Chapter 8

CAMPYLOBACTERIOSIS

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8.1 SUMMARY

Campylobacteriosis is a highly contagious and economically significant disease in sheep, most often caused by *Campylobacter fetus subspecies fetus* and *C.jejuni*. It is characterised by abortion, stillbirths, premature births, weak lambs and occasional ewe deaths due to metritis. A similar disease is recognised in goats but herd outbreaks have only rarely been reported. *Campylobacter* organisms are widespread and many animals carry them in their intestines and excrete organisms in their faeces.

A presumptive diagnosis can be made by a history of widespread abortion and typical lesions of the foetus and placenta but this should be confirmed by isolation and identification of the organism. Microscopical demonstration of typical *Campylobacter* organisms in aborted materials by specific staining techniques can also be useful in diagnosis. Although serological tests are available for the detection of antibodies against *Campylobacter* these are not used in diagnosis of abortive campylobacteriosis as antibodies are frequently found in the sera of healthy animals with no history of *Campylobacter* infections.

8.2 INTRODUCTION

Vibrionic abortion in sheep was first described in 1913 and has since been reported in many countries. The causal organism was originally assigned to the genus *Vibrio*, hence the name vibriosis, but is now in the genus *Campylobacter*. Eleven species of *Campylobacter* are currently recognised but abortion in sheep and goats is caused by infection with only one or other of two catalase-positive campylobacters *Campylobacter fetus subspecies fetus* (subsequently referred to as *C. fetus*) or *C. jejuni* (see Figure 8.1). Campylobacters are Gram-negative, non-sporing, curved or spiral rods with characteristic "S" or "seagull" shape, 0.2-0.9 μm wide and 0.5-5.0 μm long. They are microaerophilic and grow best in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% hydrogen or nitrogen. *Campylobacter* organisms are motile by means of single polar flagellum at one or both ends and have a characteristic darting, corkscrew motion.

The clinical sign of an active infection in pregnant sheep is abortion during late pregnancy. In field out breaks many ewes have a mild diarrhoea before the first abortions occur. Lambs may be carried to full term but are born dead or in a weak condition. Usually there is no indication of the impending abortion, but a few ewes may show a prior vaginal discharge. Recovery of the ewe is prompt and fer-tility in subsequent breeding seasons usually good. Occasionally, abortion is complicated by metritis and subsequent death of the ewe. *Campylobacter* abortion occurs in all breeds of sheep and all ages of ewes. Within flocks the abortion rate varies from 5 to 50% although rates as high as 80 and 90%

have been reported. Experimental evidence suggests that abortions are commoner if infection is acquired beyond the twelfth week of pregnancy and that abortion occurs between 7 and 25 days after infection, usually during the last 8 weeks of pregnancy. Both *C. fetus* and *C. jejuni* have been shown to cause abortion in goats, the clinical symptoms being similar to those in sheep. However, reported cases of field outbreaks of *Campylobacter* abortion in goats are limited.

Campylobacteriosis in sheep is transmitted orally. It occurs in many closed flocks and the source of infection is not always clear. Sheep can carry *C.fetus* and *C. jejuni* in their intestines and excrete the organisms in the faeces. It is therefore likely that some outbreaks are due to the contact of pregnant sheep with a faecal excretor which may have been introduced into a flock. The appearance of the disease in closed flocks has been attributed to contamination of pastures by wildlife vectors, as sparrows, carrion crows and magpies have been found to be intestinal carriers. After the first abortion, infection is further spread as a result of contamination of pastures from the products of this abortion and by direct contact with foetal membranes and foetuses.

The disease is self-limiting in that it is unusual for abortions to occur in a self-contained flock the year after an outbreak of abortion. Ewes which are in contact with abortions but which do not themselves abort, and non-pregnant females, do not abort at subsequent pregnancies. This development of immunity to abortion can be used to ensure that an outbreak is confined to one lambing season. Ewes which abort

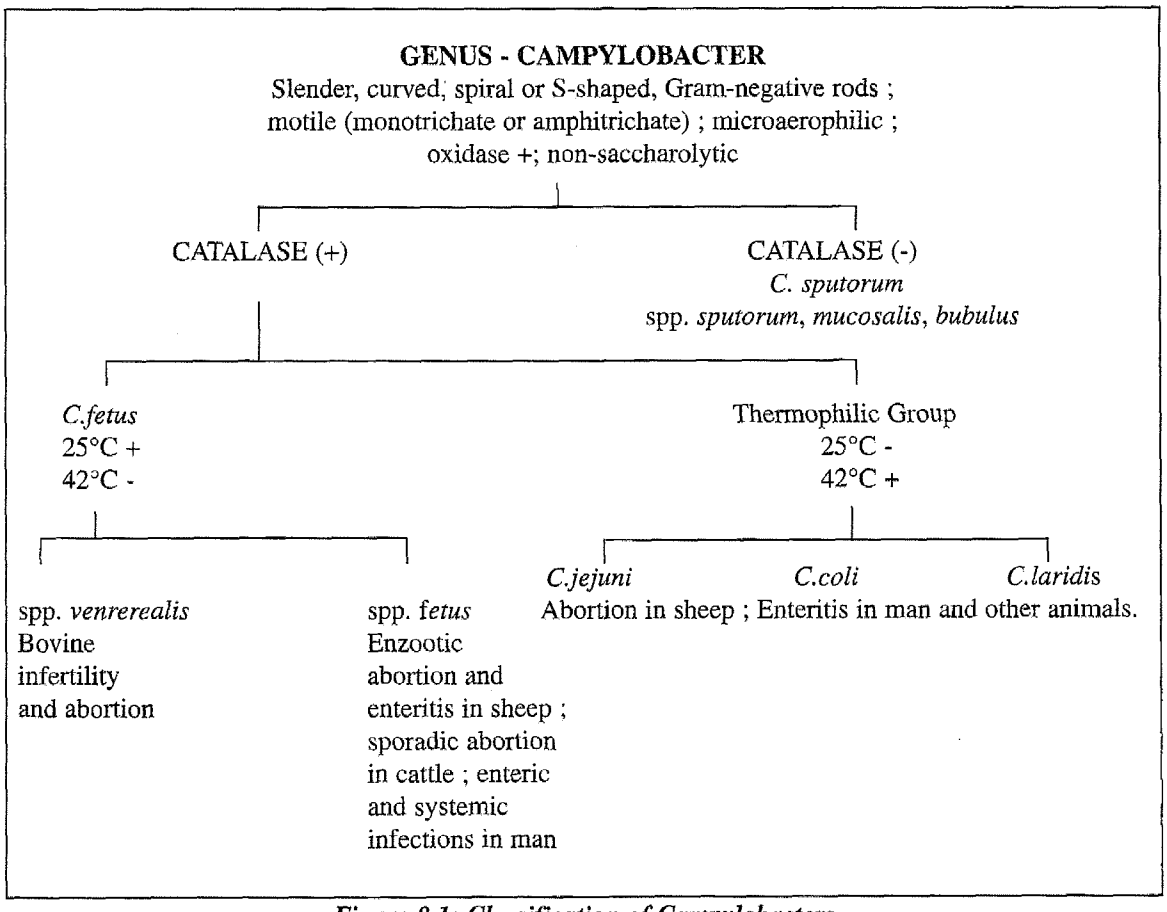

Figure 8.1: Classification of Campylobacters

should not be removed from the flock and potential additions to the breeding flock should be mixed with the infected flock. However, hygienic measures should be taken to prevent spread of infection from foetal membranes and aborted foetuses to adjacent flocks and to possible wild-life vectors. Although Campylobacters die rapidly on exposure to air, heat and desiccation, in moist conditions the organism can remain viable for several days, such as in vaginal discharge on pasture or bedding.

Treatment with penicillin and streptomycin and concurrent vaccination of in-contact ewes after the first abortion has prevented further abortions but there is no evidence that therapy alone is useful. Vaccination with disrupted, formalinized cells of *C. fetus* subspecies intestinalis in oil adjuvant has been shown to protect against experimental challenge. Also vaccination of ewes, after abortion in an experimentally-infected contact ewe, has been shown to reduce abortions. This suggests that vaccination in the face of an outbreak might be of value. The value of routine vaccination of commercial flock will depend on the prevalence of the disease. There are a number of commercial *Campylobacter* vaccines available for use in sheep in the USA but the efficacy of some of them is in doubt.

8.3 SAMPLES

Campylobacteriosis can only be confirmed by isolation and identification of the specific aetiological agent. Samples of choice are the aborted placenta, foetus and vaginal excretions. Samples should be fresh and submitted in a suitable transport medium such as FBP/glycerol and kept cool and moist during transportation to the laboratory. Stomach contents from the aborted foetus is also suitable for the isolation of *Campylobacter* because it can be sampled aseptically with a syringe.

Histopathological examination of aborted tissue samples is also advised for positive diagnosis. Small representative sections of tissue of no more than 0.5cm thickness should be taken as soon as possible after abortion or death and placed in a suitable fixative. Fixed tissues are best left for at least two days before further processing.

Preparation of reagents

- FBP/Glycerol Medium

Nutrient broth No. 2 (Oxoid, Basingstoke, UK).

0.12% w/v agar (Difco).

15% glycerol.

0.1% w/v yeast extract (Oxoid, Basingstoke, UK).

Double strength FBP enrichment supplement (Oxoid, Basingstoke, UK).

Dissolve the nutrient broth, agar and glycerol by gentle heat. Autoclave at 121°C for 15 minutes and allow to cool to 50-55°C in a water bath. Add the FBP enrichment supplement aseptically and mix gently. Dispense 4.0ml aliquots into sterile Bijou bottles.

8.4 RISKS TO HUMAN HEALTH

Campylobacters, particularly *C.jejuni*, have been recognised as important human pathogens which cause both acute bacterial gastroenteritis and extraintestinal infections. Individuals of all ages may be infected but infections are most frequent in young children and young adults. *Campylobacter jejuni* infections usually occur following ingestion of improperly handled or improperly cooked food, pri-

marily poultry products.

Due to the zoonotic potential of Campylobacters all materials from aborted ewes and does should be handled with the same precautions as any biological materials that are potentially dangerous to human health. Infection of pregnant women with *Campylobacter* can lead to abortion therefore pregnant women should not assist with lambing ewes and kidding goats nor be allowed to handle potentially infected materials in the laboratory.

8.5 DIRECT DIAGNOSIS

The placental and foetal lesions associated with *Campylobacter* abortion are specific and provide good evidence of infection by this organism. However, there is wide variation in the lesions and it is necessary to have bacteriological confirmation of the diagnosis.

8.5.1 Gross pathology of the placenta and foetus

The principal lesion in *Campylobacter* abortion is a placentitis. The placenta is oedematous and the cotyledons are pale and necrotic and separate easily from the caruncles.

In some aborted foetuses, most frequently those close to term, the liver is enlarged and haemorrhagic and shows typical gray, necrotic foci varying from 1-3 cm in diameter. Usually the foetus is oedematous and the body cavities contain a blood-stained serous fluid. There may also be a mild to moderate fibrinopurulent bronchopneumonia.

8.5.2 Isolation of *Campylobacter*

Principle

Campylobacter usually grow on most non-selective culture media especially when blood is added. A wide range of selective media have also been developed providing increased sensitivity and specificity of isolation mainly in response to the requirement to detect Campylobacters in human faeces. The majority of these media have been developed for isolation of *C. jejuni* and are rarely suitable for the isolation of *C. fetus*, therefore having limited application to veterinary samples. 5% blood agar is still a suitable media for culture of both *C. fetus* and *C. jejuni* from samples collected from aborted ewes in which *Campylobacter* numbers are high and contamination with other bacteria is minimal. However, a pre-culture membrane filtration stage is recommended to minimise any contaminants. Optimum atmospheric growth conditions (5% oxygen, 10% Carbon dioxide, 85% hydrogen or nitrogen) can be obtained by the use of commercially available gas-generating kits specifically for *Campylobacters* (Oxoid Ltd, Hampshire, UK) in conjunction with standard anaerobic jars, use of a Tri-Gas incubator or evacuation and replacement of an anaerobic incubator with the approximate gas mixture.

Materials and reagents

- 5% sheep or horse blood agar plates.
- 0.65 µ millipore membrane filters (Sartorius, Gottingen, Germany).
- Gas jars.
- Gas packs (Oxoid gas generating pack for Campylobacters, Basingstoke, UK).

Procedure

1. Centrifuge macerated tissue samples or foetal stomach contents at 100 xg for 10 minutes. Aseptically remove the supernatant and incubate at 37°C for 1 hour.

2. Place a 0.65 μ membrane filter on the surface of each isolating agar plate

3. Place between five and ten drops of the incubated supernatant fluid onto the center of each filter and incubate at 37°C for 1 hour.

4. Remove and discard the filters and spread filtrates over the agar surface. Allow plates to dry.

5. Incubate the plates in an atmosphere of 5% O_2, 10% CO_2, 85% H_2 or N_2 at 37°C for up to 72 hours.

3. Examine by a plate microscope for typical colonies of *Campylobacter* species after 48 and 72 hours incubation.

Reading and interpretation of results

Following culturing on blood containing solid media at 37°C, colonies will vary in appearance from small, round and convex ones in the case of the *C. fetus* group to fairly large mucoid colonies with *C. jejuni / C. coli*. Occasionally, *C. jejuni* and *C. coli* produce large and small colonies co-existing on the same plate, which when mature often become tanned. Strains of *C. jejuni* can develop a metallic sheen over the culture surface. Generally coccoid forms of *Campylobacters* are invariably due to long incubation and may be considered degenerative and non-viable. However, *C.jejuni* cultures frequently produce coccoid forms as early as 48 hours.

Haemolysis is not observed on blood agar.

Preparation of reagents

- Blood agar

Columbia blood agar base (Oxoid-Unipath or Difco).

5% sterile defibrinated sheep or horse blood.

Suspend blood base agar according to manufacturers instructions in distilled water and heat to boiling. Autoclave at 15 psi for 15 minutes. Bring to 45-50°C and aseptically add 5% sterile sheep or horse blood. Mix well and pour into sterile Petri dishes. Place in plate drier for a short period and store at 4°C wrapped or in sealed boxes.

8.5.3 Identification of *Campylobacter* species

Colonies that appear to be *Campylobacter* species on agar plates can be confirmed as such and catalase positive and negative strains differentiated using three simple tests :

1. Gram stain.
2. Oxidase reaction.
3. Catalase reaction.

C. fetus and *C.jejuni* can be distinguished from other *Campylobacter* species on the basis of further simple biochemical reactions:

1. Growth temperature studies.
2. Antibiotic sensitivity.
3. Production of hydrogen sulphide (H_2S).
4. Hippurate hydrolysis.

The expected result profiles for these tests for the various species of *Campylobacter* of interest are shown in Table 8.1.

8.5.3.1 Gram stain

Principle

This technique consists of showing, by specific staining, the presence of the bacteria on smears taken from the cotyledons and stomach contents of a freshly-aborted foetus. *Campylobacter* cells are often difficult to see on conventional Gram staining and it is recommended that strong (Zeihl-Neelsen's) carbol fuschin be used.

Materials and reagents

- Ammonium oxalate-crystal violet solution (mix 2ml of a 10% alcoholic

Table 8.1 : Biotyping of catalase positive Campylobacters

Test	*C.fetus* spp *fetus*	*C. fetus spp venerealis*	*C. jejuni* biotype 1	*C. jejuni* biotype 2	*C. coli*	*C. laridis*
Catalase	+	+	+	+	+	+
Relative size	Medium	Long	Short	Short	Short	
Swarming on moist media	-	-	+	+	±	
Rapid coccal formation	-	-	+	+	-	-
Growth at 25°C	+	+	-	-	-	-
Growth at 42°C	-	-	+	+	+	+
Naladixic acid sensitivity	R	R	S	S	S	R
Cephalothin sensitivity	S	S	R	R	R	R
H_2S production	-	-	-	+	-	+ (slow)
Hippurate hydrolysis	-	-	+	+	-	-

R : resistant ***S : sensitive***

solution of crystal violet, 18ml of distilled water and 80ml of a 1% aqueous solution of ammonium oxalate).

- Gram's iodine solution (dissolve 2g of potassium iodide in 100ml of distilled water and add 1g of iodine).
- 95% ethanol.
- Counterstain (15 parts 1% neutral red: 1 part carbol fuchsin).

Procedure

1. Remove colonies from a 48 or 72 hour culture and smear on a microscope slide. Fix by flame.
2. Stain with ammonium oxalate-crystal violet solution for 1 minute.
3. Rinse slide with water.
4. Stain with Gram's iodine solution for 1 minute.
5. Rinse slide with water.
6. Rapidly apply 95% ethanol until no further colour appears to flow from the sample.
7. Wash slide with water.
8. Apply counterstain for 30 seconds.
9. Dry slide and examine microscopically.

Reading and interpretation of results

Gram-positive organisms appear blue-black, Gram-negative organisms appear red.

Campylobacter cells are short Gram-negative rods and have a distinctive curved or spiral appearance.

Preparation of reagents

- Carbol Fuchsin

Basic fuchsin	40g
Phenol	80g
Absolute alcohol	200ml
Distilled water	1 litre

Dissolve the basic fuchsin in the alcohol and the phenol in the water. Mix the two solutions thoroughly and leave to stand at room temperature overnight. Filter through wet paper before use.

Caution : phenol is an extremely hazardous reagent and precautions should be taken during assay.

8.5.3.2 Oxidase test

Principle

Certain bacteria possess "oxidases" that are involved in electron transfer from electron donors. If the redox dye tetra-methyl-p-phenylene diamine is used as electron acceptor, this will be reduced, and the reduced dye has a deep blue colour.

Materials and reagents

- Platinum loop.
- 1% tetra-methyl-p-phenylene diamine hydrochloride (aqueous solution).

Procedure

1. Place some dye solution onto a filter paper.
2. Using a platinum loop remove a representative colony from a 48 or 72 hour culture and smear onto the still wet dye.

Reading and interpretation of results

A deep purple colour will form within 10 seconds with oxidase positive cultures.

8.5.3.3 Catalase test

Principle

This test demonstrates the presence of the enzyme catalase which catalyses the release of oxygen from hydrogen peroxide.

Materials and reagents

- 3% hydrogen peroxide.

Procedure

1. Remove a representative colony from a 48 or 72 hour culture and place on a microscope slide.
2. Place a drop of 3% hydrogen peroxide on the colony and mix gently.

Reading and interpretation of results

Bubbling indicates a positive catalase test.

Note

• Care should be taken when removing colonies from blood containing media to prevent removal of any media with the sample as this may result in a false positive catalase reaction.

8.5.3.4 Growth temperature and antibiotic sensitivity tests

Materials and reagents

- 5% sheep or horse blood agar plates.
- Gas jars.
- Gas packs (Oxoid gas generating pack for Campylobacters, Basingstoke, UK).
- Naladixic acid and cephalothin disks (30µg).

Procedure

1. Make a single streak of sample colonies removed from 72 hour cultures across the surface of three blood agar plates.
2. Incubate one of the plates at 25°C and one at 42°C in an atmosphere of 5% O_2, 10% CO_2, 85% H_2 or N_2 for up to 72 hours.
3. Place the naladixic acid and cephalothin disks one at each end of the streak of inocula on the third plate. Incubate this plate at 37°C in an atmosphere of 5% O_2, 10% CO_2, 85% H_2 or N_2 for up to 72 hours
4. Examine all plates for typical colonies of *Campylobacter* species after 48 and 72 hours incubation.

Reading and interpretation of results

Campylobacter fetus species will grow at 25°C and 37°C but not at 42°C.

Thermophilic species of *Campylobacter* (including *C.jejuni*) will grow at 37°C and 42°C but not at 25°C.

Isolates susceptible to either antibiotics, naladixic acid or cephalothin, will show a zone of inhibition around the relevant disk.

8.5.3.5 Production of H_2S

Principle

Production of H_2S is shown by a technique using the *Campylobacter* enrichment supplement (FBP; Oxoid, Hampshire, UK) which contains ferrous sulphate ($FeSO_4.7H_2O$), sodium metabisulphate and sodium pyruvate each at a concentration of 2.5 mg/ml. When a strain produces hydrogen sulphide, the sulphur combines with the iron to form a black precipitate of iron acetate.

Materials and Reagents

- FBP/Glycerol medium (see section on differential diagnosis of infectious abortion in small ruminants).

Procedure

1. Prepare the FBP/glycerol medium.
2. Heavily inoculate the medium with colonies removed from a 48 or 72 hour culture.
3. Incubate at room temperature for several hours in the dark.

Reading and interpretation of results

Production of H_2S will be indicated by the formation of black precipitate in the medium.

8.5.3.6 Hippurate hydrolysis

Principle

When hippurate is hydrolysed, glycine and benzoic acid are formed. A ninhydrin tube test can be used to detect glycine production or gas-liquid chromatography (GLC) can be used to detect benzoic acid. The ninhydrin test is simple to conduct but can occasionally be difficult to interpret whereas GLC is more reproducible but is technically more difficult and requires specialised equipment. One variation of the ninhydrin tube test is described [1].

Materials and reagents

- Sodium hippurate solution (1% v/w in distilled water).
- Ninhydrin solution (3.5% ninhydrin in a 1:1 mixture of acetone and butanol).

Procedure

1. Remove a large loopful of 24 or 48 hour inoculum grown on thioglycolate agar containing 5% sheep blood or brucella agar containing 5% sheep blood and emulsify with 0.4ml of sodium hippurate solution in a suitable tube.
2. Incubate for 2 hours at 37°C in a water bath.
3. After 2 hours add 0.2ml ninhydrin solution with a pipette down the side of the tube to form an overlay.
4. Incubate for 10 minutes at 37°C in air and then examine tube by eye.

Reading and interpretation of results

Production of a deep purple colouration indicates a positive reaction. No colour or a weak colour reaction are considered negative.

8.5.4 Further typing methods

A number of commercial systems have been developed as an aid to identifying selected *Campylobacter* species to genus level. Two latex agglutination assays (Meritec Campy jcl, Meridian Diagnostics, Ohio, USA [2] ; Campyslide, BBL Microbiological Systems, Cockeysville, USA [3]) can detect *C. jejuni* and *C. coli* but

cannot differentiate between them. Many typing systems have been devised to study epidemiology of *Campylobacter* infections; they vary in complexity and ability to discriminate between strains. These methods include serotyping, biotyping, bacteriocin sensitivity, detection of preformed enzymes, auxotyping, lectin binding, phage typing and multilocus enzyme electrophoresis. The most frequently used system is serotyping which tends to be performed in only a few reference laboratories because of the time and expense needed to maintain quality serotyping antisera. Commercially available heat-labile serotyping reagents are generally of poor quality.

8.5.5 DNA analysis

The requirement for rapid and sensitive methods of detection of *Campylobacter* in human faeces following outbreaks of food poisoning has led to the development of molecular-based methods such as restriction endonuclease analysis, ribotyping and polymerase chain reaction (PCR), in particular for the thermophilic *Campylobacter* species. A DNA probe directed against Campylobacter RNA sequences is available commercially (Accuprobe, Gen-Probe Inc, California, USA) for detection of *C. jejuni*, *C. coli* and *C.laridis* [4]. The probe has been shown to be 100% sensitive but also hybridised with some strains of *C. hyointestinalis*. At present such methods may be useful for confirming *Campylobacter* species if other tests are not conclusive. Investigations into the use of PCR techniques to detect and differentiate different strains of Campylobacters have also been reported [5].

The use of such techniques in veterinary diagnosis and development of probes specific to *C. fetus* have still to be evaluated.

8.6 INDIRECT DIAGNOSIS

8.6.1 Histopathology

Principle

A suitable method for histopathological preparation of tissue samples (placenta and foetal liver) and haematoxylin and eosin staining is described in the chapter on Toxoplasmosis.

Immunohistochemical labeling methods can also be applied to histological tissue sections and smears of foetal stomach contents to detect *Campylobacter* antigens using the techniques described in the chapter on *Toxoplasmosis*. The ABC indirect immunoperoxidase method is available commercially in kit form (VECTASTAIN® ABC Kit 4001, Vector Laboratories, USA) combined with an appropriate primary antibody. Alternatively the peroxidase anti-peroxidase (PAP) technique can be carried out as described in pages 104-105 using suitable primary and binding antibodies.

Reading and interpretation of results

Histologically, changes found in the placental tissue include large areas of necrosis in the chorionic villi, arteriolitis and thrombosis in the hiliar zone of the placentomes, abundant necrotic debris and high numbers of leukocytes within the stroma. Bacterial antigen can be detected within the placental trophoblasts lining most chorionic villi and within the subadjacent stroma, vascular endothelium and lumens.

The histological appearance of lesions in the foetal liver are of multiple, focal areas of necrosis.

8.7 FUTURE WORK

Abortion resulting from infection by *C. fetus* or *C. jejuni* in sheep and goats can only be confirmed by isolation and identification of the causal organism in aborted tissues and vaginal secretions. Campylobacters can be difficult to isolate and current techniques of isolation and identification are not ideal. Recent interest in improving techniques has mainly centered on *C. jejuni* and other species pathogenic to humans rather than *C. fetus*. Improvements to techniques to date therefore have limited application to veterinary samples. However, application of such improvements to veterinary materials will hopefully lead to :

- Improvements in the sensitivity and specificity of isolation media.
- Simplification of biochemical tests for species identification such as the ninhydrin test.
- Introduction of other typing systems such as serotyping, biotyping, bacteriocin sensitivity, detection of preformed enzymes, auxotyping, lectin binding, phage typing and multilocus enzyme electrophoresis.
- Application and expansion of molecular biological techniques such as PCR.

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