medium.

The inoculated containers should be clearly marked. Information regarding the nature of the sample, animal identification 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. It is important to avoid freezing and refrigeration of inoculated containers or exposure to temperatures above 37°C.

When Lander's and Clark's TEM were compared in terms of ability to isolate *C. fetus* from field samples, we found that Lander's TEM was superior to Clark's TEM but was not as successful as direct culture using either Clark's Selective Agar (CSA) or Modified Skirrow's Agar (MSA).

3.1.3. Laboratory Procedures

3.1.3.1. Samples Received in Transport Enrichment Medium

Clark's TEM is incubated for four days at 37°C. The lid is then removed and the liquid portion of the medium is plated directly onto selective agar (see 8.4 for details of composition). The selective agar plate is then incubated in a microaerophilic atmosphere (5% oxygen, 5% carbon dioxide and 90% nitrogen) at 37°C and examined at three and seven days. Lander's TEM is incubated for three days in a microaerophilic atmosphere at 37°C. An aliquot of the medium (about 0.1 mL) is then plated onto Modified Skirrow's Agar (see 8.5.), and the plate is incubated in a microaerophilic atmosphere at 37°C and examined at three and seven days.

3.1.3.2. Fresh Samples

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

Foetal tissues may be sampled aseptically and inoculated onto blood agar and selective agar plates, and a pool of the tissues inoculated into TEM. Foetal stomach contents should be examined by dark ground or phase contrast microscopy and smears made and stained using Gram's stain and the modified Ziehl-Neelsen method (see 8.6.).

The TEM cultures are processed as in 3.1.3.1.

3.1.3.3. Identification procedures

Suspect Campylobacter colonies are 1–3 mm in diameter and are translucent or grey to buff coloured. The organism is an oxidase positive Gram-negative spirally curved rod. In older cultures there may be a large number of coccoid forms.

Differentiation from other *Campylobacter* spp. may be achieved by standard biotyping methods (Lander and Gill, 1985). A suitable growth medium for biochemical tests is brucella broth with (Oxoid) 0.16% agar.

3.2. Antibody Detection

An enzyme linked immunosorbent assay (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.

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

A project is in progress to extend the application of the IgA ELISA for herd infertility investigation.

3.2.1. Collection of Vaginal Mucus

3.2.1.1. Equipment needed

(a) 4.5 mL sterile PBS containing 0.05% Tween 20 (PBST).

(b) Sterile swab (15 cm or longer).

Sampling: as per 3.1.1.1. After sampling, the cotton head of the swab should be cut and placed in the PBST.

3.2.2. Forwarding Samples to the Laboratory

The samples are kept chilled prior to and during transport to the laboratory. Time is not critical but samples should reach the laboratory within two or three days.

3.2.3. Laboratory Procedures

3.2.3.1. Preparation of vaginal mucus

The samples are agitated for one hour, centrifuged at 1500 *g* relative centrifugal force (RCF) and stored at 4°C until examined. The approximate dilution factor of mucus in PBS is 1:45.

3.2.3.2. Antigen preparation and coating

Campylobacter fetus subsp. *venerealis* is grown on Columbia blood agar (Oxoid) in microaerophilic conditions at 37°C for three days. The plates are checked for purity, and colonies are transferred to 0.5% formol saline for one hour, centrifuged at 17000 *g* RCF, washed twice with PBS (pH 7.5) and then resuspended in carbonate buffer (0.05 mol/L pH 9.6). The final optical density (OD) is adjusted to 0.21 at 610 nm. 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, filled with PBST, left for five minutes, rinsed twice with distilled water and then tapped gently to remove moisture.

3.2.3.3. Enzyme-linked immunosorbent assay

Diluted vaginal mucus (100 µL) is added to each well and incubated at 37°C for two hours. The plates are then washed as before and 100 µL of rabbit antibovine IgA (see 8.7.) added. After two hours incubation the plates are washed and 100 µL of goat antirabbit IgG conjugated to horseradish peroxidase (see 8.7.) is added to each well. After a further two hours incubation the plates are washed and 100 µL of substrate is added [(5 amino-salicylic acid 0.8 mg/µL pH 6.0, immediately activated by 2% of 1 mol/L hydrogen peroxide (H_2O_2)]. The plates are left at room temperature for 30 min and the reaction is then stopped by 50 µL of 3 mol/L sodium hydroxide (NaOH). The OD is measured on a Titertec Multiscan reader at 450 nm and expressed as ELISA values (EV).

Each sample is tested in duplicate and 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:

EV = [(OD of sample − OD of negative control)/ (OD of positive control − OD of negative control)] x 100

3.2.3.4. Interpretation of results

The test is considered to be positive if the EV is above 40.

Vaccinated animals will not react to IgA ELISA, their vaginal mucus contains only IgG isotype antibodies.

4. Prevention and Control

BVC can be controlled by vaccination. Bivalent vaccines (see 8.8.) containing *C. fetus* subsp. *venerealis* biotype venerealis and biotype intermedius, injected twice four weeks apart have a preventive and may have a curative effect.

Recommendations on vaccine usage are different in infected and non-infected herds.

4.1. Infected Herds

All breeding animals including bulls, cows, and heifers are vaccinated in the herd following the diagnosis of BVC. Additional antibiotic treatment of an infected bull at the time of the second vaccination is recommended because vaccine may not be effective in all cases to terminate established infection. The treatment consists of a parenteral injection (25 mg/kg) and a preputial infusion (5 g) of aqueous Streptomycin sulfate solution.

The next year bulls and replacement heifers are vaccinated and from the third year bulls are vaccinated annually.

4.2. Non-infected Herds

Bulls are vaccinated annually.

5. Acknowledgments

We would like to thank C. Quinn for her help in developing the IgA ELISA, J. Brunner for comparing TEMs and direct isolation methods and C. Fokker for her skilful typing of the manuscript.

6. Addendum

The Regional Veterinary Laboratory, Armidale, is willing to receive difficult isolates and to provide second opinions.

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8. Appendixes

8.1. Appendix 1 — Cleaning and Sterilisation of Equipment

(Plastic and insemination pipettes, polythene tubing and rubber bulbs).

After use, thoroughly clean and rinse pipettes with warm water and immerse overnight in tap water containing 30 mL of sodium hypochlorite per litre (using household bleach which contains 1% free chlorine). Then rinse pipettes thoroughly with tap water to remove traces of chlorine and wrap in clean paper or store in suitable containers. Sterilise the bulbs by boiling for 10 min.

Table 2. Additives for Clark's Transport Enrichment Medium

Additive	*Final concentration/ mL*
5-Fluorouracil	300 µg
Polymixin B Sulfate	100 units
Nalidixic acid	3 µg
Cycloheximide	100 µg
Brilliant green	50 µg

8.2. Appendix 2 — Clark's Transport Enrichment Medium (Australian TEM)

(a) To sterile ox serum add the following (Table 2).
(b) Mix well.
(c) Aseptically remove a small aliquot of the medium and measure the pH. This should be in the range 7.4–7.6. If necessary, adjust the pH of the medium using a suitable volume of 1mol/L hydrochloric acid (HCl) or sodium hydroxide (NaOH).
(d) Dispense 10 mL volumes of the medium into sterile bottles — suitable containers are 20 mL vaccine bottles, 25 mL bottles with screw caps and rubber injection septa, and 28 mL. Universal containers with a 1 mm hole in the lid.
(e) Heat the bottles in a boiling water bath until the medium has solidified.
(f) Break the solidified medium into small pieces with a sterile spatula or other suitable instrument. Leave the lids loose.
(g) Place the bottles in a McIntosh and Fildes jar and evacuate to about 50 cm mercury. Gas the jar with a mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen (see 3.1.3.1.)
(h) *Either* remove the bottles from the jar immediately, secure the lids and leave for one week at 4°C; *or* store the jar at 4°C for one week, then remove the bottles and secure the lids.
(i) The prepared media are stable at 4°C for at least four weeks and up to three months. Discard any media which are not deep green.

8.3. Appendix 3 — Lander's Transport Enrichment Medium (Weybridge TEM)

To 1 L of Mueller–Hinton Broth (Oxoid) add 5 g of bacteriological charcoal. The mixture is autoclaved at 121°C for 15 min and allowed to cool. The following are then added aseptically:

Lysed horse blood	70 mL
FBP supplement (e.g. Campylobacter Growth Supplement — Oxoid Ltd)	2 vials
Vancomycin stock solution (4 mg/mL)	10 mL
Polymixin B sulfate (1000 IU/mL)	10 mL
Cycloheximide (10 mg/mL)	10 mL
Trimethoprim (2 mg/mL)	10 mL
5-Fluorouracil (10 mg/mL)	50 mL

The medium is dispensed in 10 mL volumes in 28 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 three weeks.

8.4. Appendix 4— Clark's Selective Agar

8.4.1. Basal medium

Bacto peptone (Difco)	10 g
NaCl	5 g
Lab Lemco beef extract(Oxoid)	5 g
Agar	15 g
Distilled water	1 L

Add all ingredients except the agar, heat if necessary to dissolve; adjust the pH to 7.4–7.6; add the agar and heat to dissolve. Autoclave at 121°C for 15 min. Cool to 55°C. Add defibrinated sheep or ox blood to 10% and the following antibiotics (final concentrations/mL):

Bacitracin	15 IU
Polymixin B sulfate	1 IU
Sodium novobiocin	5 µg
Cycloheximide	10 µg

8.5. Appendix 5 — Modified Skirrow's Agar

Melt sterile blood agar base (Oxoid), cool to 55°C and add the following aseptically (final concentrations):

Lysed horse blood	7%
Vancomycin	20 µg/mL
Trimethoprim	10 µg/mL
Polymixin B sulfate	5 IU/mL
Cycloheximide	100 µg/mL

The vancomycin, trimethoprim and polymixin may be obtained as a commercially prepared supplement, e.g. Oxoid SR69 (Skirrow).

8.6. Appendix 6 — Modified Ziehl–Neelsen Stain

Stain for 10 min with 10% carbol fuchsin. Decolourise for 10 s with 1% acetic acid (CH_3CO_2H).

Counterstain for 30 s with 1% malachite green or methylene blue.

8.7. Appendix 7 — Immunoglobulins

Rabbit antibovine IgA (Cat No. 64175)
Goat antirabbit IgG (Cat No. 61202)

8.7.1. Manufacturer

INC Immunobiologicals, PO Box 1200, Lisle IL 60532, USA.

8.7.2. Supplier

Miles Laboratories Aust. Wellington Road, Mulgrave, Vic. 3170, Australia.
Cat No. 64175.

8.7.3. Comment

The optimal concentration of the rabbit antibovine IgA and the enzyme conjugate is assessed by chequerboard titration of positive and negative control samples at various concentrations. We found the optimal concentrations to be 1:200 and 1:2000 for IgA and the enzyme conjugate, respectively.

8.8. Appendix 8 — Vaccines

8.8.1. Vibriosis (Campylobacter) vaccine

Manufacturer: Arthur Webster Pty Ltd, 226 Windsor Road, Northmead, NSW 2152, Australia.

8.8.2. Vibrovax Bivalent
Manufacturer: Commonwealth Serum Laboratories, 45 Poplar Road, Parkville, Vic. 3052, Australia.

8.9. Appendix 9 — Suppliers

Difco Laboratories. Detroit, Michigan, USA. Supplied by Helena Laboratories (Aust.) Pty Ltd, 2 Hardner Rd, Mt Waverley, Vic. 3150 Australia. Tel. (03) 543 7299, (008) 033137; Fax (03) 543 7542.

FSE Pty Ltd. 47–49 Overseas Drive, Noble Park, Vic. 3174, Australia. Tel. (03) 795 0077; Fax (03) 7901900.

Oxoid (Aust.) 1046 North Road, Heidelberg West, Vic. 3081. Tel. (03) 4581311, (008) 331163; Fax (03) 458 4759.