AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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# Akabane Disease

## *Histopathology, Virology and Serology*

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## 1. Introduction

Major epizootics of a congenital disease of cattle, characterised by arthrogryposis (AG) and/or hydranencephaly (HE), and associated with infections with Akabane virus, have occurred in a number of geographical areas. Major outbreaks have occurred in Israel in 1969–70 (Markusfeld and Mayer, 1971; Nobel *et al.*, 1971), in Japan in 1972–74 (Omori *et al.*, 1974) and in Australia in 1974 (Della-Porta *et al.*, 1976; Shepherd *et al.*, 1978). Akabane virus infections of the foetus were associated with these epizootics when serum neutralising antibodies to Akabane virus were found in presuckling serum samples from the affected calves (Miura *et al.*, 1974; Omori *et al.*, 1974; Hartley *et al.*, 1977). Since then Akabane disease (the name given to congenital defects caused by Akabane virus; Inaba *et al.*, 1975) has been described for cattle, sheep and goats and its viral aetiology confirmed by experimental infection studies (Inaba *et al.*, 1975; Parsonson *et al.*, 1977; Kurogi *et al.*, 1977*a*, 1977*b*; Narita *et al.*, 1979).

Akabane virus is a member of the virus family *Bunyaviridae*, in the Simbu serological subgroup (Bishop and Shope, 1979; Porterfield and Della-Porta, 1981). There are at least 23 Simbu serogroup viruses, seven of these being found in Australia. The virus contains three segments of single-stranded ribonucleic acid surrounded by a nucleoprotein, which principally carries the group-specific antigens, and then an envelope containing two glycoproteins, which represent the type-specific antigens involved in neutralising and haemagglutination-inhibiting antibody responses. There is also a small number of molecules of a large protein, associated with the nucleoprotein, probably involved in replication of the viral nucleic acid.

## 2. Epidemiology

Akabane virus was first isolated in Japan by Oya *et al.* (1961), in Australia by Doherty *et al.* (1972), and in Kenya and South Africa (Porterfield and Della-Porta, 1981). Serological studies (Porterfield and Della-Porta, 1981) have further extended the known distribution of the virus to Israel, Cyprus, Thailand and possibly Taiwan, Vietnam, Indonesia, Malaysia and the Philippines. Serological evidence suggests that Akabane virus is not present in Papua New Guinea (Della-Porta *et al.*, 1976; Cybinski *et al.*, 1978). It would seem likely that the distribution of Akabane virus stretches from Australia, through South-east Asia to Japan. Possibly it is also present in India and across the Middle East to Israel and Africa. Congenital disease associated with Akabane virus is found principally at the extremities of its normal distribution.

In Australia, congenital bovine AG/HE has been observed as epizootics in south-eastern New South Wales, at least since the mid-1940s (Blood, 1956). Epizootics were observed in 1951, 1955, 1960, 1964, 1968, 1974 and 1983 (Porterfield and Della-Porta, 1981; Murray, 1987; P. Kirkland, unpublished data 1988). Similar, but smaller, epizootics have been observed in the Hunter Valley and the New England Tablelands (Coverdale *et al.*, 1978).

Australia-wide serological surveys for the presence of neutralising antibodies against Akabane virus in cattle blood (Della-Porta *et al.*, 1976; Cybinski *et al.*, 1978) have shown that about 80% of the animals in northern Australia become infected early in life. The distribution of seropositive animals has been linked with the distribution of the biting midge, *Culicoides brevitarsis*. The disease has been associated with an extension of virus-infected insects beyond their normal distribution, due to extended warm, humid summers, to areas where susceptible pregnant animals are present. These areas are either at the extreme southern distribution limits for *C. brevitarsis*, particularly the south-eastern coast of New South Wales, or in elevated areas where the insects are not usually present, for example, the New England Tablelands.

## 3. Clinical Signs

There is no evidence that Akabane virus produces ill effects in cattle, sheep and goats infected after birth although virus replication, as indicated by viraemia, occurs (Kurogi *et al.*, 1977*a*, 1977*b*; Parsonson *et al.*, 1977). Akabane disease is characterised by the development of lesions in the foetus of susceptible animals. In Japan the disease was first seen as an increase in the number of abortions. The first syndrome recognised in Australia, in the 1974 epizootic, was AG which reached its peak incidence around August (Shepherd *et al.*, 1978) while the peak incidence of HE in the same epizootic occurred in September. The susceptible pregnant animals were probably infected with Akabane virus during the late summer or early autumn.

An epizootic of Akabane disease in cattle may be first noticed by an increased rate of abortions; followed some months later by the birth of incoordinate calves, calves with mild to severe AG and sometimes cervical scoliosis, torticollis and kyphosis; and finally calves with HE sometimes associated with AG, and other minor forms of brain damage. The progression in which these syndromes are seen in the field represents the developmental stages of the foetus at which Akabane virus infection occurred, or greater awareness and better reporting as the epizootic progresses. Often the various pathological syndromes seen in Akabane disease overlap, with many occurring concurrently in the same animal.

Dystocia is usually associated with AG affected foetuses resulting in their death and necessitating delivery by embryotomy or caesarean section. In AG the affected limb joints cannot be flexed or extended through the normal range of movement. Calves born with HE may survive for many months if hand reared but they never thrive. They may show signs of ataxia and lack of coordination, blindness and dysphagia, and regurgitation of food.

## 4. Pathology

The gross pathology of Akabane disease in cattle, sheep and goats is similar, AG and HE being the main lesions seen in all species (Porterfield and Della-Porta, 1981). However, these lesions can differ in their severity, the time at which they occur, and in their detailed histopathology.

AG is seen in about 25–40% of the progeny of cows infected between four and six months of pregnancy. In affected animals the joint surfaces appear normal whereas the bulk of the muscle is usually smaller and paler in colour than normal. Microscopically there is a severe loss of myelinated fibres in the lateral and ventral funiculi of affected areas of the spinal cord. There is loss of ventral horn neurones and marked loss of nerve fibres in the ventral spinal nerves. The lesions are associated with a moderate to severe atrophy and/or adipose tissue replacement of the skeletal musculature supplied by the ventral spinal nerves.

HE is seen in about 25–40% of affected animals. It can be severe when the cerebrum is almost totally replaced by fluid, or less severe and consist of extensive cavitation (porencephaly) of the white matter of the dorsal and ventrolateral cerebral hemispheres with dilation of the lateral ventricles.

Other pathological findings have been reported for calves with Akabane disease (Hartley *et al.*, 1977; Porterfield and Della-Porta, 1981; Kirkland *et al.*, 1988). Calves have been found early in the outbreaks which are incoordinate or unable to stand at birth but without gross pathological lesions. Microscopically there was a mild to moderate non-suppurative acute encephalomyelitis, most evident in grey matter of the mid and posterior brain stem. Towards the end of the 1974 epizootic of Akabane disease in Australia there were a number of calves with additional congenital abnormalities which included micrencephaly accompanied by a thickening of the cranial bones with a resultant reduction in size of the cranial cavity. Some calves had a reduction in the size of the cerebrum which microscopically showed cavitation and collapse of the cerebral white matter. However, serological evidence suggested that a number of these calves with micrencephaly were not infected with Akabane virus.

## 5. Other Simbu Group Viruses

The Simbu serogroup of viruses is found in all continents except Europe and the Antarctic (Porterfield and Della-Porta, 1981). Oropouche and Shuni viruses are associated with human infections. Aino, Akabane, Douglas, Faceys Paddock, Peaton, Thimiri and Tinaroo viruses have been isolated in Australia. There is serological evidence (precolostral sera) that Aino virus may be associated with congenital bovine AG/HE (Coverdale *et al.*, 1978). There is also some serological evidence, from the presence of serum neutralising antibody in precolostral serum, that Tinaroo virus may also be associated with *in utero* ovine infections (T.D. St. George, pers. comm. 1979). As yet, the role of Simbu group viruses, except Akabane virus, in congenital infections has to be conclusively demonstrated.

The serological relationships between the Australian members of the Simbu group viruses have not been fully defined. In general, they are clearly separable by virus neutralisation (VN) tests. Low VN antibody titres in animals (≤8 in the microtitre VN test or < 10 mm in the plaque-inhibition test) need to be treated with caution since low level cross-reactions may occur. Non-specific inhibitions are found in some sera, with low titres (usually < 4), and these can usually be removed by treatment of sera with Kaolin.

All members show some cross-reactivity in the complement fixation (CF) test, although it is not always to equivalent levels. In the agar gel immunodiffusion precipitin (AGID) test, Aino and Peaton shown some cross-reactivity and Akabane virus AGID antigen will detect antibodies in cattle formed against other Simbu group viruses. The enzyme-linked immunosorbent assay (ELISA) shows similar cross-reactivity to that seen in the AGID test. Hence, interpretation of all serological tests needs to be made with care.

## 6. Diagnosis

### *6.1. Introduction*

Diagnosis of Akabane disease is usually based on serology. Attempts to isolate virus from animals at term are likely to prove unfruitful, as most animals possess virus neutralising antibodies at birth (Hartley *et al.*, 1977; Kirkland *et al.*, 1988). The use of pre-colostral serum samples from the affected offspring, and a serum sample taken at the same time from the dam, has proved most useful for providing serological evidence of *in utero* infection by Akabane virus. Serum, including badly haemolysed samples, pleural and pericardial fluids, and/or cerebrospinal fluid (CSF) collected from calves born dead or calves that have to be delivered by embryotomy, are suitable in AGID tests to confirm Akabane infection *in utero*.

### 6.2. Virus Isolation

Isolation of Akabane virus from animals or from insects has usually been accomplished by intracerebral inoculation of one- to two-day-old suckling mice (Oya *et al.*, 1961; Doherty *et al.*, 1972; ). If mice do not develop paralysis by the tenth day after inoculation their brains are harvested and the material repassaged, at a 1:10 dilution of a 10% brain preparation, by intracerebral inoculation of another set of suckling mice. Mice usually become paralysed and die during the initial passage of Akabane virus. Isolations have also been made in cell cultures (St. George *et al.*, 1978; P. Kirkland, unpublished data 1988).
Comparison of the tissue culture isolation systems with suckling mice (Kurogi *et al.*, 1976; Porterfield and Della-Porta, 1981) indicated that HmLu-1, Vero, SVP and BHK21 cell lines were almost as sensitive as mice. A sensitive isolation system is the inoculation of blood onto C6/36 mosquito cells and the subsequent passage onto BHK21 cells (G.P. Gard, unpublished data 1981). Some care needs to be taken on passage because Akabane virus is prone to interference.

Akabane virus has been isolated from two naturally infected bovine foetuses (Kurogi *et al.*, 1976). In one case the foetus was removed from an infected pregnant cow at 92 days gestation and examined for pathological changes and for the presence of virus. The foetus showed signs of AG/HE. Virus was isolated from brain, cerebral fluid, spiral cord muscles, foetal placenta, and amnion, but not from viscera pool, intestine, amniotic fluid or foetal blood. In the other case virus was isolated from a foetus, aborted at 134 days gestation. Virus was isolated from brain but not from heart (the only tissues examined).

The virus has also been isolated from two naturally infected lamb foetuses (Della-Porta *et al.*, 1977). One foetus taken at 89 days gestation, showed gross pathology of AG micrencephaly and the virus isolated from the placentomes, foetal fluids and foetal membranes but not from the lung, muscle, thymus, heart, kidney, spleen, liver, stomach contents and cerebrospinal fluid. The other foetus taken at 74 days of gestation had microscopic lesions of porencephaly and virus was isolated from the placetomas, foetal fluids, foetal membranes, lung, muscle and cerebellum but not from the thymus, heart, kidney, spleen and liver.

Samples from the cerebral remnants and placenta of aborted foetuses should be taken for virus isolation. Other tissues which may prove useful are cerebellum, spinal cord, heart, spleen, CSF and muscle. The tissues are made into a 10% suspension in nutrient broth containing antibiotics (200 units of penicillin, 200 µg streptomycin, and 5 µg fungizone for each mL) and clarified by low speed centrifugation. A sample of the material is stored below -70°C for confirmation of any virus isolation. The fresh material is inoculated intracerebrally (i.c.) (0.05 mL) into a litter (up to six) one- to two-day-old suckling mice for each sample, or into tissue culture tubes of one of the above cell lines (0.1 mL per tube) and rolled at 37°C. The brain is harvested from paralysed mice, or after 10 days, and is repassaged through suckling mice.

If a cytopathic effect (CPE) develops, the cells and fluid are harvested and repassaged (after storing a sample below -70°C) through another set of tissue culture tubes. If no CPE develops, the material is repassaged after five to seven days (depending on the condition of control, non-inoculated tubes always run at the same time). Virus isolates are typed as a Simbu group isolate by FA tests and then identified as Akabane virus by virus neutralisation tests in comparison with prototype strains.

### 6.3. Histopathology

Samples of brain and the cervical, thoracic and lumbar regions of the spinal cord and other tissues should be fixed in 10% neutral formalin (HCHO) for histological examination. All tissues are embedded in paraffin, and sections stained with haematoxylin and eosin (H and E). All central nervous system (CNS) sections are also stained with luxol fast blue. The histological examination should reveal lesions similar to those described under pathology of Akabane disease (see 4.). Pathology similar to Akabane disease does not necessarily confirm Akabane virus as the causative agent. Virus isolation and/or serology are the only tests which identify the causative virus.

### 6.4. Virus Neutralisation Test

All isolates of Akabane virus found so far appear to be serologically identical. The most common serological test is the virus neutralisation (VN) test performed with BHK21, HmLu-l, SVP or Vero cells by the quantal VN test using either tube cultures or microtitre trays or a quantitative VN test, the plaque-inhibition (PI) VN test (Parsonson *et al.*, 1981). Tube cultures or microtitre trays are used with 100 $TCID_{50}$ doses of Akabane virus, respectively (Della-Porta *et al.*, 1976; Kirkland *et al.*, 1983). The virus is mixed with dilutions of the test sera and incubated at room temperature (20–25°C) for one hour. The test is read five days post inoculation when 75–100% of the cell sheet should be expected to be destroyed by the virus. The 50% endpoints ($VN_{50}$) are calculated by the method of Reed and Muench (1938) and titres >4 are considered positive. Non-specific inhibitors are found in some sera, with titres up to four. A detailed description of the method used for the microtitre VN test is in 9.1.

### 6.5. Agar Gel Diffusion Precipitin Test

The agar gel diffusion precipitin (AGID) test offers a means of rapidly screening large numbers of field sera for the presence of antibody against Akabane virus. It is not as sensitive as the VN test and will detect antibodies produced against other related Simbu serogroup viruses (G.P. Gard, unpublished data, A.J. Della-Porta and M.D. Murray, unpublished data 1979). Studies using hyperimmune mouse ascites tumour fluids against Australian Simbu serogroup viruses (McPhee and Della-Porta, 1981) showed that Akabane virus AGID antigen could detect antibodies against Akabane, Aino, Tinaroo and Peaton viruses, whereas Aino virus AGID antigen could only detect antibodies against Aino, Tinaroo and Peaton viruses. AGID antigens are concentrated from infectious cell fluid using a dialysis sac and polyethylene glycol (20 000 m.w.) or ultrafiltration (McPhee and Della-Porta, 1981). The antigen is placed in the centre well surrounded by six outer wells, reference serum and test sera being placed in alternate wells. The test is read after 48 and 72 hours of incubation in a moist chamber at room temperature and the test sera evaluated against the reference antiserum. Details of the AGID test are given in 9.2., the procedure being a modification of the method described by Littlejohns and Snowdon (1980) for mucosal disease.

### 6.6. Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) systems can be adapted for both detection of Simbu serogroup viruses in cell culture and the antibody response to these viruses in serum of host animals. A thorough evaluation of the ELISA test is required before it can be adopted as a standard diagnostic procedure. As this test offers many advantages, it is described in 9.3. so that laboratories can evaluate the test. Virus-specific antibody detection can be achieved utilising an indirect ELISA system. Purified antigen (McPhee and Della-Porta, 1988) is bound directly to the wells of a microtitre tray followed by successive incubations with appropriately diluted test and control sera and a species Ig-specific antibody conjugated to the enzyme horseradish peroxidase (Ide *et al.*, 1989; J.R. White, unpublished data 1987). The extent of specific binding of the conjugate is revealed by addition of an appropriate substrate (e.g. O-phenylenediamine) to yield a chromogenic reaction which can be read spectrophotometrically in an ELISA plate reader. Alternatively, specific antibody may be detected in a 'capture' ELISA system (J.R. White, unpublished data 1987) where microtitre trays are first coated with a Simbu serogroup reactive rabbit antiserum followed by addition of concentrated tissue culture supernatant from virus-infected cells. Subsequent test serum and conjugate addition steps are identical to the indirect ELISA method. The capture ELISA methodology increases the number of steps required and therefore lengthens total test time, but antigen preparation is much simpler than for the indirect ELISA (McPhee and Della-Porta, 1988; Ide *et al.*, 1989). Detection of Simbu serogroup viruses can also be achieved by using the capture ELISA format, with a known positive antiserum being applied instead of test serum. Both antibody and antigen detection ELISA systems are essentially serogroup reactive. It has been observed that particular Simbu viruses and the antisera raised to them (most notably Douglas virus) can show some degree of restriction in their ability to detect heterologous viruses and antibodies (J.R. White, unpublished data 1987). However, Akabane virus isolates and antiserum to this virus exhibit sufficient cross-reactivity with other Australian Simbu viruses and antisera (i.e. Tinaroo, Douglas, Peaton and Aino viruses) to provide a reliable serogroup reactive test. The antigen detection ELISA can be made specific for Akabane virus by incorporating neutralising monoclonal antibodies raised to isolates of this virus (McPhee *et al.*, 1987; Hyatt *et al.*, 1988) as detector antibody in the capture ELISA format.

Detailed descriptions of all ELISA methodologies are provided in 9.3. It should be noted that these are not to be regarded as standard diagnostic procedures but are included for completeness.

### 6.7. Haemagglutination Inhibition Test

Haemagglutination with the Simbu group viruses is both pH and sodium chloride molarity dependent (Goto *et al.*, 1978). The method of Clarke and Casals (1958) was modified by using a diluent with 0.4 mol/L sodium chloride, 0.2 mol/L phosphate at pH 6.0–6.2 for haemagglutination and haemagglutination inhibition (HI). An HI test has been developed for detecting antibodies in domestic animals and gave results which correlated closely with the neutralising antibody titres of the same sera (Goto *et al.*, 1978). The HI test involves antibody reaction with the external virus glycoproteins and is virus type-specific and not group-reactive. Hence it is of use in detecting antibodies which are specific for Akabane virus. A thorough evaluation of the HI test is required before it can be introduced for diagnostic testing, but it offers many potential advantages as a rapid and inexpensive serological test.

### 6.8. Complement Fixation Test

The complement fixation (CF) test has principally been used for comparisons of the relationships between the Simbu group viruses (Doherty *et al.*, 1972) and is the main test used for grouping bunyaviruses into serological subgroups (Bishop and Shope, 1979; Porterfield and Della-Porta, 1981).

## 7. Differential Diagnosis

Agents other than Akabane virus can produce similar congenital deformities in cattle, sheep and goats (Porterfield and Della-Porta, 1981). These causes may be of an infectious nature, or of genetic or environmental origin, or associated with teratogenic chemicals. The cause of some deformities is unknown. The detection of elevated levels of immunoglobulins in pre-colostrum serum samples (using the radial diffusion method as described by Brandon *et al.*, 1971) from offspring may indicate whether an infective agent was involved. Diagnosis of Akabane disease can rarely be accomplished on the basis of virus isolation but depends largely on a combination of gross pathology, histopathology and serology. Note that normal calves can occasionally have antibodies to Akabane virus in their pre-colostral sera (Kurogi *et al.*, 1977*b*).

Possible causes, which need to be differentiated from Akabane virus, are: bluetongue virus, Aino virus, bovine viral diarrhoea–mucosal disease virus, Wesselsbron and Rift Valley fever viruses (in many cases associated with live attenuated vaccine strains of these viruses); toxic substances including alkaloids associated with lupins or feeding of green feed containing *Astragalus* spp., genetic inheritance of arthrogryposis and cleft palate seen in Charolais calves (Hartley and Wanner, 1974), and possibly other unknown causes.

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# 9. Appendixes

## *9.1. Appendix 1 — Microtitre Virus Neutralisation Test*

The virus neutralisation (VN) test for Akabane is carried out with sterile techniques in flat bottomed 96-well microtitre plates, which have been treated to allow for the growth of tissue culture. Two types of VN tests are performed: a screening test at a dilution of 1:4 and a titration of positive sera from 1:4.

### 9.1.1. Serum for Test

The serum is heated at 56°C for 30 min before testing. For *screening tests*, 50 µL of each serum is dispensed into two microtitre wells using a sterile disposable tip on an suitable micropipettor. The serum is diluted 1:4 in the well by adding 150 µL diluent. This dilution is done in duplicate, using two wells on the plate. Then 50 µL of the diluted serum is similarly pipetted into the next two wells, 100 µL serum is discarded, while the 50 µL of diluted serum remaining in the wells is kept as a serum control with diluent added instead of virus. The screening test uses four wells in the plate for each serum, two for the test and two for serum control, thus enabling up to 24 sera to be screened on a plate.

To *titrate a serum*, dilutions for each serum are made in duplicate from 1:4 to 1:64, using either columns 1–5 or 7–12, with columns 6 or 12 being used for a 1:4 serum control. Serum is diluted 1:4 by mixing 50 µL of diluent in wells in column 1 or 7 (as described above). Diluent (50 µL) is added to the next four wells (2–5 or 8–11) and two-fold dilutions carried out using a 50 µL multichannel pipettor. 50 µL is discarded from last dilution well (5 or 11). 50 µL of the 1:4 dilution is added to wells in column 6 or 12, to serve as the serum control and 50 µL discarded from wells in column 1 or 6. All wells now hold 50 µL of diluted serum, up to eight sera can be titrated in duplicate on a 96-well microtitre plate.

### 9.1.2. Virus

The virus to be used is Akabane (isolate R7949 or B8935), which is diluted to contain 100 $TCID_{50}$/50 µL. The virus is diluted in 10-fold steps, from $10^0$ to $10^{-4}$, in 1/2 oz McCartney bottles, and then added to the microtitre wells, four wells per dilution, using a 50 µL multichannel pipette.

### 9.1.3. Diluent

The diluent is Medium 199 + 10% foetal calf serum (FCS). The FCS is heated at 56°C for 30 min prior to use and must be free of antibody to Akabane virus and non-toxic to the cells.

### 9.1.4. Cells

The tissue culture cells are BHK21, HmLu-1 or Vero at a concentration of $3 \times 10^5$ cells/mL. The cells are gently stirred in a flask, while being added to the wells, to prevent settling.

### 9.1.5. Controls

#### 9.1.5.1. Cell control

Four wells receive cell suspension and diluent only.

9.1.5.2. Foetal calf serum, negative control
Four wells receive cell suspension, diluent and 50 µL of FCS.

9.1.5.3. Foetal calf serum, positive control
Four wells receive cell suspension, 50 µL FCS and 50 µL virus suspension.

9.1.5.4. Virus control
The virus is diluted and dispensed in sets of four wells/dilution in ten-fold steps, $10^0$ to $10^{-4}$; the concentration of virus used in the test being $10^0$.

9.1.5.5. Positive serum control
A serum with a known virus neutralising antibody titre is diluted in two-fold steps using four wells/dilution, from 1:2 to two steps beyond its expected titre. Sheep serum of known antibody titre, prepared against plaque purified virus, is available in limited stocks from Head of Laboratory, CSIRO Australian Animal Health Laboratory, PO Bag 24, Geelong, Vic. 3220, Australia. [Tel. (052) 275 000; Fax (052) 275 555).

9.1.5.6. *Individual serum control*
Two wells are inoculated with each individual serum being tested, at a dilution of 1:4 (see 9.1.1.), but without virus, to check for serum toxicity.

9.1.6. Sequence of Test
The reagents are added to microtitre plates in the following order:

(a) Serum (50 µL) (at 1:4 preliminary testing or diluted as per 9.1.1.).
(b) Virus (50 µL) (100 $TCID_{50}$).
(c) Shake.
(d) Incubate 60 min at room temperature.
(e) BHK21, HmLu-1, SVP or Vero cells (0.1 mL) ($3 \times 10^5$ cells/mL).
(f) Seal the plate with a plate sealer, or cover with a microtitre plate lid, and incubate in a carbon dioxide incubator. Alternatively, about 0.1 mL paraffin oil (sterilised by filtration not heat) can be added to each well. Incubate for five days at 37°C in standard incubator. CPE should be clearly observed in test wells.
(g) 50% endpoints are calculated using the method of Reed and Muench (1938). The control positive serum titration and virus titration should not show more than a two-fold variation from normal. If they do, then the test should be repeated.

## *9.2. Appendix 2 — Agar Gel Diffusion Precipitin Test*

9.2.1. Medium

| | |
|---|---|
| Sodium hydroxide, NaOH | 2 g |
| Boric acid, $H_3BO_3$ | 9 g |
| Sodium azide, $NaN_3$ | 0.1 g |
| Seakem agarose | 10 g |

Distilled water to volume of 1 L
Dissolve salts in approximate volume of water and check pH to 8.5–8.6. Adjust with 0.1 mol/L hydrochloric acid (HCl) or 0.1 mol/L sodium hydroxide. If necessary, add agarose, make volume to 1 L and dissolve in a boiling water bath or autoclave. Allow 10 min after agarose has dissolved and bubbles in mixture cease to be apparent. We pipette straight from autoclave and pour 14 mL per 90 mm polystyrene petri dish on a level surface. When set store upside-down in cans in cold for up to several weeks. Preferably do not use before one day old.

*N.B.* The surface characteristics of polystyrene appear to vary, presumably depending on accelerator and polymeriser conditions. Most have a hydrophobic surface allowing agarose gel to release easily and preventing wetting and under-running. If the gel sticks to the polystyrene the plates should be lightly smeared with silicone oil before medium is poured.

9.2.2. The Test
Patterns of seven wells, one central and six peripheral in hexagon pattern are cut in the agar and plugs removed. Wells are 6 mm in diameter with 2 mm spacing between wells. Care should be taken that wells are cut exactly perpendicular to the surface, that neither well walls or surface of the agar are damaged or that threads of the surface 'skin' are not trailed across the surface between wells.

Peripheral wells are identified as 1 near the perimeter of the dish and 2–6 counted clockwise from it. It should be possible to fit six patterns around the plate. Space in the centre of the plate which could accommodate a seventh pattern should not be used.

Reference antigen (50 µL) is loaded into the centre well and reference serum (50 µL) into wells 1, 3 and 5. The dilutions of these reference reagents have previously been balanced to give a sharp central precipitin line. Materials to be tested for antibody or antigen are then loaded into wells 2, 4 and 6. A 50 µL micropipette with disposable plastic tip (Oxford, Ependorf or equivalent) is used. This allows that wells 3 and 5, the most proximate between adjacent patterns, contain the same material.

9.2.3. Reading and Interpretation
The test may be read after 48 hours at room temperature in a humidified atmosphere. Negative or suspicious reactions should be re-examined at 72 hours. Reading should be done in subdued light with the dish illuminated from beneath by incident light (at an approximate angle of 45°). Antibody activity in a test material turns the reference lines inwards within the peripheral hexagon and antigenic activity causes reference lines to turn outwards between peripheral wells. In either case the reaction is roughly quantitated by comparison of the test with the reference line as follows:

>3+ denotes reactant stronger than reference
3+ denotes reactant equivalent to reference
2+ denotes reactant weaker than reference, but producing a continuous line across the face of the test well
\+ denotes a distinct turn on the end of the reference line
? denotes doubt whether a turn can be recognised rather than doubt whether a minor degree of reaction is significant or specific.

Non-specific reactions are recognised as lines of precipitation which either cross, or fail to establish a line of identity with the reference line.

#### 9.2.4. Preparation of Reference Antisera

Highly reactive antisera can be produced in cattle or in sheep using the following protocol. Mouse brain grown Akabane virus, a clarified suspension containing $10^7$ plaque-forming units (PFU) in Hanks balanced salt solution (pH 7.4), is emulsified in an equal volume of Freund's complete adjuvant. First, ensure that a pre-immune bleed is negative for reactivity. The animals are then given at least five subcutaneous inoculations ($10^7$ PFU) of at least 1 mL volume. One month later, the animals are given an intramuscular inoculation of mouse brain grown Akabane virus ($10^7$ PFU), followed by a similar inoculation one month later. The animals are bled weekly and when a strong Akabane positive AGID antiserum is obtained, a large collection of blood is made to prepare the reference antiserum. The serum should be examined rigorously for specificity and carefully balanced by dilution to react in optimum proportion with the standard antigen. The serum should be stored frozen at or below -20°C or lyophilised.

Some reference bovine antisera against Akabane virus and against Aino virus are available from the Head of Laboratory, CSIRO, Australian Animal Health Laboratory, Division of Animal Health, PO Bag 24, Geelong, Vic., 3220, Australia. (Fax 052 275555).

#### 9.2.5. Preparation of Antigen

Large roller vessels of BHK21 cells are used as soon as possible after they become confluent. Cells are maintained in Medium 199 + 0.2% BSA + 30 mmol HEPES (pH 7.4). The cells are inoculated with plaque purified Akabane virus (isolate R7949 or B8935), at a virus dose of about 0.01 PFU/cell, and incubated on a roller apparatus at 37°C. The medium is harvested when the cells show total CPE (usually after three to five days) and clarified of cellular debris by low speed centrifugation (6000 *g* for 30 min). The clarified tissue culture fluid is added to a large dialysis sac and concentrated at 4°C using polyethylene glycol (PEG 20 000), the sac being suspended in a large beaker containing the PEG which is stirred on a magnetic stirrer. The fluid needs to be concentrated about 25–50-fold. Alternatively, the antigen may be concentrated by ultrafiltration using an Amicon stirred cell with a PM10 membrane (McPhee and Della-Porta, 1981).

### *9.3 Appendix 3 — Enzyme-linked Immunosorbent Assay Systems*

The Akabane virus ELISA is *not* an approved standard diagnostic test. It is included for completeness. The AGDP and virus neutralisation tests are the standard procedures. The ELISA gives similar cross-reactivity to the AGDP tests.

#### 9.3.1. Materials and Reagents

##### 9.3.1.1. Round-bottomed 96-well polyvinyl microtitre trays (Cooke, Dynatech).

##### 9.3.1.2. Purified Virus

Akabane virus is grown in Vero cells (m.o.i. 0.01–0.1) using Medium 199 + 5% inactivated FCS. Medium is harvested after development of CPE and the antigens concentrated (x30) in an Amicon ultrafiltration cell (model 402) containing an XM-50 membrane. Purification is as according to McPhee and Della-Porta (1988). Briefly, concentrated medium is purified by centrifugation in a linear sucrose gradient (20–50% w/v) in TES buffer (0.01 mol/L Tris-HCl pH 7.6 + 0.001mol/L EDTA + 0.1 mol/L sodium chloride) plus 0.2% BSA. The antigens are centrifuged at 24 000 rpm for four hours in a Beckman SW28 rotor. Virus particles are re-diluted in TES + 0.2% BSA and pelleted at 24 000 rpm for two hours.

##### 9.3.1.3. Precoat buffer [0.05 mol/L Tris-HCl + 0.15 mol/L NaCl (pH 9.)]

(a) Solution A, Tris-base 6.05 g, sodium chloride 8.78 g, water up to 100 mL.
(b) Solution B, Tris-hydrochloric acid 1.58 g, sodium chloride 1.76 g, water up to 200 mL.

Add 90 mL of solution A with 10 mL of solution B.

##### 9.3.1.4. Substrates

9.3.1.4.1. O-phenylenediamine (OPD). About 40 mg of OPD is dissolved in 1 mL of methanol ($CH_2OH$) and added to 49 mL of citrate-phosphate buffer pH 5.0 [24.85 mL 0.2 mol/L disodium hydrogen phosphate ($Na_2HPO_4$) + 24.15 mL 0.2 mol/L citric acid ($C_6H_8O_7$)]. Immediately before using in the assay, 5 µL of 30% (v/v) hydrogen peroxide ($H_2O_2$) is added and stirred rapidly.

9.3.1.4.2. 3,3,5,5 Tetramethylbenzidine (TMB). 0.2 mL of 42 mmol/L TMB in DMSO + 18 mL water + 2 mL citrate–acetate buffer [100 mL of 1 mol/L sodium acetate ($CH_3CH_2Na$) adjusted to pH 5.9 using 1 mol/L citric acid]. About 25 µL of 3% hydrogen peroxide (v/v) is added and rapidly mixed just prior to use.

### 9.3.2. Indirect ELISA for Detection of Simbu Serogroup Specific Antibody

#### 9.3.2.1. Antigen attachment

Purified antigen is diluted in precoat buffer at a dilution pre-determined by checkerboard titration. 100 µL of the solution is added to the wells of a polyvinyl microtitre tray. Plates are incubated overnight at 4°C.

#### 9.3.2.2. Test sample preparation

The antigen solution is removed by suction and the plate washed 2x five minutes with phosphate buffered saline containing 0.05% Tween 20 (PBST). Test serum samples are titrated in PBST + 0.1% gelatine (minimum dilution 1/100, followed by two- or four-fold dilution steps) and duplicates of each dilution added to positive and control antigen wells on the plate (50 µL/well). The plate is incubated for one hour at 37°C.

#### 9.3.2.3. Conjugate addition

Serum samples are removed and the plate washed 5x two minutes with PBST. The conjugate (an antibody raised to the serum antibodies under test which is linked to an enzyme, horseradish peroxidase) is diluted to a pre-optimised level and 50 µL added to all wells on the plate. The plate is then incubated for one hour at 37°C.

#### 9.3.2.4. Substrate addition

Conjugate solution is removed and the plate washed 4x two minutes with PBST and 2x two minutes with distilled water. 50 µL of substrate solution (TMB or OPD) is added to all wells on the plate and allowed to react at room temperature for up to 10 minutes. The reaction is stopped with 50 µL/well of 1 mol/L sulfuric acid ($H_2SO_4$). The extent of reaction is quantified by using a 450 nm (TMB) or 492 nm (OPD) filter in an ELISA plate reader.

#### 9.3.2.5. Controls

(a) Known positive serum;
(b) a known negative serum;
(c) a serum control (test serum on control antigen); and
(d) a conjugate control (diluent only at serum addition step).

### 9.3.3. Capture ELISA for Detection of Simbu Serogroup Specific Viruses and Virus Specific Antibodies

#### 9.3.3.1. Precoat antibody attachment

Hyperimmune rabbit antiserum to Akabane virus is diluted to an appropriate level (usual range 1/800 to 1/3200) in precoat buffer and 100 µL/well added to the plate and incubated overnight @ 4°C. The plate is washed 2 x five minutes with PBST just prior to conjugate addition.

#### 9.3.3.2. Antigen detection

9.3.3.2.1. Test virus addition. Concentrated supernatant from uninfected and test virus infected cell cultures are diluted in PBST (about 1/100 for a $10^6$ PFU/mL preparation) and 100 µL added to appropriate wells on the plate. The plate is then incubated for one hour at 37°C.

9.3.3.2.2. Detector antibody addition. A known positive antiserum to Akabane virus is diluted appropriately (usual range 1/200–1/1000) in PBST + 0.1% gelatine and 50 µL added to plate wells. The plate is incubated for one hour at 37°C.

9.3.3.2.3. Conjugate addition. As for the Indirect ELISA.

9.3.3.2.4. Substrate addition. As for the Indirect ELISA.

9.3.3.2.5. Controls. As for the Indirect ELISA.

#### 9.3.3.3. Antibody detection

9.3.3.3.1. Antigen addition. A standardised preparation of Akabane infected concentrated tissue culture supernatant and of an uninfected cell control are diluted appropriately in PBST and 100 µL added to suitable wells. The plate is incubated for one hour at 37°C.

9.3.3.3.2. Test serum addition. The plate is washed 4x two minutes in PBST then test sera is diluted in PBST + 0.1% gelatine (minimum dilution 1/100) and 50 µL of each dilution added in duplicate to the plate. The plate is incubated for one hour at 37°C.

9.3.3.3.3. Conjugate addition. As for the Indirect ELISA.

9.3.3.3.4. Substrate addition. As for the Indirect ELISA.

9.3.3.3.5. Controls. As for the Indirect ELISA.

### 9.3.4. Analysis of ELISA Results

#### 9.3.4.1. Plate blanking

Microplate optical density (OD) values are blanked against the conjugate control on the plate.

#### 9.3.4.2. Non-specific reactions

Positive control and test sample OD values are corrected by substraction of their OD values on control antigen.

#### 9.3.4.3. Negative 'cut-off' value

Where the negative serum or antigen control OD is <0.10, a corrected test serum OD value of >0.20 is regarded as positive. When the negative serum or antigen OD value is >0.10 a corrected test sample OD value which is > x 2 the negative OD is regarded as positive.

#### 9.3.4.4. Quantifying positive values

9.3.4.4.1. Titrated samples. Test serum or antigen samples are fully titrated in two-fold or four-fold steps and the highest dilution which remains positive is quoted as the titre for the sample.

9.3.4.4.2. Use of a standard curve. A standard positive serum or antigen.