

Ruminant Pestivirus Infections

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Part 1. Diagnostic Overview

Summary

Pestivirus infections are important in the livestock industries, with infection occurring in cattle, sheep and pigs. Pestiviruses are considered to be the most important viral pathogens of cattle in Australia and New Zealand. There can be significant economic loss through the effects of the virus on reproduction, especially causing conception failure, embryonic mortality and early foetal infections that lead to abortion or the birth of persistently infected animals that have a markedly reduced life expectancy and poor production. Acute infections can also cause respiratory and enteric disease. Persistently infected animals provide a reservoir of the virus in the population. Infection of sheep occurs intermittently and there is little economic impact. The porcine virus, classical swine fever virus, is exotic. The diagnostic methods that are used most frequently aim to detect persistently infected animals, either for the investigation of disease or for health certification for regulatory purposes. Enzyme-linked immunosorbent assays or virus isolation in cell cultures are the most frequently used tests to detect persistently infected animals. The agar gel immunodiffusion test or virus neutralisation test are usually used to detect antibodies to these viruses. The detection and exclusion of pestivirus from a range of biological materials that are used for diagnostic or therapeutic purposes is important to ensure that test results are reliable and to prevent the spread of the virus to other animals.

1. Introduction

Pestiviruses are the most important viruses to infect cattle in Australia and New Zealand. The viruses are widespread and common. In contrast, pestivirus infections of sheep and goats are relatively uncommon. The emphasis in this document is on infections in cattle but other species are mentioned where relevant.

2. Aetiology

Bovine pestivirus or bovine viral diarrhoea virus (BVDV) is ubiquitous in the cattle population. The virus is also known as mucosal disease (MD) virus although this name is misleading because the virus causes a much broader disease spectrum than is represented by mucosal disease. Although related pestiviruses usually cause specific disease syndromes in sheep (border disease virus — BDV) and pigs (classical swine fever or hog cholera virus — CSFV), the bovine virus can also cause disease in these species¹ and also in other ruminant species (for example, goats, deer, camelids).

In Australia and New Zealand, all strains of BVDV are believed to belong to the Type I genotype, with relatively limited genetic variation. Strains belonging to the Type II genotype have not been found in Australia or New Zealand but are widely distributed in North America and Europe, along

with Type 1 strains. Viruses found in the northern hemisphere show a broad genetic and antigenic diversity, with implications for both diagnosis and disease control. There have been recent trends towards the description of subtypes within the main Type I and Type II genotypes but this appears to be largely of scientific interest with no apparent practical implications for diagnosis or disease control.

3. Clinical Signs in Cattle

In cattle in Australia and New Zealand, pestivirus is predominantly a pathogen of the conceptus and is a significant cause of reproductive wastage, producing conception failure, embryonic mortality, abortion, congenital defects, stillbirths and perinatal mortality. Cattle that are persistently infected (PI) with BVDV (an outcome of foetal infection between 30-90 days of gestation) and survive to term may show a variety of clinical signs. Virtually all PI animals have a markedly reduced life-expectancy and can present with illthrift, scouring, lameness and a range of bacterial diseases that are secondary to the severe immunosuppression that the virus induces. Clinical signs are most frequently observed in cattle from 6-24 months of age but animals can be affected outside this age range. Mucosal disease, with ulceration of the buccal mucosa and epithelium of the alimentary tract, is a sequel to the infection of a PI animal with a second mutant virus that is described as cytopathogenic due to its properties in laboratory cell cultures. Very few PI animals show signs of mucosal disease, with most succumbing from secondary infections due to reduced immune function.

Postnatal infection of cattle with BVDV is usually asymptomatic but this virus can be a primary cause of respiratory disease and, rarely, acute enteric disorders (BVD). The virus may also produce a degree of immunosuppression, which increases susceptibility to other infectious agents. This is particularly important as a cause of the bovine respiratory disease (BRD) complex seen in feedlot cattle. Strains belonging to the Type II genotype can cause severe postnatal disease, in both calves and adult cattle. Signs include a severe mucosal disease-like syndrome and sometimes a haemorrhagic disease syndrome.

4. Epidemiology

This virus is highly contagious and commonly infects susceptible cattle, usually without producing overt disease unless an animal is pregnant. Maintenance of the virus in the population depends on the persistently infected 'carrier' animal, a consequence of foetal infection between 30 and 100 days of gestation. In the general cattle population, the prevalence of carrier animals is about 1%. The management of the persistently infected animal has a critical influence on the epidemiology of infection, development of immunity, and occurrence of disease in a herd. The epidemiology, disease manifestations and control measures for pestivirus infections in cattle have been extensively covered in reviews.^{2,3,4}

5. Occurrence and Distribution

BVDV is widely distributed throughout the cattle populations of Australia and New Zealand. A moderate to high prevalence of seropositive animals can be found in most beef and dairy herds. However, the spread of BVDV within and between herds can be greatly influenced by herd management. Some herds that have been 'closed' and have not introduced animals may have a very low prevalence of seropositive animals. There may also be marked variations in antibody prevalence within a herd if individual groups of animals have been either mixed with or segregated from a persistently infected animal for long periods of time. In beef herds and in some seasonally calving dairy herds, there is a greater likelihood of finding a very high prevalence of antibody because of the increased potential for long periods of contact between newborn calves and breeding animals. In contrast, in year-round calving herds, newborn calves, the greatest source of virus, are born in small segregated groups and are soon removed from the cows, limiting spread. In Australia

there is also a lower prevalence of seropositive animals in the large extensive beef herds of northern Australia, probably as a result of a lower survival rate of persistently infected animals in this harsh climate. At any point in time, there is active virus transmission in only a moderate to low proportion of herds due to the limited lifespan of persistently infected animals. However, within a herd or group of animals in which there has been recent virus spread, depending on the coincidence between virus spread and critical stages of pregnancy, the incidence of persistently infected animals can range from 1-2% to as high as 50%.

6. Clinical Signs in Other Ruminants

While BDV infection occurs in sheep in Australia and New Zealand, unlike BVDV in cattle, it is extremely uncommon. Infection of pregnant sheep can cause a range of clinical manifestations that are similar to the bovine equivalent with reduced fertility, abortion and the birth of congenitally infected lambs.⁵ These lambs are often small, weak and many have neurological defects so that their viability under natural conditions is usually threatened without human intervention.

Infection of goats in Australia and New Zealand is relatively uncommon but does occur.⁶ It is more likely that infection will be due to bovine strains of the virus. The main clinical manifestation in goats is reproductive disease. Abortion and a mucosal disease-like syndrome have been described in camelids but little is known about the syndrome.

7. Pathology

With the exception of the mucosal disease complex, there are few gross or histological changes that are pathognomonic for pestivirus infections. However, when severe lymphoid depletion, crypt cell necrosis, herniation of crypts into Peyer's patches and vasculitis in submucosal vessels are detected histologically, a pestivirus infection should be considered. With reproductive disease, an aborted foetus is infrequently presented for examination and is often autolysed. Of the congenital defects that occur, cerebellar hypoplasia is the change that is most suggestive of BVDV infection, especially when associated with cavitating defects of the adjacent cerebrum. Cerebellar hypoplasia is an important lesion to differentiate between BVDV and Akabane virus infection in areas where Akabane virus may be present. Lesions observed in BVDV-induced respiratory disease are invariably those associated with and resulting from the secondary infections that occur. With cases of mucosal disease, grossly there is ulceration of the mucosa of the gut and varying, but often severe, lymphoid depletion is observed on microscopic examination.

8. Diagnostic Tests and Specimens

Specimens are likely to be examined for evidence of pestivirus infection for:

- (a) diagnosis of specific disease incidents;
- (b) certification of the health status of animals;
- (c) screening of materials of animal origin used in assays and the preparation of various biologicals, for example, vaccines.

Health certification may be required for a diversity of commercial purposes. These range from sale and introduction of animals to herds of known disease status through to selection for artificial breeding programmes, including selection of both donors and recipient cows for embryo transfer and bulls for entry to artificial insemination centres.

Crucial to accurately diagnosing pestivirus infections is an understanding of the pathogenesis of foetal infection, the occurrence of virus persistence and the development of the immune response. Infection of the foetus at less than about 100 days of gestation will result in persistence of the virus in most foetal tissues including blood, while antibody will be found in blood and body fluids following later infection. The possible occurrence of either virus or antibody in specimens (and the selection of appropriate tests) must not be overlooked, especially when testing specimens for disease diagnosis.

8.1 Disease Diagnosis

Due to differences in the viruses that may infect sheep and goats compared with cattle, and also difference in the levels of virus in small ruminants, the requirements for sheep and goats should be considered separately to cattle.

8.1.1 Cattle

In the live animal, disease may be associated with either persistent or acute transient infection. Transient infection is usually involved in cases of reproductive or respiratory disease.

For the diagnosis of persistent pestivirus infection in a live animal, samples of blood (either clotted or unclotted) are usually sufficient and testing is directed at the detection of either virus (live virus or viral RNA) or viral antigen. Some laboratories limit virus or antigen detection to animals that are seronegative or have low antibody titres [agar gel immunodiffusion (AGID) reaction of 1 or virus neutralising (VN) titre <20]. However, a small proportion of animals, especially those in the terminal stages of disease, may have higher antibody titres. Consequently, testing should ideally proceed regardless of antibody status.

Virus isolation by cultivation in cell cultures can be undertaken using serum, the blood clot or buffy coat (white blood cell fraction) from unclotted blood. Plasma from unclotted blood is less suitable because of the potential for residual anticoagulant to adversely affect cell cultures. Viral RNA is detected using the polymerase chain reaction (PCR) and can be undertaken on either serum or white blood cells. Detection of antigen uses the antigen capture ELISA. Extraction of antigen from buffy coat cells is preferred to blood clot or serum. Either heparin or EDTA is a suitable anticoagulant. Each of these assays will detect both Type I and Type II strains of BVDV. When animals have circulating antibody, either maternally derived from colostrum or produced to a related virus as occurs in the terminal stages of BVDV infection, testing of the clot or serum is less satisfactory. The antibody can block capture of antigen in the ELISA. Cells, however, contain significant amounts of antigen in addition to that incorporated into infectious virions and intact cells can be washed to remove the inhibiting antibody.

When testing calves under about five months of age, maternal antibody may mask the seronegative status encountered in most carrier animals and may interfere with virus isolation. If virus isolation is being used, the latter problem can usually be overcome by collecting eye and nose swabs, or by washing of white blood cell preparations. The antigen (ELISA) test has also largely overcome the problem of maternal antibody masking persistent infection and will detect antigen in most animals, irrespective of antibody titre, provided washed white cells are used. Maternal antibody should not interfere with testing by PCR.

When transient postnatal pestivirus infection is suspected (for example, in cases of enteric or respiratory disease), nasal or ocular swabs should be collected into viral transport medium (for example, phosphate buffered gelatin saline [PBGS]). While virus isolation in cell culture or PCR will demonstrate viraemia in these cases, the antigen ELISA, due to its lower sensitivity will invariably give a negative result. Alternatively, seroconversion may be demonstrated by testing acute and convalescent sera. The latter should be collected three to four weeks after the onset of illness. The detection of antibody is also of significance in the colostrum-deprived neonate or in fluids from a stillborn or aborted foetus that has been infected in the second half of pregnancy.

At necropsy of either the foetus or postnatal animal, samples of pleural or pericardial fluid are preferred to heart blood for serology to circumvent problems associated with postmortem haemolysis. Antibody titres in these fluids are not significantly different to serum. The preferred specimens for antigen detection by ELISA or for PCR are spleen, lung and mesenteric lymph nodes. For virus isolation fresh lung or spleen are preferred. When respiratory disease is present, an advantage of submitting lung for virus isolation is the potential for detection of other viral

pathogens. Scrapings of intestinal mucosa, especially adjacent to erosive lesions, can be submitted for antigen detection in the AGID test.

Several other diagnostic methods have been used for BVDV screening or disease diagnosis overseas. However, these methods are not in routine use in Australia or New Zealand. These include:

- immunoperoxidase staining on formalin fixed tissue sections. The key monoclonal antibody for such testing is not available in Australia. This method is relatively expensive and has been limited to research applications but does allow confirmation of BVDV infections when only fixed tissue is available.
- detection of BVDV antigens in skin biopsy samples by immunoperoxidase staining or antigen ELISA, usually for large-scale detection of PI animals. The most frequently collected sample is a small portion of skin from the ear pinna, often taken at the same time as the application of an ear tag. In addition to the unavailability of key reagents, testing by immunoperoxidase staining is not cost-effective for large scale screening. These biopsy samples can be readily tested by antigen ELISA but there has not been validation of tests for use in Australia and such tests may not distinguish between persistent and transient infections, even on repeat sampling at intervals of longer than 4 weeks. There has been some evaluation of this test in New Zealand but it has not been adopted for routine use.
- testing of bulk milk samples by PCR to detect the presence of one or more PI animals in a dairy herd.

The types of serological tests commonly used for testing for pestivirus antibody are:

- (a) The AGID test;
- (b) The VN test;
- (c) The antibody ELISA.

Each of these tests has a role in the diagnosis of pestivirus infection. The choice of one or more of these tests depends to a large degree on the purpose for the testing. The AGID test is recognised to be group reactive and will reliably detect antibody to all strains of pestivirus, whether of bovine, ovine or porcine origin. In contrast, the VNT is less broadly reactive and reflects a degree of serotype specificity. It is, however, usual for a significant degree of cross-reactivity to be detected between most strains of pestivirus in the VNT. Choice of an endemic strain of virus as antigen for the VNT should usually allow the detection of neutralising antibody. The indirect ELISA has been used less extensively than the AGID or VN tests but is similar in specificity to the VNT. The Complex-trapping Blocking (CTB) ELISA⁷ has high sensitivity and has the advantage that it can be used to identify antibodies against specific viral proteins by varying the monoclonal antibody that is used. Use of this assay has however been limited to research applications.

An important difference among the three main serological tests is their ability to detect antibodies at different times after the cessation of viraemia in animals undergoing acute transient infection (in comparison with the persistently infected animal). In the first two weeks of seroconversion, the AGID test and VNT are slightly more sensitive than the ELISAs but after long periods (greater than nine months) the VNT and ELISA are more sensitive than the AGID test. The VNT and ELISA are then of similar sensitivity. The strength of the reaction in the AGID test can be used as an indicator of whether or not infection occurred recently. Reactions of '3' generally do not persist for more than about six to nine months in most animals. Similarly, reactions of '>3' are rarely found at times other than from one to three months after infection (see Table 1). Seroconversion can be demonstrated equally well with each of these tests if the acute sample is collected very early in infection and the convalescent sample is collected about three weeks later. At other times after infection, the VNT most reliably reflects changes in antibody levels, with titres rising for at least three months after infection has occurred.

The preceding comments relate to herds and populations in which vaccination against BVDV has not been undertaken. Vaccination has been practised in New Zealand for some years using several different inactivated vaccines. An inactivated vaccine was also released in Australia in October 2003 and is in relatively widespread use. Antibodies induced by vaccination will not be detected by the AGID test. As a result, it is unlikely that vaccination will interfere with the use of this test for diagnostic purposes. However, when the VNT or antibody ELISA is used to estimate the prevalence of antibody in a herd, the results should be interpreted in the light of the use of vaccine in the herd under test.

In the persistently infected carrier animal, antibodies can sometimes be detected. This is because the immunotolerance is specific to the antigens of the strain of virus responsible for the persistent infection. If the animal encounters an antigenically remote strain of virus, it may produce antibodies to the antigens that differ from those of the persistent strain. Such reactions are often detected in the AGID test but are less common in the VNT and the titres are very low.

8.1.2 Sheep and goats

Generally the same diagnostic tests and sampling strategies that are used for cattle apply to sheep and goats. However, due to the very low levels of virus that are often found in persistently infected sheep, virus isolation is the most appropriate test for the detection of persistently infected animals. If an ELISA is used to detect antigen in samples of blood, it is essential to test white blood cell preparations. Testing of serum or clotted blood will often yield negative results for a persistently infected animal. Further, the selected ELISA should have proven pestivirus group reactivity, by specific evaluation of samples from BDV-infected sheep.

8.2 Health Certification

Cattle: Testing for this purpose is aimed almost exclusively at the identification and exclusion of the persistently infected 'carrier' animal. The submission of a clotted blood sample for the preparation of serum is usually adequate for virus isolation. With the use of the antigen ELISA, white blood cell preparations are preferred to achieve maximum sensitivity. While some commercially available antigen ELISA kits advocate the use of whole blood or serum as a specimen, such specimens are not recommended where certification for freedom from BVDV is required because of the reduced sensitivity and also interference from antibody. If young animals, which are likely to have residual maternal antibody, are to be assessed in cell culture, eye and nose swabs should also be collected for virus isolation. In some countries, especially the USA, samples of skin (usually as punch biopsies from the ear) are collected for testing either by antigen ELISA or by immunoperoxidase (IPX) staining. It is probable that such samples will overcome the problem of interference from maternal antibodies in young animals.

While pestivirus can be detected in the germ plasm (semen, ova, embryos) of carrier animals, the certification of such materials can be most effectively achieved by the initial testing of the donor animal to demonstrate freedom from persistent infection. If this is not possible, virus isolation is the preferred method. Care should be taken to ensure that a sufficient quantity of material is examined (for example, at least 5 straws of semen) and specimens are prepared to minimise toxicity. Care should also be taken in assessing the status of pregnant animals. The presence of a high antibody titre does not exclude the possibility that these animals have become infected during the first trimester of pregnancy and hence could be carrying a persistently infected foetus.

Sheep and goats: The same testing considerations that are followed for cattle also apply to small ruminants but virus isolation is the preferred method for the detection of persistent infections. When the antigen ELISA is used, only white cell preparations should be tested and the test should have a proven ability to detect BDV.

8.3 Specimen Storage

8.3.1 Blood and serum samples

Blood and serum samples should be chilled during transport to the laboratory and can be stored for about four weeks at 4°C without significant decline in virus, antigen or antibody titre. Freezing at -20°C or lower is preferred for longer storage but repeated freeze-thaw cycles should be avoided. Note, however, that if white cell preparations are being made for antigen extraction, the buffy coat should be separated within a few days of, and not more than 1 week after, collection. When it is necessary to freeze unclotted blood, the buffy coats should be removed and the cell pellets frozen separately.

8.3.2 Swabs

Swabs should be placed in phosphate buffered gelatin saline (PBGS) immediately after collection and chilled while being transported to the laboratory. On receipt at the laboratory, the cotton tipped swab should be removed from the PBGS and the fluid frozen at -20°C or lower if virus isolation cannot be commenced within a few days. Freezing and thawing more than once may result in a significant reduction in the virus titre.

8.3.3 Tissues

When virus isolation is to be conducted, if not examined within a few days of receipt, tissues should be stored frozen at -20°C or, preferably, lower. Tissues can be stored for several weeks at 4°C if they are to be tested by antigen ELISA or PCR.

Ruminant Pestivirus Infections

Part 2: Diagnostic Test Methods

1. Detection of Bovine Viral Diarrhoea Virus

Specimens are likely to be examined for evidence of pestivirus infection for:

- (a) diagnosis of specific disease incidents;
- (b) certification of the health status of animals;
- (c) screening of materials of animal origin used in assays and the preparation of various biologicals (for example, vaccines).

For diagnostic testing, it may be necessary to test for either the virus (or its antigens or RNA) or for antibodies. Testing for health certification almost exclusively requires detection of persistently infected animals using virus (or antigen or RNA) detection methods. When various biological materials of animal origin are being screened for pestivirus, on most occasions testing is limited to ensuring freedom from infectious virus but occasionally there may also be a requirement to test for antibodies to BVDV.

The regular occurrence in cattle of foetal infection with bovine pestivirus has had a major impact upon the reliable diagnosis of pestivirus infections. Virus isolation procedures for pestivirus rely on the use of bovine or ovine cells and usually bovine serum. Extreme care must be taken to ensure that both cells and serum are screened for freedom from adventitious virus. Serum should also be free of antibody to pestivirus. Inadvertent use of contaminated culture components is likely to have a profound effect on both virus isolation and serology results, producing either false positives or false negatives. However, other than animal inoculation, virus isolation by growth in cell culture is the only method that will allow the detection of infectious virus. To detect very low levels of virus it may be necessary to passage material for many weeks. As a result, virus isolation can be slow and relatively expensive. The antigen capture ELISA has the marked advantages of being quick, relatively inexpensive and not being dependent on cell culture, but does not have the sensitivity of virus isolation or PCR and cannot discriminate between residual non-infectious antigens in tissues and the presence of live virus. PCR has similar limitations to the antigen ELISA in not being able to discriminate between the presence of residual RNA and live virus. It has relatively high sensitivity compared with the antigen ELISA and takes a similar length of time but is more expensive.

1.1 Virus Isolation Procedures

The reliable diagnosis of pestivirus infection by virus isolation depends on the availability of cell cultures and medium supplements, especially serum, that are free of adventitious virus and specific antibody. Different batches of primary bovine cells and cells of different types vary in their susceptibility to virus infection and should be checked prior to use for virus isolation. Contamination of the virus isolation system with adventitious virus or antibody can result in false positive or false negative results as well as reducing the sensitivity of the procedure.

1.1.1 Selection of Cell Culture Components

Although it is essential to confirm the lack of adventitious contamination of both serum and cell cultures, there is no substitute for starting with 'raw' materials from donor animals of known freedom from pestivirus.

Adult bovine serum from a pestivirus-free donor herd is usually adequate for the growth of most cell types used for pestivirus work. The free status of such a herd should be checked as close to the time of collection as possible. If new-born or foetal calf serum is required, it should preferably be collected from the progeny of cows of known pestivirus-free status. If collections are to be made from calves of known status at abattoirs, *extreme* care should be taken to minimise the likelihood of accidental contamination of the serum during collection. Commercially collected foetal calf serum *should not* be used for pestivirus diagnosis or research because it is inevitably contaminated. Gamma-irradiation of serum may remove most, or all, of the infectious virus. However, there is usually antibody present, often at levels that escape routine detection methods but still sufficient to affect virus isolation. Commercially collected horse serum is also under suspicion because of the possibility of cross-contamination from foetal calf serum during processing in the commercial laboratory.

Tissues for the preparation of cell cultures should be obtained only from newborn animals of known pestivirus-free status (or from a foetus of a cow or ewe of known status). Cell lines grown in foetal calf serum should be viewed with great suspicion. Established cell lines from commercial sources should be considered as contaminated until exhaustive testing proves otherwise.

1.1.2 Screening Procedures

In order to reliably screen new culture components, it is essential to have available serum and cell cultures that are free of pestivirus. The recommended procedures to be followed are as follows.

1.1.2.1 Cell cultures

The cultures to be screened should be allowed to grow to confluence. After removal of the culture medium, the monolayer should be washed thoroughly with phosphate buffered saline (PBS). The cells are then subcultured for five passages in medium containing pestivirus (and antibody) free serum. Duplicate cultures should be available for *in situ* immunoperoxidase (IPX) staining at five to seven days after each subculture. At the first subculture, portion of the cells are also taken and used to inoculate susceptible indicator cell cultures. These cultures are screened for evidence of infection by IPX staining for at least three passages. Known pestivirus-free cultures should be run in parallel to provide uninfected controls.

The cells are generally considered to be free of pestivirus if they pass both of the above screening procedures. If additional confirmation of freedom is required, 10^8 washed cells from the first passage can be used to inoculate a pestivirus and antibody-free calf that is held in isolation from other livestock. Serum should be collected at the time of inoculation and four weeks later. If the calf fails to seroconvert, the cells are considered to be free of pestivirus.

1.1.2.2 Medium components

Serum or serum-derived medium supplements should be tested for the presence of both antibody and virus. When testing for antibody, the virus neutralisation test (VNT) should be used. An antibody ELISA may also be used for additional screening but should not be a substitute for the VNT. In the VNT, the test serum should give a negative result when tested in a two-fold dilution series commencing at 1/2. If the test serum passes the ELISA and VN tests, final confirmation of freedom should be provided by quantal assay. This is carried out by using the test serum as the serum supplement (10%) in growth medium for a virus assay. As a control, a duplicate assay should be run in parallel using reference antibody-free serum. The single dilution of virus used should give virus growth in 50–80% of wells in 1 or more 96-well microplates. The virus titre in

the test plate should not be significantly lower than the control plate when virus titres are calculated.^{8,9}

Testing serum for the presence of adventitious virus is similar to the protocol described for the screening of cells (see 1.1.2.1). In this case, serum samples are examined with pestivirus-free cells as follows:

- (a) Cells are grown for five passages in the test serum with screening by IPX 5-7 days after each subculture.
- (b) Monolayers are inoculated with the test serum and subcultured for three passages. The test serum should be allowed to adsorb at 37°C for one to two hours, after which the culture medium is changed to medium containing known BVDV free serum. The indicator cells should show no evidence of pestivirus infection after the third passage. Uninfected control cultures, grown in pestivirus free serum, should be tested in parallel. This approach is taken to minimise the inhibitory effects of antibodies that may also be present in the serum.

Note that both of the above screening methods should be used for testing of bovine serum.

1.1.3 Selection of Cell Type

A range of bovine and ovine cells are suitable for pestivirus isolation and serology. When a new stock of cells is being produced, after passing quality control criteria for freedom from adventitious agents, they should be tested for susceptibility to infection with pestivirus. For this testing, it is necessary to have proven cells and a virus stock of known titre. The test cells are then used to titrate the virus stock to confirm they give the titre obtained in the cell standard. If the titre is more than 0.5 log(10) lower than in the standard cells, the test cells are rejected. A similar procedure can be used to determine the yield of virus from a new batch of cells.

Cell types (bovine or ovine) routinely used for pestivirus testing include the following:

1.1.3.1 Primary cells

Primary cells are usually testis, lung or kidney cells. These are generally obtained from a foetus or neonate. The tissues are diced, trypsinised and then held frozen for subsequent end-use without previous culture.

1.1.3.2 Secondary cells

Secondary cells are commonly testis, kidney or lung cells. As these cells are subcultured three to six times prior to use, there is the advantage of being able to produce a much larger (and hence longer lasting) batch of cells than primary cells. Generally these cells are derived from the same source as primary cells. Limitations may be required on the number of subcultures permitted. Secondary testis cells, for example, become less susceptible to infection after the fifth passage. Similarly, secondary testis cells, irrespective of passage level, frequently do not demonstrate cytopathic effect (CPE) well. The cell type should be selected for the specific application in mind. The passage limits for all secondary cell types should be established by confirming the sensitivity of the cells to a virus preparation of known titre.

1.1.3.3 Continuous cells

Continuous bovine cells have been derived from turbinate tissue (commercially available bovine turbinate line), kidneys (Madin Darby Bovine Kidney, MDBK) or lung (not available commercially). When obtained from commercial sources, extreme care should be taken to ensure freedom from adventitious virus. Like secondary cells, CPE is not easily seen with some cytopathogenic isolates. There are a number of continuous ovine cell lines but these are rarely used for pestivirus work.

1.2 Virus Isolation Methods

As most isolates of pestivirus are not cytopathogenic in cell culture, replication of the virus is usually monitored by antigen detection methods. The isolation of pestivirus in cell culture can be usually considered in two stages:

- (a) inoculation and passage of the specimen in susceptible cell cultures; and
- (b) screening of cultures for the presence of antigen.

1.2.1 Reagents for virus isolation

Antibiotics – cell culture grade:

Penicillin
Streptomycin
Amphotericin B

Phosphate buffered saline (PBS)

NaCl	8.0 g
Potassium chloride, KCl	0.2 g
Disodium hydrogen phosphate, Na ₂ HPO ₄	2.9 g
Potassium dihydrogen phosphate, KH ₂ PO ₄	0.2 g

Make up to 1 L with water and adjust pH to 7.4.

Phosphate buffered gelatin saline (PBGS)

Phenol red	0.4 mg
Gelatin	5.0 g
PBS (pH 7.4)	1 L

Dissolve gelatin in PBS at 56°C, add phenol red, dispense in 5 mL volumes and autoclave at 121°C for 20 min.

1.2.2 Preparation of Specimens

Serum, pleural fluid or pericardial fluid - No preparation is needed.

Blood clots – Virus isolation from blood clot was practised routinely in the past but is not essential. Serum is now preferred because of the ease of culturing. It has been shown that serum and blood clot usually contain similar amounts of infectious virus. If clots are to be tested, freeze and thaw, then add a volume of penicillin and streptomycin solution (50,000 units/mL) equal to 10% of the volume of the specimen. After mixing, the specimen is ready to be cultured.

Tissues - Fresh spleen, lung, or lymph nodes are the preferred tissues. Prepare a 10% (w/v) homogenate of the tissue in cell culture growth medium (no serum) or PBGS containing 1000 units/mL of penicillin and streptomycin, and 4 ug/mL of amphotericin B. Centrifuge at 800 g for 10 min at approximately 4°C, discard the cell pellet and centrifuge the supernatant again. The supernatant is then ready to be cultured.

Swabs - After removal of the swab from the transport medium (PBGS is preferred), centrifuge at 800 g for 10 min at approximately 4°C. The supernatant is then ready to be cultured.

Semen - For each individual batch of semen that has been submitted, a minimum of 5 straws of extended semen is required. If there are multiple collections from the same animal on different days, these requirements still apply, ie 5 straws are needed from each semen batch. Just prior to examination, thaw each straw of semen from the batch(es) to be examined and remove the contents of each of the 5 straws. Pool all of the semen from a single collection batch and estimate the volume (this should be approximately 1 mL).

Dilute the pooled semen 1/10 in serum-free cell culture medium (suitable for the cell type to be used). The cell culture medium should contain double strength antibiotics and an antifungal agent. The total volume of diluted semen should be approximately 10 mL. Centrifuge the diluted semen at 1500g for 20 min at 4°C. Collect the supernatant and discard any pelleted material. Filter through a 0.45 µm filter.

The supernatant can be stored at 4°C for up to 48 hours. Once the cell cultures have been inoculated (see below) the residual supernatant should be stored frozen at approximately -70°C.

1.2.3 Inoculation of Cultures

Generally, specimens are inoculated onto slightly subconfluent monolayers of the cell type offering maximum sensitivity to BVDV infection. Traditionally these cells have been grown in sealed tubes rather than microplates. Tubes offer the advantages of specimen biosecurity, minimising the potential for cross-contamination between specimens, and permit easy manipulations of the culture especially facilitating medium changing after inoculation with potentially cytotoxic specimens.

For health certification, where fresh serum is the specimen, if the likelihood that specimens contain potentially zoonotic or other dangerous pathogens is low, cultures can be grown and inoculated in 96-well microplates. Microplates are not recommended for primary passaging of samples for disease diagnosis. Care should be taken to avoid contamination of adjoining wells. Monolayers that have been seeded 24 hours earlier and are approaching confluence offer maximum sensitivity. The specimen is inoculated directly into the growth medium, which is not changed at any time. Multichannel pipettes can be used to passage specimens from plate to plate.

All cultures should be incubated for 5 to 7 days after inoculation, frozen and thawed and then passaged to the cell cultures, which will be used for the final antigen detection. This second passage should be incubated for five days prior to staining. Although most virus infected specimens could be detected if screening was employed at the end of the primary passage, there are occasional specimens where a second passage is required to achieve maximal sensitivity. Further, problems of non-specific staining from residual inoculum can occasionally be encountered if the primary culture is screened without the second passage.

Known positive (virus infected) and uninfected specimens should be run as controls with each batch of specimens. When using microplate cultures for health certification, it is preferable that controls are included on each plate. Culturing of uninfected specimens as a routine negative control provides a further control to check for adventitious contamination of any of the cell cultures or reagents.

1.2.3.1 Inoculation of Tube Cultures

- (a) Inoculate 0.2 mL of sample (fluid, tissue homogenate, serum or blood clot) to duplicate tubes. Tube cultures should be slightly subconfluent, with tissue culture medium freshly changed to maintenance medium prior to inoculation. There are special requirements for semen samples. For semen samples, the specimen has been diluted 1/10 during sample preparation. Prepare a further 1/10 dilution of this material (ie a 1/100 dilution of the original fluid) in serum-free cell culture medium immediately prior to inoculation of tube cultures. Remove the growth

medium from each of 8 cell culture tubes and rinse the monolayer with serum-free cell culture medium. To each of 4 tubes add respectively 1 mL of either the 1/10 diluted semen supernatant or the 1/100 dilution of supernatant.

- (b) After rolling the tubes for two hours the medium is again changed, decanting tissue culture fluid into separate containers for each specimen to prevent cross-contamination of tubes. Add new maintenance medium and incubate tubes at 37°C.
- (c) At six days after inoculation, freeze tube cultures at -20°C prior to sub-culturing into microplates to screen for antigen. For routine virus isolation, this will result in 2 passages of the specimen in cell culture. Additional passages are usually limited to testing of biological materials such as vaccine components.
- (d) Pool the contents of all tubes inoculated with the same specimen. Passage 50 uL of pooled culture fluid to each of 4 wells of a microplate containing almost confluent cells. Cultures that have shown evidence of the presence of a cytopathogenic agent should be handled carefully, and perhaps separately, to cultures that show no evidence of the presence of an infectious agent.
- (f) After incubation (37°C with 5% carbon dioxide) for another 5-7 day period, prepare the monolayers for antigen detection (see 1.2.4 below)

1.2.3.2 Inoculation of Microplates for Virus Isolation from serum

Note: the method described below is specifically intended for screening of serum from animals for health certification purposes.

- (a) Prepare 96-well microplate cultures of the appropriate cells (bovine or ovine origin for cattle samples; only ovine cells for sheep and goat samples). Seed plates at a sufficient density that cells are almost confluent at the time of use (24 hours after seeding).
- (b) Add 50 uL of serum to each of 4 wells.
- (c) Add 50 uL of pestivirus free bovine serum to each of 4 wells to serve as a negative control and, lastly, 50 uL of a non-cytopathogenic strain of BVDV (or BDV for sheep samples) to each of 4 wells to serve as a positive control. Add the positive control carefully to avoid any splashing or aerosol spread to adjacent wells.
- (d) Incubate plates in a 5% CO₂ incubator at 37°C. Check the plates after 24-48 hours to check for any cytotoxicity or microbial contamination.
- (e) After 5-7 days incubation, freeze the plates at or below -20°C.
- (e) After thawing contents of plates, passage 50 uL of culture fluid from each well to the corresponding wells of a new plate containing almost confluent cells.
- (f) After incubation for another 5-7 day period, prepare the monolayers for antigen detection (see 1.2.4 below)

1.2.4 Screening of Cultures for Antigen

For the detection of pestivirus antigen in cell cultures, screening usually employs an antibody to identify infected cells. The most commonly adopted techniques use an indirect detection method with a second antispecies antiserum that is either coupled to a fluorescent dye¹⁰ or is enzyme-labelled to react with a chromogenic substrate that will stain the infected cells. The more common method is the use of peroxidase labelling¹¹, which often can be read macroscopically or microscopically with a conventional light microscope. In addition, a permanent record, in the form of the stained cultures, can be kept for future reference. The greatest problem with immunofluorescence is the need to use a fluorescent microscope, and the lability of the fluorescent

dye. Further, immunoperoxidase staining is ideally suited if there is a need to examine cells *in situ* in a larger vessel such as a cell culture flask.

The primary antibody must be broadly reactive with pestivirus group antigens. A specific polyclonal antiserum of high titre may be used but has largely been replaced with monoclonal antibodies. Monoclonal antibodies with proven pestivirus group reactivity are now available and should be used whenever possible. These antibodies offer the advantages of minimal background staining and the availability of a continuous supply of reagent of uniform quality. If the screening passage is carried out in 96-well culture plates, the cells can be fixed *in situ* and the subsequent washing and staining steps automated similar to an ELISA. The antigen detection method is as described below.

1.2.4.1 Equipment

- 96-well tissue culture microplates
- Automatic plate washer

1.2.4.2 Reagents for immunoperoxidase staining

- Monoclonal antibodies

These are available commercially (see Part 3). The supplier will usually indicate an approximate working dilution. However, new batches of antiserum should be titrated by checkerboard titration with known infected and uninfected cells to determine optimal dilutions before use.

- Peroxidase conjugated second antibody

Use any commercially available, good quality horseradish peroxidase conjugated anti-mouse antiserum with monoclonal antibodies. New batches of conjugate should be titrated by checkerboard titration with known infected and uninfected cells to determine optimal dilutions before use.

- Formaldehyde/NP40 solution (3% formaldehyde, 0.1% NP-40)

- Skim milk powder

- Gelatin

- Phosphate buffered saline (PBS) – see 1.2.2

- Phosphate buffered gelatin saline (PBGS) – see 1.2.2

- 3-Amino-9-ethylcarbazole substrate

3-Amino-9-ethylcarbazole (AEC)	10 mg
Dimethylsulfoxide (DMSO)	3 mL
Sodium acetate (20 mmol/L), pH 5	50 mL

H₂O₂, 30%

40 uL

Dissolve AEC in the DMSO. Immediately before use add the AEC/DMSO solution to the acetate buffer and then add hydrogen peroxide.

1.2.4.3 Immunoperoxidase Staining Cell Cultures

The method described below is for the staining of cells in a 96-well microplate but can be adapted for the *in situ* staining of cells in any vessel.

- (a) Fix the microplates for 10 min by adding 200 uL of 3% formaldehyde (1:10 dilution of 30% formaldehyde stock solution in PBS) containing 0.1% NP-40 to all wells. Add this fixative to the plate without removing the tissue culture medium from the wells.
(Note: if there may be any need to passage the specimens further, or to retain any potential virus isolates, an appropriate quantity of culture supernatant should be removed before addition of the formaldehyde solution).
Discard the fixative/culture supernatant after 10 min. Although 20% (v/v) acetone (CH₃COCH₃) may be used as an alternative fixative to formaldehyde, it should not be used if plates are to be washed in an automated plate washer as the cells are often washed off the plate.
- (b) Wash the fixed monolayers 3 times using an automatic microplate washer with 0.05% Tween 20/water. Other wash solutions may be suitable but should first be checked against proven methods.
- (c) Block the monolayers with 5% skim milk powder in PBS (150 uL/well) at 37°C in a humidified chamber for one hour. (Note that, when monoclonal antibodies are used for staining, this step may be omitted if there is minimal non-specific binding).
- (d) Wash five times as in (b).
- (e) To each well add 50 uL of a mixture of group reactive monoclonal antibody (MAb) mixture (at an appropriate dilution in 1% gelatin/PBS) and incubate at 37°C in a humidified chamber for 90 min. The optimum concentration should be determined for each batch of MAb mixture by 'checker-board' titration against reference positive and negative controls.
- (f) Wash five times as in (b).
- (g) Add 50 uL/well of peroxidase conjugated rabbit antimouse immunoglobulin at the optimum dilution in 1% gelatin/PBS and incubate at 37°C in a humidified chamber for 90 min. The optimum concentration should be determined for each batch of conjugate by 'checker-board' titration against reference positive and negative controls.
- (h) Wash five times as in (b).
- (i) Develop with 3-amino-9-ethylcarbazole (AEC) substrate (100 uL/well) and read after 30 min by examination with an inverted microscope. Infected cells have reddish-brown cytoplasmic staining and uninfected cells are unstained.

1.2.4.4 Interpretation

Specific staining of cells in the screening culture is *prima facie* evidence of persistent infection in the test animal. Virus isolation methods will occasionally detect an animal that is not a carrier and is undergoing acute transient infection. This can be confirmed by retesting the animal at any time three weeks after the collection of the original sample. At this time, an animal undergoing an acute primary infection will no longer be viraemic and will have seroconverted. However serological testing of paired sera will usually provide the quickest result.

1.3 Detection of Pestivirus Antigen

The introduction of the pestivirus antigen capture ELISA^{12,13} has revolutionised the identification of animals with persistent pestivirus infection. The method does not rely on cell culture and, therefore, is less expensive and is relatively quick, allowing a diagnosis to be made within 1-2 days rather than several weeks. It also allows much larger numbers of samples to be examined.

The test is based on combinations of antibodies to capture the antigen(s) onto the plastic surface of a 96-well ELISA plate and another set of antibodies to detect the bound antigen. Some tests¹² use a broadly reactive polyclonal antiserum to capture the antigen and a mixture of several group reactive monoclonal antibodies to detect the bound antigen. Other tests may use monoclonal-monoclonal^{13,14} or polyclonal-polyclonal combinations for the capture-detector combinations. Because of the use of these mixtures of antibodies, the test is highly specific for pestiviruses but is also broadly reactive within the pestivirus group. A correctly configured ELISA will detect all known isolates of BVDV (both Type I and Type II strains), BDV and the porcine pestivirus, classical swine fever virus (CSFV). In some tests, each sample is tested against an irrelevant monoclonal antibody to confirm that non-specific binding of reagents has not occurred. It is also possible to vary the combinations of monoclonal antibodies to configure a test that is specific for an individual pestivirus species, that is, to be BVDV, BDV or CSFV specific.

A variety of specimens are suitable for testing in the ELISA, with a preference for specimens that contain a high proportion of cells from the live animal, either blood clot or peripheral blood leukocytes (PBLs or buffy coat cells) from unclotted blood (EDTA or heparin), though the latter are preferred for cattle and are essential for sheep. PBLs provide maximum sensitivity for testing from the live animal due to the very high concentration of antigen containing cells in the specimen. A further advantage of PBL preparations over blood clot (and serum, see below) is the potential to wash the cells to remove interfering antibodies. It is also possible to test serum¹⁵ in some antigen ELISAs but the assay is not as sensitive as a test based on detection of antigen from cells and is more prone to interference from antibodies (for example, colostral antibody in young calves). At necropsy a range of specimens are suitable but lung, spleen and mesenteric lymph nodes are preferred. The tissues that are least satisfactory are liver, kidney and heart, largely due to non-specific binding that interferes with the assay. Specimens can be held refrigerated (for up to 4 weeks) or frozen prior to testing, but buffy coat preparations should be separated from the red cells prior to freezing or if they are to be stored chilled for more than 1 week. Processing of specimens for ELISA testing is outlined in section 1.3.2.

Although the pestivirus antigen ELISA is now the preferred test for detection of carrier animals and for the diagnosis of disease associated with persistent infection, its limitations must be recognised. While the test has high sensitivity for the detection of persistently infected animals, it will not detect transiently infected animals (eg for the investigation of respiratory disease in feedlots) and is not suitable for the certification testing of biological materials (for example, vaccines, foetal calf serum).

A number of commercial kits are available. Some kit protocols indicate that serum or whole blood may be tested. However, it must be recognised that to obtain maximum sensitivity, washed buffy coat preparations are essential. This is important for regulatory testing (entry to AI centres, export) and export protocols should be carefully examined to confirm the type of specimens that are permitted. Details of the commercial kits and reagents used in Australia and New Zealand are described in Part 3 of this document. Because there can be considerable variation between the performance (sensitivity and specificity) of different commercial kits, evaluation data must be submitted to SCAHLS for approval prior to use for either regulatory or diagnostic purposes.

1.3.1 Reagents for Sample Preparation.

- 1% NP-40 in PBS

5 mL Nonidet P-40 detergent (analytical grade)

495 mL PBS

Store in a sterile 500 mL bottle at 4°C and label with the date. Do not keep longer than 1 month.

- Phosphate Buffered Saline (PBS) – see section 1.2.1.

1.3.2. Sample Preparation

Prior to testing, the antigen is extracted from the specimen with a non-ionic detergent. The detergent extract supernatants can be stored at 4°C for up to two weeks before testing or stored indefinitely at -20°C. The methods are as follows:

1.3.2.1 Peripheral blood leucocytes

- (a) Centrifuge 10 mL heparin or EDTA blood specimens at approximately 1500 g for 20 min.
- (b) Carefully remove buffy coat using a plastic transfer pipette. *N.B.* Contamination with some red cells does not matter. Place white blood cells into a 10 mL centrifuge tube and add 5 mL ice cold (4°C) 0.17 mol/L ammonium chloride (NH₄Cl). Mix cells and leave for 10 min to lyse any red cells.
- (c) Fill centrifuge tube with cold PBS and mix. Centrifuge at approximately 1000 g for 10 min.
- (d) Discard supernatant and add 1 mL of 1% NP-40 in PBS to the pellet of white cells. Mix by shaking thoroughly and leave at room temperature for two hours with occasional mixing or at the time specified by the kit manufacturer.
- (e) Centrifuge at approximately 1000 g for 10 min and decant supernatant for assay.

1.3.2.2 Blood clots

- (a) Chop the clot from 10 mL of blood into small pieces (about 2–5 mm) using sterile scissors and forceps and place in a 10 mL centrifuge tube. Alternatively, macerate clots that have not been frozen by forcing through a 10 mL syringe. Add 2 mL of 1% NP-40 in PBS and mix thoroughly. Leave at room temperature for two hours (or as specified by the kit manufacturer), mixing occasionally.
- (b) Centrifuge at approximately 1000 g for 10 min and decant supernatant for assay.

1.3.2.3 Tissues

The preferred tissues are spleen, mesenteric lymph node and lung. Tissues can be stored at 4°C for up to one month before processing provided they are sterile and freshly collected. The test will usually work with poorer quality samples but the optical densities (ODs) may be lower.

- (a) Chop about 2 g of tissue into small pieces (about 2–5 mm) in a suitable centrifuge tube using forceps and scissors. Add 5 mL of 1% NP-40 in PBS and shake well. Leave at room temperature for two hours, shaking well about every 30 min.
- (b) Centrifuge at approximately 1000 g for 10 min and decant 1.0–1.5 mL of the supernatant into a microcentrifuge (Eppendorf) tube. Place in a microcentrifuge at 10,000 rpm for two minutes. Use the clarified supernatant for the assay.
- (c) Some antigen ELISA assays includes a negative (irrelevant) monoclonal antibody to detect non-specific reactivity. When specimens give a high optical density against the negative

monoclonal (indicative of non-specific binding), the sample extract should be retested after microcentrifuging 10,000 rpm for two minutes. Test the supernatant from the re-centrifuged specimen extract in the assay and also dilute this re-spun supernatant 1:5 with 1% NP-40/PBS and test this diluted material as well.

1.3.3 Pestivirus Antigen Enzyme-linked Immunosorbent Assay

The antigen ELISA kits and reagents that are available in Australia are described in Part 3 of this document. All kits must be used strictly in accordance with the manufacturer's instructions and results interpreted according to the criteria defined by the manufacturer. Any variation from the manufacturer's instructions must be supported by a full set of validation data that is generated locally. Note the comments at Section 1.3 above regarding the selection and testing of different types of specimens.

1.4 Detection of Nucleic Acid

The polymerase chain reaction (PCR) is sometimes used for the detection of nucleic acid from pestiviruses. Because it is necessary to first synthesize complementary DNA (cDNA) from viral RNA, a reverse transcription (RT) step is necessary. This is then followed by the PCR amplification of the cDNA. To achieve sufficient sensitivity, it is also usual to employ a second ('nested') amplification step. Consequently, a pestivirus-specific PCR is relatively expensive. Currently this test is only used for diagnostic or research purposes and in its current form is not practical for the screening of large numbers of samples for regulatory purposes (for example, cattle for export). However, the test is relatively quick, has high sensitivity and has the advantage of being able to detect nucleic acid from virus that is either defective or is no longer infectious. This can be an advantage for the diagnosis of pestivirus in an aborted foetus. Nevertheless, some of these features can also be disadvantageous. For example, the test will detect residual viral nucleic acid in materials of biological origin (such as foetal bovine serum) after the material has been treated to inactivate the contaminating virus. Consequently, the test cannot be used to certify freedom from infectious virus. Extreme care must be taken to ensure that there is not cross-contamination of materials or reagents or between different steps of the procedure as a result of the release of amplified nucleic acid into the environment. It is strongly recommended that the different steps in the test are conducted in completely separate laboratories. Staff involved in the procedure should move systematically between laboratories during a working day, starting in 'clean' areas (where there is no PCR product generated) and moving to areas that are progressively more likely to be contaminated with PCR product (for example, electrophoresis areas) last. It is also essential that sufficient controls are included in the assay to monitor for contamination and false positive results. The use of 'closed tube' systems and 'real time' PCR help to reduce these problems.

1.4.1 Reverse Transcriptase Nested PCR (rt-PCR) for Pestiviruses

There is no standardised rt-PCR method for pestiviruses and individual laboratories have developed 'in house' preferences. However, it is generally accepted that primers based on key segments of the 5'UTR region of the virus will consistently generate a positive result with all strains of BVDV.¹⁵ With the use of appropriate primers it is also possible on most occasions to discriminate between viruses of Type I and Type II.¹⁷ Care should be taken interpreting results based on a PCR for BVDV Type II because some primer sequences are known to generate positive results with some Australian viruses that are known to belong to Type I (as all Australian viruses appear to be). Examples of suitable methods for the rt-PCR are included in the publications listed.^{16,17,18}

1.4.2 'Real Time' PCR for Pestiviruses

'Real-time' PCR technology has the advantage of very high sensitivity and does not require detection by electrophoresis of amplified nucleic acid. Further, it is possible to conduct these assays in a 'closed tube' system, greatly reducing the risk of cross-contamination. Examples of suitable methods are included in the publications list.^{19,20,21}

2. Detection of Antibodies to BVDV

The types of serological tests commonly used for testing for pestivirus antibody are:

- (a) The AGID test;
- (b) The VN test;
- (c) The antibody ELISA.

Each of these tests has a role in the diagnosis of pestivirus infection. The choice of one or more of these tests depends to a large degree on the purpose for the testing. The AGID test (section 2.1 below) is recognised to be group reactive and will reliably detect antibody to all strains of pestivirus, whether of bovine, ovine or porcine origin. In contrast, the VN test (section 2.2) is less broadly reactive and reflects a degree of serotype specificity. It is, however, usual for a significant degree of cross-reactivity to be detected in the VNT between most strains of pestivirus within a species group (for example, between BVDV strains). This cross-reactivity is, however, much less marked between Type I and Type II strains of BVDV. Choice of an endemic strain of virus as antigen for the VNT should usually allow the detection of neutralising antibody. Originally the VNT employed a cytopathogenic reference isolate²², but this may be antigenically distant from local isolates. As non-cytopathogenic strains of pestivirus are more widespread in the population, the choice of a non-cytopathic isolate is preferred. A very reliable VNT based on non-cytopathogenic virus is described in the peroxidase-linked neutralisation assay.²³ The indirect ELISA test (section 2.3) has been used less extensively than the AGID or VN tests but is similar in specificity to the VNT.

2.1 Agar Gel Immunodiffusion Test

This method is adapted, with minor amendments, from that described in the original ASDT.²⁴

2.1.1. Preparation of Medium

- (a) Add 7 g of SeaKem ME Agarose (FMC Bioproducts, Cat no. 50014) to 700 mL of borate buffer. The recipe for borate buffer as follows:

NaOH	2.0 g
Boric acid, H ₃ BO ₃	9.0 g
Sodium azide, NaN ₃	1.0 g

 Add 1 L of type 1 (distilled) water and adjust pH to 8.6.
- (b) Heat in a microwave oven on the highest power available for 10 min.
- (c) Cool to 56°C in a waterbath and pour 15 mL into 9 cm diameter plastic petri dishes.

2.1.2. Preparation of Antigen

- (a) Infect bovine cell monolayers in large culture vessels (eg, roller bottles or 'cell factories'). For best antigen production, the cells should be grown to confluence in medium with foetal calf serum and changed to maintenance medium with serum free of BVDV antibody prior to the

addition of the inoculum. The inoculum should contain a virus dose of about 1 TCID₅₀ (tissue culture infective dose 50%) per cell.

- (b) After 4 days pour off the tissue culture fluid and discard. If a cytopathogenic strain of virus is being used, harvest very early when there is early (1+) evidence of CPE. Harvest cells with trypsin/versene solution and pellet by centrifugation at 800 g for 10 min.
- (c) Resuspend the cells in a small quantity of the trypsin/versene solution and freeze at -20°C. The antigen is ready for use when thawed.
- (d) The antigen is tested at dilutions against a standard reference antiserum to establish the dilution that reacts in optimum proportion and as an unknown antigen in a standard test to establish its specific activity.

2.1.3. Reference Antiserum

There is no specific protocol for the preparation of highly reactive precipitating antiserum. Hence, reference sera are usually collected opportunistically from animals that develop adequate antibody after natural or experimental infection. The serum should be examined rigorously for specificity and carefully balanced by dilution to react in optimum proportion with a standard reference antigen. If serum of adequate reactivity is not available then a reference antibody may be prepared from a weaker serum by total globulin precipitation by half-saturated ammonium sulphate and dialysis against 0.9% (w/v) (0.15 mol/L) NaCl.

2.1.4. Agar Gel Immunodiffusion Test

The method described below was originally developed as a standardised method for the AGID to provide optimal sensitivity. Any variation from this method (eg smaller well sizes, spacing) should be extensively evaluated prior to routine application.

- (a) Cut wells in the agar in the petri dishes. Patterns of seven wells, one central and six peripheral, in a hexagonal pattern, are cut exactly perpendicular to the agar surface. The agar plugs should be removed carefully, so that well walls and the agar surface are undamaged and not touched. The wells should be 5–6 mm in diameter and 2 mm apart.
- (b) The peripheral wells are identified as No 1 near the perimeter of the dish and 2–6 counted clockwise from it. Six patterns should fit around the perimeter of a dish. The space left in the centre, which would accommodate a seventh pattern, must not be used.
- (c) Using 50 µL of solution per well, load reference antigen in the central well and reference serum in wells 1, 3 and 5. Specimens to be tested for antibody or antigen should be loaded into wells 2, 4 and 6. This arrangement is important as it prevents reactivity between different patterns. Any unused wells on the plate should be filled with borate buffer.

2.1.5. Reading and Interpretation of Results

- (a) Read the test after holding the plates for 24 hours at room temperature in a humidified atmosphere. Negative or suspicious reactions should be re-examined at 48 hours.
- (b) Examine the petri dishes in subdued light with the dish illuminated from beneath by a narrow beam of light (eg, an egg-candling light) at an angle of about 45°.
- (c) Antibody activity in the test sample turns the reference lines within the peripheral hexagon and antigen causes reference lines to turn outward between peripheral wells. Quantitation is done by comparison of the test with the reference lines.
- (d) The following scale is used for reporting results (Table 1.)

Table 1. Scale for reporting the results of the agar gel immunodiffusion test

<i>Antibody</i>	<i>Antigen</i>	<i>Comments</i>
>3	++++	Denotes sample stronger reactivity than reference. Care should be taken with sera of very high antibody titre that inhibit precipitation on adjacent reference lines. They should be retested at dilutions.
3	+++	Denotes sample reactivity equivalent to reference
2	++	Denotes sample weaker in reactivity than reference but producing a continuous line across the face of the test well
1	+	A distinct turn on the end of the reference line
Non-specific		Lines of precipitation which either cross or fail to establish a line of identity with the reference line

2.2. Virus Neutralisation Test — Peroxidase Linked Neutralisation Assay

Originally the VNT employed a cytopathogenic reference isolate²¹, but this may be antigenically distant from local isolates. As non-cytopathogenic strains of pestivirus are more widespread in the population, the choice of a non-cytopathic virus isolate is preferred. The preferred method to detect virus neutralising antibodies against pestiviruses is the peroxidase linked neutralisation assay. This assay uses non-cytopathogenic virus, the biotype that is most widely encountered in the field. The neutralising peroxidase linked assay is similar to a conventional virus neutralisation test except that the challenge virus is not cytopathogenic.^{21,22} The growth of the challenge virus is detected by immunoperoxidase staining. This allows the use of a virus isolate that is more likely to be representative of most isolates infecting local cattle, as most isolates are non-cytopathogenic. It also allows a wider range of cell cultures to be used, (for example, secondary bovine testis cells, which do not show CPE clearly). If a cytopathogenic virus is to be employed it should be selected from the Australian livestock population and be shown to have antigens that are common to a majority of isolates.

2.2.1. Virus Neutralisation Test

- Inactivate sera to be tested by heating at 56°C. Dilute sera in 96-well tissue culture microplates. Sera are tested in duplicate starting at a 1:4 dilution. Allow 2 sets of wells for the neutralisation test and a third set of wells as a 'serum control' to monitor for toxicity of the test samples. Add 75 uL of diluent (complete growth medium) to the first row of wells and 50 uL to all other wells. Make doubling dilutions, using a multichannel pipette, by adding 25 uL of test serum to the first row, discarding tips and transferring 50 uL to the next row to finally give 50 uL of diluted serum per well. Discard 50 uL from the last row of wells. Include as a positive control a serum with a known neutralising titre.
- Add the noncytopathogenic virus (50 uL/well), diluted in tissue culture medium to contain 100 TCID₅₀/50 uL. The final dilution will vary depending on batch of virus stock and cell type used. The titre of the stock virus should be measured in the same cell type used for the VNT. The 'Trangie' strain of BVDV has been used in many laboratories as an Australian reference non-cytopathogenic virus.
- Incubate at 37°C for one hour.

- (d) In each test, titrate the virus used to check the amount of challenge virus used per well. Leave four to eight wells uninfected as a cell culture control. A known positive serum should also be included in each test for standardisation purposes.
- (e) Add cells in growth medium (100 uL/well) at the appropriate seeding rate to achieve confluence in about 24 hours.
- (f) Incubate plates at 37°C in 5% carbon dioxide, 85% relative humidity, for five days.
- (g) Immunoperoxidase stain the monolayers in the microplates as described in Section 1.2.4.3 (above). Read endpoints as the highest dilution at which there is no staining in at least 50% of the wells. Any focal staining, whatever the extent, should be scored as positive, indicating no specific antibody. If a viraemic animal is tested, this status will be confirmed by the presence of staining in the serum control wells.

2.2.2. Acceptance Criteria

- (a) The control virus titration should be correct to within 0.5 log₁₀ (preferably within 0.3 log₁₀) of the preferred challenge dose (2.0 log₁₀) for the results to be accepted.
- (b) If the titration exceeds 10^{2.5}, positive results can be accepted but the actual titres may be higher than currently observed.
- (c) If the titre is too low (<10^{1.5}), only negative results should be accepted and tests of all other sera repeated.
- (d) The positive reference serum should not vary by more than four-fold.

2.2.3. Notes

- (a) Use a cell line that supports growth of pestivirus to high titre, such as primary or limited passage bovine testis or foetal bovine kidney cells.
- (b) Stock virus should be prepared and stored in small volumes at -70°C, then diluted before use.

2.3 Indirect Enzyme-linked Immunosorbent Assay for Antibody

This test is usually used only for research or perhaps diagnostic purposes where the antibody status of a large number of animals needs to be determined. A number of ELISA kits are now commercially available and are often preferred. Minimal validation testing has been undertaken in Australia. The relative sensitivity and specificity of kits from different suppliers should be determined by extensive testing with a large collection of sera of known status, both positive and negative, in the VNT. Any data already generated in Australia should be reviewed in the light of the introduction (October 2003) of an inactivated vaccine for pestivirus as this may affect the 'background' reactivity and the determination of test 'cut-off' values. It may be possible, with the selection of an appropriate assay, to distinguish between naturally infected and vaccinated animals.

2.4 CTB ELISA

The Complex-Trapping Blocking (CTB) ELISA⁷ is a very sensitive assay for antibody. It is effectively based on a variation of the antigen capture ELISA and has the advantage that it can be used to detect antibodies specific for individual viral proteins (for example, anti E2, anti NS3). This epitope-specific reactivity is determined by the individual monoclonal antibody that is used in the assay. At present, this assay is only used for research purposes in Australia, but a test based on this method is widely used overseas for detection of antibodies to classical swine fever virus.

3. Diagnosis of Border Disease Virus Infection

While all of the assays described above for the diagnosis of BVDV infection can be used for the diagnosis of Border disease infection in sheep, attention should be given to the following issues.

3.1 Virus isolation

The general principles and precautions outlined in Sections 1.1 & 1.2 should be closely adhered to with the additional points to be noted:

- Cell type and control: Ovine cells with proven high sensitivity to BDV should be used, with an isolate of BDV as a positive control;
- Antigen detection: While the monoclonal antibody(ies) used for immunoperoxidase staining should be pestivirus group reactive, they must have proven reactivity with a true BDV isolate.

3.2 Antigen Detection by ELISA

Due to the antigenic variation of BDV isolates compared with BVDV, any antigen capture ELISA used for testing of sheep samples must be validated for its sensitivity to detect BDV isolates by testing of both a range of cell culture isolates and field specimens. At the time of completion of the review of this procedure (March 2006), while there are a number of antigen ELISAs that have proven group reactivity, they have been optimised for the detection of BVDV in cattle samples. Few of the commercially available assays have validation data for the testing of sheep samples. Due to the lower levels of virus and antigen in BDV infected sheep, compared with BVDV in cattle, if ovine blood samples are to be tested in an antigen ELISA, it is imperative that white cell preparations are used as the test sample. Of the kit reagents that have been shown to react with BDV viruses that have been amplified in cell culture, none has an adequate body of data generated from the testing of clinical specimens (eg blood samples) from sheep.

3.3. Antibody detection

The AGID and VNT are both suitable for testing of sheep sera. Within the limits of sensitivity described in Section 2 (above), the AGID test will readily detect antibody in sheep infected with BDV. When the VNT is required, a BDV isolate should be used as the test (challenge) virus.

4. References

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Part 3: Reagents and Test Kits for Pestivirus Diagnosis in Australia and New Zealand

The list of suppliers provided below may not be exhaustive but includes all materials (complete kits and other reagents) that have been evaluated and found to be suitable for use under Australian or New Zealand conditions. These reagents and kits have been approved only for use in Australia or New Zealand for the purposes and within the limits described below. Other suppliers who have materials that may be used for the purposes described below are welcome to submit reagents and kits evaluation by contacting the Executive Officer, SCAHLS <www.scahls.org.au>.

1. Antigen ELISA Kits

A number of antigen ELISA kits developed both in Australia and overseas are now available commercially in Australia and New Zealand. However, these vary in sensitivity, largely as a result of the specificity and reactivity of the monoclonal and polyclonal antibodies used, recommended specimen types and sometimes modified incubation procedures. These kits should be thoroughly evaluated on a wide range of samples of known pestivirus status, including a number of samples containing pestivirus-specific antibodies (both maternal and naturally acquired, if possible). For regulatory purposes, it is essential that buffy coat samples are prepared to achieve maximum sensitivity and the most reliable results. Tests that use either serum or whole blood as the preferred specimen are generally prone to blocking from antibody (and hence production of false negative results). While these tests offer convenience from the perspective of a need for minimal or limited processing of samples, a failure to detect a persistently infected animal in a herd can have significant consequences.

The kits that have been evaluated for use in Australia and New Zealand are manufactured by IDEXX Scandinavia AB (Osterbybruck, Sweden), Institute Pourquier (Montpellier, France) and Synbiotics (Europe) (Lyon, France). Reagents for the antigen ELISA are also available in bulk from the Elizabeth Macarthur Agricultural Institute (see 3 below). The respective kits and their performance characteristics are as follows:

1.1 IDEXX

a) IDEXX Herdchek BVDV Ag/Leukocytes

This kit is intended to be used for testing of antigen extracted from tissues, nasal swabs, leucocytes and cell cultures. The test detects several of the different viral proteins and has high sensitivity and specificity. Washing of leucocytes collected from the blood of young animals will minimise the adverse effects of colostral antibody. This test is based on a combination of polyclonal antibodies and will detect all strains of BVDV. The kit has also been shown to react with a wide range of strains of BDV, based on the testing of BDV viruses propagated in cell culture but the reliability of this kit to detect BDV antigen in clinical specimens from sheep is unknown.

b) IDEXX Herdchek BVDV Ag/Serum Plus

This kit is intended to be used for direct testing of serum, plasma, whole blood and skin biopsy (ear notch) samples. With a monoclonal antibody as the detector, the assay exclusively detects the protein E^{ms} (previously known as Eo). The test has relatively high sensitivity and specificity and is claimed to be comparable in sensitivity to the Herdchek BVDV Ag/Leukocytes assay. However, the presence of antibody, either maternal or produced after postnatal infection, may result in a false

negative result. The test has very poor sensitivity in detecting strains of BDV and could not be recommended for testing of sheep samples, even for diagnostic purposes. This kit is suitable for testing of skin biopsy samples but it should be noted that it has not been evaluated for use for this purpose in Australia. Preliminary testing would suggest that test cut-off values may need to be adjusted to avoid generation of false positive results.

1.2 Institut Pourquier

a) *Institut POURQUIER ELISA BVD/MD Total Antigen Screening (P00621E)*

This kit is intended to be used for testing of antigen extracted from tissues, leucocytes, blood clots, whole blood and cell cultures. The test detects several of the different viral proteins. Although the test has moderately high sensitivity, it will generate some false positive results. Washing of leucocytes collected from the blood of young animals will minimise the adverse effects of colostral antibody. This test is based on a combination of polyclonal antibodies and will detect all strains of BVDV. The ability of this kit to detect BDV antigens is not known.

In New Zealand, this kit may also be used for the detection of BVDV antigens in serum, plasma, milk, urine and mucosal fluids. However, in Australia, use of the kit for testing these samples is prohibited by a patent.

b) *Institut POURQUIER ELISA BVD/MD Antigen Mix Screening (P00623)*

This kit is intended to be used for testing of antigen extracted from leucocytes. The test detects the NS2/3 (formerly p80) and E^{ms} (formerly E⁰) viral proteins. Although the test has high moderately high sensitivity, it will generate some false positive results. Washing of leucocytes collected from the blood of young animals will minimise the adverse effects of colostral antibody. This test is based on a combination of polyclonal antibodies and will detect all strains of BVDV. The ability of this kit to detect BDV antigens is not known.

1.3 Synbiotics

a) Serelisa BVD p80 Ag Mono Indirect (ASBVD6)

This kit is intended to be used for testing of antigen extracted from tissues, leucocytes, blood clots and whole blood. The test detects the NS2/3 (formerly p80) viral protein. The test has moderately high sensitivity, but may generate some false positive results. Washing of leucocytes collected from the blood of young animals will minimise the adverse effects of colostral antibody. This test is based on a combination of monoclonal and polyclonal antibodies and will detect all strains of BVDV. The kit will also detect antigens from CFSV and BDV but for maximum sensitivity for the detection of BDV antigens, washed white blood cell extracts should be tested. The sensitivity for the detection of BDV is not as high as virus isolation in cell culture and is not suitable for testing for regulatory purposes.

In New Zealand this kit may also be used for the detection of BVDV antigens in serum, plasma, milk, urine and mucosal fluids. However, in Australia, use of the kit for testing these samples is prohibited by a patent.

2. Antibody ELISA Kits

While the suppliers of antigen ELISA kits listed above and also Svanova Biotech AB (Uppsala, Sweden) each has kits available for the detection of antibodies to BVDV, only two kits have been evaluated for use in New Zealand. These are the ELISA Antibody P80 Screening Serum & Milk, P0645 (supplied by Institut Pourquier) and the HerdChek BVDV Ab (supplied by IDEXX Laboratories).

None of these kits has been evaluated for use for regulatory purposes in Australia. If a laboratory chooses to use a kit for diagnostic purposes, it should undertake a comparison with the VNT to determine its suitability for use. There are both indirect and blocking ELISA kits available. Some will allow the discrimination between the use of an inactivated vaccine and natural infection.

3. Reference Sera, AGID and other Reagents

The following reagents are available from the Elizabeth Macarthur Agricultural Institute:

3.1.1. Cell culture

Pestivirus-free bovine serum.

3.1.2. Virus isolation and Antigen Detection

Antiserum for antigen detection by immunoperoxidase or immunofluorescence. Both polyclonal and monoclonal antibodies are available but the latter are preferred.

3.1.3. Antigen capture enzyme-linked immunosorbent assay

All reagents for this test are available either in complete 'kit' form or individually. Supply of reagents in kit form is recommended as all reagents have been calibrated for optimal reactivity. This kit has proven reactivity against pestiviruses from all species, including ruminants, pigs and wildlife. The kit remains in the original prototype format¹², without the shortening of certain steps that has occurred in commercially available kits. These reagents are designed to detect pestivirus antigens from tissue extracts, peripheral blood leukocytes (buffy coat) and blood clot. The test is does not have sufficient sensitivity to consistently detect antigen in serum. These reagents can also be used to produce a CTB ELISA.

3.1.4. Agar gel immunodiffusion test for antibody

Both antigen and reference serum are available.

3.1.5. Virus neutralisation tests

Reference viruses and control sera (positive and negative) are available. Monoclonal antibody for peroxidase staining can be supplied.

4. Suppliers of Reagents and Kits.

The commercial distributors of the above ELISA kits and reagents for pestivirus diagnosis are:

4.1 Australia:

Elizabeth Macarthur Agricultural Institute – AGID, BVDV free serum, monoclonal antibodies, reference virus, control sera.

Virology Laboratory,
EMAI,
PMB 8,
Camden, NSW 2570,
Tel. (02) 4640 6338;
Fax (02) 4640 6429.

IDEXX kits – Antigen ELISA kits

IDEXX Laboratories Pty Ltd,
Unit 20/38-46 South St.,
Rydalmere NSW 2116
Tel: 02 9898 7300 or 1800 655 978
Fax: 02 9898 7302 or 1800 634 409

Institute Pourquoi – Antigen ELISAs

Laboratory Diagnostics
Unit 8, 106 Canterbury Rd.,
Bankstown NSW 2200
Tel: 02 8707 4222 or 1800 023 623
Fax: 02 8707 4200

Svanovir – Antibody ELISA

Australian Laboratory Services
PO Box 328,
Sydney Markets NSW 2129
Tel: 02 9764 4055 or 1800 252 286
Fax: 02 9764 3533

Synbiotics (Europe) – Antigen ELISAs

Laboratory Diagnostics
Unit 8, 106 Canterbury Rd.,
Bankstown NSW 2200
Tel: 02 8707 4222 or 1800 023 623
Fax: 02 8707 4200

4.2 New Zealand:

IDEXX kits – Antigen and Antibody ELISA kits

IDEXX Laboratories Pty Ltd
Unit 20/38-46 South St.,
Rydalmere NSW 2116

Tel: NZ Freecall 0800 102 084

Fax: NZ Freecall 0800 448 443

Institute Pourquoi – Antigen and Antibody ELISAs

Diagnostic Bioserve

PO Box 5246

Mt Maunganui, NZ.

Tel: 64 7 542 2325

Fax: 64 7 542 2326

Synbiotics kits – Antigen ELISAs

Alpha-Scientific,

C/- Gribbles,

57 Sunshine Ave, Hamilton, NZ.

Tel: 64 7 850 0777