Chapter 12

OTHER BACTERIAL CAUSES OF ABORTION

M. PEPIN, R. SANCHIS, M. CALAMEL,
M. LAMBERT, P. RUSSO & C. VITU

12.1 SUMMARY

A large number of bacteria can cause occasional abortion either by colonising the placenta during a septicaemia phase or when the immune defences of the dam are low. When repeated abortions cannot be attributed to any of the major abortive organisms, the presence of other bacteria can be investigated using the techniques presented briefly in this chapter.

12.2 INTRODUCTION

Apart from the major pathogenic bacteria responsible for abortion in small ruminants (*Chlamydia*, *Coxiella*, *Brucella*, *Listeria*, *Campylobacter*, *Salmonella* abortusovis and *Leptospira*), other bacteria can occasionally be implicated in abortion outbreaks (Table 12.1).

These occasional abortions can arise in two, not unrelated, circumstances:

1) Abortions that arise from a systemic bacterial infection; in essence, all bacteria that occur in sheep and goats can potentially, at the time of gestation and during a period of septicaemia, colonise the placenta and foetus and cause abortion.

2) Abortions caused by opportunistic organisms which take advantage of a lowering of immune defences in the sheep or goat.

Eventhough the importance of these outbreaks remains limited and they are usually confined to a particular geographical area, it is acknowledged that certain of these "minor" pathological agents could become important in the future (the concept of "new diseases"). The relative importance of these secondary infections has been assessed in two studies, one in the USA with 1784 cases [4] and one in France with 1614 cases [7]. These studies showed that "minor" pathogens caused 24% and 5%, respectively, of occasional abortions (i.e. abortions not attributed to the major pathogenic agents) out of the infectious abortions diagnosed. These figures probably under-estimate the real percentage of occasional abortions due to "minor" pathogens since the cause of 56% and 57%, respectively, of the abortions was

Table 12.1 : List, in order of decreasing importance, of the minor pathogenic agents responsible for abortions in small ruminants

Minor pathogenic agent	Reference
Actinomyces pyogenes	[4, 7]
Yersinia	[2, 4, 7, 12, 14]
Y.enterocolitica	
Y.pseudotuberculosis	
Pasteurella	[4, 7]
P.haemolytica	
P.multocida	
Escherichia coli	[4]
Bacillus sp	[4]
Flexispira rappini	[3 - 5]
Pseudomonas sp	[4]
Fusobacterium nucleatum	[4]
Steptococcus sp	[4]
Staphylococcus sp	[4]
Clostridium sp	[4]
Enterobacter sp	[4, 13]
Histophilus ovis	[6]
Erysipelothrix rhusiopathiae	[7]
Corynebacterium sp	[4]

not established. These results also illustrate the large variability in the causes of abortion in small ruminants, whether due to major or minor pathological agents, between countries [4, 7] and within the same country [7, 10].

12.3 ABORTIONS DUE TO ENTEROBACTERIACEAE

12.3.1 Bacteria of the *Yersinia genus*

Bacteria of the *Yersinia* genus, family Enterobacteriaceae, have been associated with abortions in sheep. Three species have been identified, *Yersinia enterocolitica*, *Yersinia intermedia* and *Yersinia pseudotuberculosis*. In general, bacteria of the *Yersinia* genus are associated with intestinal infections of ungulates but they can infect a large number of other species including man (particularly *Y.enterocolitica*).

In cases of suspected infection with *Yersinia*, the bacteria can be detected directly in all sample types by use of selective media (CIN, Oxoid). Colonies grown on CIN media can be rapidly identified as *Yersinia* by the urease test and acid production from the carbohydrates, cellobiose, melibiose, rhamnose and saccharose [11]. Yersinia can also be isolated and identified using conventional agar (blood and McConkey: the bacteria are McConkey positive and lactose negative) or after enrichment at 4°C (the sample is placed in a phosphate buffer [0.1 M], held under refrigeration for 3 weeks and tested for Yersinia each week on McConkey or CIN media). Identification can then be undertaken using conventional biochemical tests or by using identifying kits such as AP120E (BioMerieux) [9].

Abortions caused by *Y.enterocolitica* or *Y.intermedia* occur at the end of gestation. The affected foetus shows no distinctive external signs but the internal organs show signs of a general bacterial infection. One study carried out with 120 isolates of *Y.enterocolitica* and *Y.intermedia*, recovered at the time of abortions, showed that the isolates that could be typed belonged to serogroups O:6, 30 and 7 [1]. The ability of these isolates to cause abortion was verified at the time by experimental infections [1, 2]. It should be noted that *Y.enterocolitica* possesses a common antigen with *Brucella* resulting in cross reactions during indirect serological diagnosis [9].

Yersinia pseudotuberculosis can cause abortion in sheep and goats, probably as a result of an initial intestinal infection. Placental cotyledons may have opaque off-white centres associated with suppurative microscopic lesions and the aborted dam may develop a suppurative pneumonia [12, 14].

12.3.2 Enterobacter cloacae and other enterobacteria

Enterobacter cloacae, a lactose positive bacteria, was originally described in abortive episodes in goats. Abortion occurs towards the third month of gestation and is associated with the expulsion of the foetus from which the causal agent can be isolated in pure culture [13].

Other enterobacteria, in particular *Escherichia coli* and *Salmonellae* other than *Salmonella abortusovis*, can be isolated at the time of abortion in small ruminants, generally following a febrile illness.

12.4 ABORTIONS DUE TO ACTINOMYCES PYOGENES

Actinomyces pyogenes, previously *Corynebacterium pyogenes*, is the cause of a number of suppurative infections in sheep and goats (mastitis, navel-ill,

arthritis). This bacteria can be isolated at the time of abortion, mainly in sheep [4, 7]; it can be cultured aerobically or in an atmosphere of 5-10% CO_2 on blood agar. After 48 hours very small colonies about 1mm diameter are visible surrounded by a narrow zone of haemolysis and on microscopic examination curved, Gram positive bacillus are seen. Identification of *A.pyogenes* can be confirmed by a positive CAMP test with *Staphylococcus aureus* [9].

12.5 ABORTIONS DUE TO OTHER BACTERIA

- *Pasteurella* (*Pasteurella haemolytica and P.multocida*), responsible for respiratory infections (acute or subacute, non-progressive pneumonias), have been recovered at the time of abortion in sheep and goats in a number of cases; such abortions illustrate the concept of opportunistic bacterial infection [4, 7].

- *Histophilus ovis* is a Gram negative, facultative anaerobic coccobacillus, close to the genus *Haemophilus*, belonging to the family of Pasteurellacae. This bacteria has been associated with a number of ovine pathologies (mastitis, epidydimitis, synovitis, meningoencephalitis) and has recently been identified as a cause of abortion in sheep [6]. *Histophilus ovis* grows slowly in small colonies on blood and chocolate agar incubated in an atmosphere of 10% CO_2. Identification of the bacteria, oxidase positive, catalase negative, nitrite positive, indole positive, can be made using the AP150CH kit (BioMerieux) [6].

- Flexispira rappini is an anaerobic, flagellated bacteria, recognised as a possible cause of abortion in small ruminants since 1984 [5]. This bacteria resembles *Campylobacter* and can be detected by culture on blood agar in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% CO_2) after about 7 days incubation. In recent published cases the proportion of abortions that had occurred by the end of gestation remained low: 2-4% [3, 5].

- Other bacteria have very occasionally been identified as the cause of abortion in small ruminants (Table 12.1), following recovery in pure cultures from aborted foetuses or placental samples.

12.6 BACTERIA RESPONSIBLE FOR IMPORTANT DISEASES IN SMALL RUMINANTS THAT DO NOT CAUSE ABORTION

It is important to note that several bacteria cause important diseases in ovines and caprines that do not lead to abortions except in very exceptional cases during general illnesses. Amongst these bacteria and the corresponding illness, it is necessary to mention :

- *Corynebacterium pseudotuberculosis*, the causal agent of caseous lymphadentitis [8];

- infections due to anaerobic bacteria of the genus *Clostridium*, responsible for enterotoxaemias, gaseous gangrene, tetanus ;

- *Dilechobacter* (*Bacteroides*) *nodosus*, the causal agent of footrot ;

- Mycobacteria : *Mycobacterium tuberculosis* (tuberculosis) and *Mycobacterium paratuberculosis* (paratuberculosis).

These non-abortive infections are the result of bacteria that have pathogenic characteristics or a specific tropism.

12.7 CONCLUSION

The difficulty of diagnosing abortions due to the less important bacteria in small ruminants is linked to the number of possible causal agents. To minimise the amount of testing involved, it is best to analyse samples for the main pathogenic agents responsible for abortions. Only if the results are negative (and before abortions are repeated), should tests for the bacteria of lesser importance be carried out (see Chapter 2 on differential diagnosis of abortions in small ruminants).

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Chapter 13

BORDER DISEASE

P. F. NETTLETON & J. BREBNER

13.1 SUMMARY

Border disease (BD), caused by the pestivirus Border Disease virus (BDV), is a congenital disease of sheep characterised by barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show tremor of skeletal muscles, abnormal body conformation, hairy fleeces (so-called "hairy-shaker" or "fuzzy" lambs), defective myelination of the central nervous system, inferior growth and a variable degree of skeletal deformity. The virus produces similar symptoms in goats but disease outbreaks are rarely reported. Although BDV is principally a cause of congenital disease it can also cause acute and persistent infections. BDV spreads naturally among sheep and goats by the oronasal route and by vertical transmission. Sheep and goats may also be infected following close contact with cattle excreting the closely related bovine virus diarrhoea virus (BVDV).

The diagnosis of BD presents little difficulty if typical "hairy shaker" lambs are born although laboratory confirmation is advisable. Histological examination of the central nervous system can confirm BD but should be supported by virus isolation from blood and tissues or the demonstration of viral components by specific immunological or reverse transcriptase-polymerase chain reaction methods.

13.2 INTRODUCTION

Border Disease (BD) is a congenital virus disease of sheep first reported in 1959 from the border region of England and Wales. It has subsequently been reported from most major sheep-producing countries of the world. Prevalence rates vary from 5% to 50% between countries and from region-to-region within countries. The disease occurs naturally primarily in sheep and occasionally in goats.

BD virus (BDV) is a pestivirus in the genus Flaviviridae and is closely related to classical swine fever and BVDV. Nearly all isolates of BDV are non-cytopathogenic (ncp) in cell culture although occasional cytopathic viruses have been isolated. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity and three distinct antigenic groups have been identified.

Healthy newborn and adult sheep exposed to BDV experience only mild or inapparent disease. The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is subclinical or mild the consequences for the foetus are serious. Foetal death may occur at any stage of pregnancy but is commoner in foetuses infected early in gestation. Small dead foetuses may be resorbed or their abortion pass unnoticed since the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of larger foetuses, stillbirths and the premature births of small, weak lambs is seen.

During lambing, an excessive number of barren ewes may become apparent but it is the diseased live lambs that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The nervous symptoms of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the muscles of the hind legs and back to barely detectable fine trembling of the head, ears and tail. Fleece abnormalities are most obvious in smooth-coated breeds which have hairy fleeces especially on the neck and back. Abnormal brown or black pigmentation of the fleece may also be seen in BD-affected lambs. Deformities of the skeleton include abnormally shortened long bones, a reduction in crown-rump length and the long axis of the skull which results in lambs appearing more compact and short-legged than normal.

With careful nursing a proportion of BD lambs can be reared although deaths may occur at any age. The nervous symptoms gradually decline and can have disappeared by 3 to 6 months of age. Weakness, swaying of the hind-quarters together with fine trembling of the head may reappear at times of stress. Affected lambs often grow slowly and under normal field conditions many will die before or around weaning time. Occasionally this is the first presenting sign of disease when losses at lambing time have been low and no lambs with obvious symptoms of BD have been born.

Some foetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated, and lambs with such lesions frequently have high concentrations of serum antibody to BDV. Most lambs infected in late gestation are normal and healthy and are born free of virus but with BDV antibody. Some such lambs, however, can be stillborn or weak and may die in early life.

In sheep foetuses infected before the onset of immune competence viral replication is uncontrolled and 50% foetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear to be tolerant to the virus and have a persistent infection usually for life. Typically, there is no inflammatory reaction and the most characteristic pathological changes are in the CNS and skin. At all levels in the CNS there is a deficiency of myelin which is responsible for the nervous symptoms. In the skin there is an increased size of primary wool follicles and fewer secondary wool follicles causing the hairy or coarse fleece.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Rams that are persistently infected usually have poor quality, highly infective semen and reduced fertility. Persistently viraemic sheep are a continual source of infectious virus to other animals, excreting the virus in nasal secretions, saliva, urine and faeces. Viraemia is readily detectable at any time except in the first two months of life when virus is masked by colostral antibody and in animals older than 4 years old some of which develop low

levels of anti-BDV antibody.

In goats, foetal death is the major outcome of infection of the pregnant doe and infections prior to 60 days of gestation almost invariably result in reproductive failure. Persistently infected "shaker" kids and clinically normal kids are born with infections around 60 days of gestation but are very rare. The caprine foetus develops immune competence against pestiviruses between 80 and 100 days of gestation.

There is no standard vaccine for BDV but a commercial killed whole virus vaccine has been produced. Ideally such a vaccine should be able to be administered to females before breeding to prevent transplacental infection. The use of BVDV vaccines has been advocated but this would be irresponsible given current knowledge on the antigenic diversity of BD viruses. BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.

Control strategy lies in attempting to engender a flock immunity and to avoid exposing sheep or goats to infection during pregnancy. Persistently infected animals are a continuous source of infection and are best identified and culled. However, persistently infected animals can be run with the flock when it is not pregnant, particularly with replacement stock, in an attempt to produce infection and immunity prior to gestation. Close herding for at least 3 weeks, preferably indoors, is necessary for BDV to spread effectively and exposure should stop two months before the start of the breeding season when the persistently infected animals should be removed. In most flocks a serious outbreak of disease is followed by minor disease in subsequent years, the flock developing immunity in the initial outbreak. In flocks that are free from infection consideration should be given to blood testing all brought-in replacements to ensure none are persistently infected.

13.3 SAMPLES

The diagnosis of BD on clinical grounds presents little difficulty when typical "hairy-shaker" lambs are born, but laboratory confirmation is frequently necessary to distinguish BD from sway-back, hypothermia, "daft-lamb" disease, bacterial meningo-encephalitis and focal symmetrical encephalomalacia. Since placentas and foetuses aborted due to BD infection have no distinguishing characteristics, laboratory confirmation is required to differentiate BD from other known causes of abortion. The specimens required by the laboratory to confirm BD are summarised in Table 13.1.

13.3.1 Virus isolation

13.3.1.1 Placental and foetal tissues

Five or six placental cotyledons and the associated intercotyledonary membranes and tissues from aborted foetuses and newly dead "hairy shaker" lambs should be collected aseptically and placed in a suitable sterile container holding virus transport medium (VTM). On arrival at the laboratory samples should be frozen at -70°C or treated as follows:

1. Macerate tissue in VTM by a suitable method e.g. a "stomacher" to form a homogeneous suspension.
2. Centrifuge the suspension at 3,000 xg for 10 minutes.
3. Pass the supernatant through a sterile 0.45μ filter and retain the filtrate.
4. If not analysed immediately store the filtrate at -70°C.

Table 13.1 : Samples to be collected for the laboratory confirmation of Border Disease as the cause of abortion in small ruminants

Clinical Signs	Diagnostic Technique	Samples From:	
		Live Animal	Dead Animal
Abortion	Virus Isolation	Heparinised blood from the dam	Thyroid, kidney, spleen, brain, placenta in VTM
	Antigen detection	Heparinised blood from the dam	Thyroid, kidney, spleen, brain, placenta
	Histology	--	Brain and spinal cord in fixative
	Serology	Blood from the dam	Foetal fluids
"Hairy shaker", Weak Poorly , Thriving or Scouring Lambs	Virus Isolation	Heparinised blood from the lamb and dam	Thyroid, kidney, spleen, brain, gut, lymph nodes in VTM
	Antigen detection	Heparinised blood from the lamb and dam	Thyroid, kidney, spleen,, brain, gut, lymph nodes
	Histology		Brain and spinal cord in fixative
	Serology	Blood from the lamb and dam	

13.3.1.2 Blood samples

Blood samples can be used for virus isolation or can be analysed for the presence of BD antigens or antibodies.

Blood samples from aborted ewes are best collected at the time of abortion. Pre-colostral blood samples from "hairy-shaker", weak, poorly thriving or scouring lambs should be collected, although, there are practical difficulties in obtaining such samples.

Two blood samples should be collected from each animal, one in a sterile, evacuated glass blood tube containing no anti-coagulant for antibody detection and one in a sterile, evacuated, heparinised glass blood tube (e.g. Vacutainers) for virus isolation and antigen detection. On arrival at the laboratory, heparinised blood samples should be processed as follows to recover leukocytes:

1. Centrifuge samples at 3,000 xg for 10 minutes. Plasma may be retained for serological analysis.

2. Remove the buffy coat with minimum amounts of plasma and erythrocytes and divide equally between:

a) 1 ml of serum-free maintenance media (see Section 13.5.1) for virus isolation

b) 1ml of Tris-ammonium chloride for antigen detection by ELISA.

3. Following addition of Tris-ammonium chloride, rotate tubes gently for 20-30 seconds to lyse any contaminating erythrocytes.

4. Pellet the leukocytes by centrifugation in a microcentrifuge (11,600 xg) for

15 seconds.

5. Discard the supernatant and re-suspend the pellet in 1.5ml phosphate buffered saline (PBS).

6. Repeat centrifugation and washing with PBS.

7. Resuspend final pellet in 1.5ml PBS containing 1% Nonidet P-40 and agitate at room temperature for 2 hours.

8. Analyse immediately or store samples at -70°C until required.

13.3.2 Antigen detection

Antigen detection can be undertaken on placental samples and samples of tissues from aborted foetuses or newly dead "hairy shaker" lambs collected aseptically and placed in sterile containers or on leukocytes recovered from heparinised blood samples as described above.

13.3.3 Histological examination

Samples of brain and spinal cord should be placed in a glass sample jar containing a suitable fixative such as calcium formol saline (0.85g sodium chloride and 1.00g calcium chloride dissolved in a mixture of 10ml 40% formaldehyde and 90ml water) with a ratio of fixative to tissue of at least 10:1 (v/v). Fixed tissues should be left for at least four weeks prior to further processing.

13.3.4 Antibody detection

13.3.4.1 Blood samples

Clotted blood samples should be centrifuged at 3,000 xg for 10 minutes and serum removed. If not analysed immediately serum should be stored at 4°C for processing within 7 days or at -20°C for later testing.

13.3.4.2 Foetal fluids

Stomach contents, heart blood, pleural and peritoneal fluids can be removed from aborted foetuses that are not too autolysed. The fluids should be aseptically removed from the foetus as soon as possible after abortion using a sterile syringe and placed in a suitable sterile container. On arrival at the laboratory, samples should be centrifuged at 3,000 xg for 15 minutes and the supernatants removed and either analysed immediately or stored frozen at -20°C.

Preparation of reagents

- Virus Transport Media

Hanks balanced salt solution (see Section 13.5.1)

1% bovine serum albumin (BSA)

Penicillin G	300 IU/ml
Streptomycin sulphate	300µg/ml
Polymixin B sulphate	150 IU/ml

- Tris Ammonium Chloride

Ammonium chloride	8.3g
Tris	20.6g
Distilled water	1.9litres

Dissolve ammonium chloride in 1 litre of water (0.16M). Dissolve Tris in 900mls of water (0.17M) and adjust pH to 7.65 with 1M hydrochloric acid.

Working solution: Combine 90mls of 0.16M ammonium chloride and 10mls of 0.17M Tris solution, mix thoroughly and adjust pH to 7.2 with 1M hydrochloric acid.

13.4 RISKS TO HUMAN HEALTH

There are no known human health risks associated with pestiviruses.

13.5 DIRECT DIAGNOSIS

Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish. The most sensitive proven method for identifying BDV is still virus isolation achieved by growing the virus in cell culture. Direct detection ELISA

and PCR methods are also used.

N.B. : It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and foetal bovine or equivalent serum which contains no antipestivirus activity and no contaminating virus.

13.5.1 Virus isolation

Principle

Border Disease virus can be isolated from blood, placental material and foetal or lamb tissues in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung). Ovine cell lines for BDV growth are rare and unobtainable. Semi-continuous cell lines derived from foetal lamb muscle (FLM) or sheep choroid plexus can be useful but different lines vary considerably in their susceptibility to virus. Detection of viral growth in cell cultures is undertaken using immune-labelling methods.

There are many variations in virus isolation procedures. All should be optimised for maximum sensitivity using reference virus preparation and, whenever possible, recent BDV field isolates. A practical tube isolation procedure with detection by indirect immunofluorescence is outlined below:

Materials and reagents

- Test tubes cultures with sub-confluent or newly confluent monolayers of a suitable primary ovine cell line e.g. foetal lamb kidney.
- UV microscope.
- Reference cytopathic and non-cytopathic BDV strains.
- Maintenance media.
- Growth media.
- Hanks BSS.
- Cold acetone.
- Phosphate buffered saline (PBS).
- BDV antiserum (Moredun Scientific Ltd., UK).
- Conjugate: Donkey anti-sheep IgG conjugated with fluorescein isothiocyanate (FITC).

Procedure

1. Wash test tube cultures with sub-confluent or newly confluent monolayers of susceptible ovine cells at least twice with Hanks BSS to remove growth medium.
2. Inoculate duplicate tubes with 200µl of each test sample.
3. Make suitable dilutions of the reference viruses in maintenance media and inoculate quadruplicate tubes with 200µl of each dilution. Adsorb for 2 hours at 37°C.
4. Wash cultures with 2ml medium, discard and add 1ml of culture maintenance medium.
5. Incubate for 5 to 7 days at 37°C. Examine microscopically on a daily basis and record evidence of cytopathic effect.
6. Freeze tubes at -70°C, and then thaw and passage as before to fresh tube cultures containing cells growing on coverslips.
7. Remove coverslips three days later and fix in cold acetone at 4°C for 15 minutes.
8. Remove coverslips from acetone and place on a staining tray.
9. Add 100µl of BDV antiserum to each coverslip and incubate at 37°C for 30 minutes in a moist atmosphere.
10. Wash coverslips three times in warm PBS for 10 minutes each wash and allow to air dry.
11. Add 100µl of FITC conjugate to each coverslip and incubate at 37°C for 30 minutes in a moist atmosphere.
12. Wash coverslips three times in warm PBS for 10 minutes each wash and allow to air dry.

13. Mount coverslips with cells facing downwards on microscope slides using buffered glycerol.

14. Examine coverslips under UV microscope.

Reading and interpretation of results

BDV is confirmed by the presence of diffuse cytoplasmic fluorescence characteristic of pestiviruses (Figure 13.1, page 96).

Preparation of reagents

- Growth Media

Medium E199 (Northumbria Biological Ltd, UK)

10% Foetal bovine serum

- Maintenance Media

Medium E199 (Northumbria Biological Ltd, UK)

2% Foetal bovine serum

Penicillin G	100 IU/ml
Streptomycin sulphate	100 µg/ml

- Hanks BSS

Sodium chloride (NaCl)	40g
Potassium chloride (KCl)	2g
Calcium chloride ($CaCl_2.2H_2O$)	0.93g
Magnesium chloride ($MgCl_2.6H_2O$)	0.5g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.5g
Sodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	0.76g
Potassium dihydrogen phosphate (KH_2PO_4)	0.3g

Dissolve salts in 5 litres of distilled water, mix thoroughly and sterilise by filtration.

- Buffered Glycerol

Sodium hydrogen carbonate ($NaHCO_3$)	0.286g
Sodium carbonate (Na_2CO_3)	0.064g
Distilled water	40ml
Glycerol	360ml

Note

- Alternatively, frozen and thawed cultures can be added to cells growing on chamber slides and stained by IFT as above.
- Immunoperoxidase staining can also be used on coverslips or chamber slides as well as microtitre plates (see method under Virus Neutralisation test, Section 13.6.2.1).
- Frozen and thawed cultures can also be tested in an antigen detection ELISA system (see below).

13.5.2 ELISA for antigen detection

The ELISA test for antigen detection is most commonly employed to identify persistently infected viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening high numbers of bloods. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA test is more effective than virus isolation in the presence of antibody, but may give false negative results in viraemic lambs younger than two months old. As well as for testing leukocytes the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to IF and IP methods, on cell cultures.

Several pestivirus ELISA methods have been published and a number of commercial kits are now available for detecting BVDV in cattle. While some of these may be suitable for use in sheep further evaluation is required.

Principle

The ELISA for pestivirus antigen detection described is a double monoclonal antibody (mab) capture system

which can be used for sheep and cattle samples [1]. Two capture mabs are bound to wells in microtitre plates and two other mabs conjugated to horse radish peroxidase serve as detector mabs.

Materials and reagents

- 96 well microtitre plates.
- ELISA plate reader.
- Carbonate buffer, pH 9.6.
- Capture mabs (VPM20 and 13G4; Moredun Scientific Ltd., Scotland).
- Phosphate buffered saline (PBS).
- Tween 20.
- Horse serum free of anti-pestivirus activity.
- Positive control antigen (Moredun Scientific Ltd., Scotland).
- Conjugate: Detection mabs VPM22 and VPM12 conjugated to horse radish peroxidase (HRP) (Moredun Scientific Ltd., Scotland).
- Orthophenylene diamine (OPD) substrate.
- 2.5M sulphuric acid.

Procedure

1. Add 100µl of a mixed suspension of the capture mab's each diluted to 1mg/ml with carbonate buffer to all wells of the required number of 96 well microtitre plates. Seal plates and incubate overnight at 4°C (or for one hour at 37°C).

2. Wash plates 3 times with PBS with 0.05% Tween 20 (PBST) and blot dry.

3. Add 100µl of PBST containing 10% horse serum (PBSTH) to all wells. Seal plates and incubate for 1 hour at 37°C.

4. Wash plates 3 times with PBST and blot dry.

5. Add 100µl of each test sample or positive control antigen diluted in PBSTH to plate wells as appropriate.

6. Seal plates and incubate for 2 hours at 37°C or overnight at 4°C.

7. Wash plates 3 times with PBST and blot dry.

8. Dilute the HRP conjugated detection mabs in PBSTH and add 100µl to all wells of the plates.

9. Seal plates and incubate for 1 hour at 37°C.

10. Wash plates 3 times with PBST and blot dry.

11. Add 100µl of activated OPD substrate to all wells and incubate at room temperature for 15 minutes.

12. Add 50µl of 2.5M sulphuric acid to all wells.

13. Read plates using an ELISA plate reader at 492nm.

Reading and interpretation of results

Calculate the mean optical densities (OD) of the two control dilutions and of each test sample. Samples with OD's greater than the highest control are positive, samples with OD's less than the lower control are negative, samples with OD's between the two controls are inconclusive and should be retested.

Preparation of reagents

- Carbonate Buffer

0.2M sodium carbonate (Na_2CO_3)	7.9ml
0.2M sodium hydrogen carbonate ($NaHCO_3$)	17.1ml
Deionised water	75.0ml

Add solutions to distilled water and mix thoroughly. Adjust the pH to 9.6 using 0.2M $NaHCO_3$.

- Orthophenylene Diamine Substrate

0.1M citric acid	24.3ml
0.2M sodium hydrogen phosphate (Na_2HPO_4)	25.7ml
Deionised water	50.0ml
Orthophenylene diamine (OPD)	0.08g

Add citric acid and Na_2HPO_4 to the

deionised water and mix thoroughly. Adjust the pH to 5.0 with 0.1M citric acid and then add OPD. Immediately before use activate by addition of 40ml of hydrogen peroxide per 50ml substrate buffer.

13.5.3 Nucleic acid detection

Ovine pestiviruses can be detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) using primers which also detect pestiviruses from other species [2]. While RT-PCR has not yet been evaluated for diagnostic purposes in sheep it is likely to be of future value. The detection of viral RNA in foetal tissues may yet be an important application since other methods are insensitive. The exquisite sensitivity of RT-PCR makes it a valuable tool for detecting low level virus contaminations as in cell culture constituents or vaccines

In-situ hybridisation has also been shown to be a sensitive method for detecting BDV but it has been used more for research than diagnostic purposes [3].

13.6 INDIRECT DIAGNOSIS

13.6.1 Histopathology of the brain and spinal cord

Principle

A suitable method for preparation and staining of tissues for histopathological examination is described in the chapter on Toxoplasmosis. For diagnosis of BD specific myelin stains such as luxol fast blue can be used as follows:

Materials and reagents

- Luxol fast blue stain.
- Ethanol (99%, 95%, 90%, 70% solutions).
- Xylene.
- Saturated lithium carbonate.
- Neutral red stain.

Procedure

1. Immerse slides holding processed tissue sections in xylene for 2x10 minutes to remove wax.
2. Hydrate tissues through a graded alcohol series (e.g. 99% ethanol for 2 x 2 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes).
3. Rinse slides thoroughly under running tap water.
4. Immerse slides in luxol fast blue stain (1% solution in ethanol) overnight at 60°C.
5. Rinse slides in 95% ethanol then in distilled water.
6. Immerse slides in saturated lithium carbonate for 5 seconds.
7. Examine under microscope: if there is clear differentiation between blue stained white matter and colourless grey matter continue to step 8; if not, immerse slides in 70% alcohol until there is a clear differentiation between the tissues.
8. Rinse slides thoroughly under running tap water.
9. Wash slides in distilled water.
10. Immerse slides in 1% neutral red counterstain (1% in distilled water) for 3 minutes.
11. Examine under microscope: if there is clear differentiation between blue stained white matter and red stained grey matter continue to step 12; if not, immerse slides in 95% alcohol until there is a clear differentiation between the tissues.
12. Rinse slides thoroughly under running tap water.
13. Dehydrate and clear slides through a graded alcohol series and xylene (e.g. 70% ethanol for 2 minutes, 90% ethanol for 2 minutes, 99% ethanol for 3 x 2 minutes, xylene for 2 x 2

minutes).

14. Mount coverslips over the stained tissue sections and examine under a microscope.

Reading and interpretation of results

The most characteristic feature of BD is a deficiency of stainable myelin.

13.6.2 ANTIBODY DETECTION

Antibody to BDV can be detected in sheep sera using virus neutralisation (VN) or an ELISA. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera from several sheep can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming acute BDV infection. Bleeds from one animal should always be tested alongside each other on the same plate.

13.6.2.1 Virus neutralisation test

Principle

The cytopathic Moredun strain of BDV can be used for the VN test with semicontinuous cells such as FLM. An outline protocol is given below.

Materials and reagents

- Cell culture grade flat-bottomed 96 well microtitre plates.
- CO_2 incubator.
- Suitable cells diluted to 2 x 10^5 cells/ml.
- Growth media (see Section 135.1).
- Cytopathic BDV (Moredun strain).
- Control positive and negative sera.

Procedure

1. Heat inactivate test sera for 30 minutes at 56°C.

2. It is usual to screen all test sera initially at a dilution of 1/4 and then to titrate out positive sera. To screen sera a minimum of four wells per sample is required: two wells to measure neutralisation activity and two wells to verify the absence of serum toxicity to the cells. To titrate positive sera, starting from a dilution of 1/4, make serial twofold dilutions of the test sera in cell culture media in 96 well microtitre plates using two wells for each sample. The lowest serum dilution of each sample should be added to two additional wells to act as a toxicity control.

3. Use 25µl volumes of the diluted serum in each well and add 25µl of media to each of the lower two control wells.

4. Add 25µl of media containing 100 TCID50 of virus to each of the test wells and 25µl of media to each of the wells for toxicity control. Include control positive and negative sera and a virus titration in every test.

5. Seal plates with non-toxic plate sealers or lids and incubate at 37°C for 1 hour.

6. Add 100µl of a cell suspension with a count of 2 x 10^5 cells/ml to every well.

7. Seal the plates or incubate in a moist chamber in 5% CO_2 for four days at 37°C.

8. Examine the wells microscopically for cytopathic effect.

Reading and interpretation of results

The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman-Karber method [5]. A seronegative animal will show no neutralisation at the lowest dilution.

Note

• Cytopathic effects in the control wells of the test sera will be due to toxicity. Further dilution of toxic sera can be attempted but it may not be possible to obtain reliable results with occasional sera.

• The choice of test virus is difficult due to antigenic diversity among pestiviruses [6]. Reference strains of cytopathic BVD viruses can be used. Oregon C24V results correlate better with Moredun BDV than results with the NADL strain. No single strain is ideal. A local strain which gives the highest antibody titre with a range of positive sheep sera should be used.

• The VN test can also be used with non-cytopathic viruses when the following immunoperoxidase staining system is used after stage 7 above.

13.6.2.2 Imunoperoxydase staining

Materials and reagents

- 95% acetone in distilled water (held at -20°C overnight).
- Phosphate buffered saline (PBS).
- BDV antiserum.
- Tween 80.
- Conjugate: anti-sheep IgG, peroxidase conjugated (Sigma/ICN-flow/Dako).
- 3-amino-9-ethyl carbazole (AEC) substrate.

Procedure

1. Remove culture medium and wash cells gently with warm PBS.
2. Air dry cells and cool plates to 4°C.
3. Fix cells by quickly adding 95% acetone (in water) previously cooled to -20°C to all wells. Hold plates at -20°C for 30 minutes. Do not stack plates or allow them to warm or etching of the plastic may occur.
4. Remove acetone and dry plates quickly in a cool environment.
5. Wash plates with PBS and blot dry. Fixed plates can be stored at -20°C before staining.
6. Add 50µl of BDV antiserum to all wells at a predetermined dilution in buffered saline with 1% Tween 80 (PBST). Incubate at 37°C for 30 min in a moist atmosphere.
7. Empty plates and wash three times with PBST.
8. Drain wells and add 50µl of conjugate at a predetermined dilution in PBST to all wells. Incubate for 30 minutes at 37°C in a moist atmosphere.
9. Empty plates and wash three times with PBST.
10. Drain wells and add 50µl of activated AEC substrate.
11. Incubate plates at room temperature and monitor known virus positive control wells for development of specific red-brown cytoplasmic staining (5-10 minutes). When staining is complete remove substrate carefully and wash wells thoroughly with tap water. Leave tap water in wells and examine microscopically for virus containing wells.

Reading and interpretation of results

The VN titre is calculated as above using the Spearman-Karber method [5].

Preparation of reagents

- 3-Amino-9-Ethyl Carbazole Substrate

Stock solution: 0.1g AEC dissolved in 15ml dimethyl formamide.

Working solution: add 0.3ml stock to 5µl 0.05M acetate buffer pH 5.0.

Activate immediately before use by addition of 25µl 30% H_2O_2.

NB *: This solution is toxic and should be handled with adequate precautions.*

13.6.2.3 ELISA

Principle

A monoclonal antibody capture ELISA for measuring BDV antibodies is described. Two pan pestivirus mabs detecting different epitopes on the immunodominant non-structural NS 2/3 protein are used to capture detergent-lysed cell culture-grown antigen. The results correlate qualitatively with the VN test [7].

a / Antigen preparation

Materials and reagents

- 225cm^3 flasks FLM cells.
- Cytopathic BDV (Moredun strain).
- Maintenance media.
- Foetal bovine serum (FBS).
- Phosphate buffered saline (PBS).
- Nonidet P40.

Procedure

1. Use eight x 225cm^2 flasks of newly confluent FLM cells, four controls and four infected. Wash flasks and infect four with a 0.01-0.1 m.o.i. of Moredun cytopathic BDV. Adsorb virus for 2 hours at 37°C.

2. Add maintenance media containing 2% FBS and incubate cultures for 4-5 days until cytopathic effect is obvious.

3. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000xg for 15 minutes to pellet cells.

4. Discard supernatants and retain cell pellets.

5. Wash flasks with 50ml PBS and repeat centrifugation as above.

6. Pool all control cell pellets in 8ml PBS containing 1% nonidet P40 and return 2ml to each control flask to lyse remaining attached cells.

7. Repeat for infected cells.

8. Keep flasks at 4°C for at least 2 hours agitating the small volume of fluid on the cells vigorously every 1/2 hour to ensure total cell detachment.

9. Centrifuge control and infected antigen at 12,000xg for 5 minutes to remove cell debris.

10. Store supernatant antigens at -70°C in small aliquots.

b / ELISA test

Materials and reagents

- 96 well ELISA grade microtitre plates.
- ELISA plate reader.
- Capture monoclonal antibodies (Moredun Scientific Ltd., Scotland).
- Bicarbonate buffer (see Section 13.5.2).
- Phosphate buffered saline with 1% Tween 80 (PBST).
- Horse serum.
- Anti-ovine IgG peroxidase conjugate.
- Orthophenylene diamine (OPD) substrate.
- 2.5M Sulphuric acid.

Procedure

1. Dilute the two mabs to a pre-determined dilution (commonly 1/4000 ascites) in 0.05M bicarbonate buffer, pH9.6. Coat all wells of a suitable ELISA grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) overnight at 4°C using 100µl volumes per well.

2. Wash 3 times in PBST, then add 100ml of a blocking solution of PBST + 10% horse sera (PBSTH) to all wells and incubate at 37°C for 1 hour.

3. Dilute the antigen to a pre-determined dilution in PBSTH and coat alternate rows of wells with 100µl of virus and control antigens for 1 hour at 37°C.

4. Wash plates 3 times in PBST.

5. Dilute test sera 1/50 in PBSTH and add 100µl of each sera to duplicate virus and duplicate control wells. Incubate for

one hour at 37°C.

6. Wash plates 3 times in PBST.

7. Dilute anti-ovine IgG peroxidase conjugate to a pre-determined dilution in PBSTH and add 100µl to all wells. Incubate for 1 hour at 37°C.

8. Wash plates 3 times in PBST.

9. Add 100µl of a suitable activated enzyme substrate, such as ortho-phenylene diamine noting the manufacturer's toxicity warning.

10. After colour development stop the reaction with 50µl of 2.5M sulphuric acid.

11. Read the optical density (OD) on an ELISA plate reader.

Reading and interpretation of results

The mean value of the 2 control wells is subtracted from the mean value of the 2 virus wells to give the corrected OD (CODs) for each serum. Results are expressed as CODs with reference to CODs of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

13.7 FUTURE WORK

While reliance on cell culture methods is essential for evaluation of most methods direct detection systems, especially those using PCR, will continue their development.

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